Doctoral (PhD) dissertation

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A transcriptomic study exploring the role of the *Vitis vinifera NAC* genes in response to stresses: case of *Botrytis cinerea*

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
ABF	ABA binding factor
ABRE	abscisic acid responsive element
AREB	ABA-responsive element
BAP	6-Benzylaminopurine
Bgh	Blumeria graminis f. sp. Hordei
CARE’s	Cis-acting regulatory elements
CDS	Coding DNA sequence
DPE	Downstream promoter elements
DRE	Dehydration-responsive elements
DREB	Dehydration responsive binding element
DREB	DRE Binding
ERE	Ethylene responsive element
ET	Ethylene
HR	Hypersensitive response
ID	Intrinsic disorder
Inr	Initiator region
JA	Jasmonates
LTREs	Low-temperature responsive elements
MiRNA	Micro- RNA
MW	Molecular weight
MYB	Myeloblastosis
MYC	Myelocytomatosis
NAA	Naphthalene acetic acid

NACRS	NAC-recognition sequence for drought response
NACs	NAM – no apical meristem, ATAF – *Arabidopsis* transcription activation factor, and CUC – cup-shaped cotyledon
NGS	Next-generation sequencing
NPR1	Natriuretic peptide receptor 1
ORF	Open reading frame
Pi	Isoelectric point
PM	Powdery Mildew
PR	Pathogen related
SA	Salicylic acid
SAGE	Serial Analysis of Gene Expression
SAR	Systemic acquired resistance
SGFP	Synthetic green fluorescent protein
SiRNA	Small interfering RNA
STRE	stress responsive elements
TF	Transcription factor
TFDB	transcription factor database
TILLING	Targeted Induced Local Lesions in Genome
TMH	Transmembrane helices
TR	Transcription regulatory
TRR	Transcription regulatory regions
TSS	Transcription start site
Vv	*Vitis vinifera* L.
WRE3	Wound-responsive elements 3

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1. INTRODUCTION

Biotic and abiotic stresses are the main factors that significantly limit growth and fruit quality of essential crops worldwide. Plants have developed the ability to cope with the harmful effects of stresses by activating a number of defense mechanisms which include phytohormone signaling networks (Masri and Kiss 2023).

Transcription factor (TFs) proteins are essential regulators of gene expression in all living organisms, with roles in plant growth, cell cycling, cell signaling, and stress response (Li et al., 2021) by binding to specific short sequence motifs mostly detected within the promoters of the target genes, known as cis-regulatory sequences (Priest et al., 2009). Thus, the interaction between them, formed a crucial functional link of gene regulatory networks in plant defence response by controlling various stresses (Kaur et al., 2016).

The NACs (**N**AM – no apical meristem, **A**TAF – *Arabidopsis* transcription activation factor, and **C**UC – cup-shaped cotyledon) are considered as one of the largest TF families that consists of N-terminus and C-terminal. The NAC domain is highly conserved, comprise of 160 amino acid residues that classified into five subdomains from A to E (Puranik et al., 2012). The researchers discovered several *NAC* genes in various plant species using genomic sequencing, including 151 in rice, 117 in *Arabidopsis*, 74 in grape (Shao et al., 2015; Ju et al., 2020).

Different roles for NAC proteins in response to abiotic and biotic stresses, as well as in developmental processes have been reported such as, leaf senescence (Breeze et al. 2011), cell division (Kim et al. 2006), seed development (Sperotto et al. 2009), fiber development (Ko et al. 2007), shoot apical meristem formation (Kim et al. 2006) and embryo development (Duval et al. 2002).

Grapevine (*Vitis vinifera* L.), a member of the *Vitaceae* family and one of the world's most valuable fruit crops with high economic value is proned to a wide range of pathogens that cause yield and quality losses (Ju et al., 2020).

Plant pathogens are categorized to different classes according to their modes of nutrition, such as: biotrophic, necrotrophic, hemibiotrophic and saprophytic pathogens.

The necrotrophic fungus *Botrytis cinerea,* is the main cause of the grey mold disease in more than 200 plants species (Elad et al., 2004) and one of the most crucial pathogens in grapevine (Haile et al., 2017) with a short biotrophic phase (Veloso and van Kan, 2018).

However, in the vineyards, the infection of *B. cinerea* targets primarily the ripe berries; the pathogen inoculation occurs during the onset of the grape berry development (Keller et al., 2003; Pezet et al., 2003).

The *B. cinerea* weakens the natural pathogen resistance system of grape berries and imposes different modifications such as lowering the mechanical resistance of the cell-wall, compacting the bunches and increasing the sugar levels and the concentration of organic acids (Prusky et al., 2013; Blanco-Ulate et al., 2015). Additionally, speeding up the ripening process or promoting the programmed cell death machinery of the grape berries can be manipulated by this pathogen during the infection (Veloso and van Kan, 2018).

Many studies have reported the role of *V. vinifera NAC* genes in response to biotic stress. For example, the *VvNAC1* gene *(*known as *VvNAC60* in Genoscope database*)* showed resistance to *B. cinerea* and *Hyaloperonospora arabidopsidis* (Le Henanff et al., 2013), *VvNAC36* gene in *A. thaliana* was up-regulated in response to powdery mildew colonization (Tóth et al., 2016).

Therefore, studying NAC transcription factor promoters can reveal useful information about the genes and signaling networks involved in abiotic and biotic stress responses. Web-based databases such as, Genomatix, PLACE and Plant CARE have provided a convenient and easy *in silico* way to search motifs and detect cis-acting regulatory elements (CAREs) of the promoters (Ibraheem et al., 2010). Recently, many studies for elucidation and annotation of gene functions have been carried out by use of *in silico* methods. Fortunately, the past decade has seen a revolution in omics technologies that have generated abundant amounts of useful data for *in silico* function predictions (Rhee et al., 2014).

1.1 Objectives

The NAC proteins play an important role in providing resistance to plants. Till now, few works have been reported on cis-acting regulatory elements present in *Vitis NAC* genes. Therefore, our aim in the present work was to perform a transcriptomic study of the *VvNAC* genes to report their responses against *B.cinerea* through an *in silico* approach by characterizing the cis-acting regulatory elements of the *Vitis vinifera* L. *NAC* genes, followed by an *in vitro* work wherever is available in order to find a link between both approaches giving results that might be complemented. This work throws light on the promoter regions of grape *NAC* genes which further can provide new ways for the plant genetic engineering technology for protection of crops against stresses and especially *B.cinerea.*

1. Characterizing of the *Vitis vinifera* L. *NAC* TF genes through an *in silico* approach.
    - Identifying the *c*is-acting regulatory elements (CAREs) of the *Vitis vinifera* L. *NAC* genes.
    - Analyzing the microarray expression profiles of the *Vitis vinifera* L. *NAC* genes in response to *B. cinerea*.
    - Interpreting the up-regulations' results by linking them to the existence of specific CAREs in the promoters of the up-regulated *VvNAC* genes.
2. Detailed interpretation of the function of the *VvNAC* genes.
    - Detecting the remarkable responses whereas up-regulation or down- regulation of the *VvNAC* genes is induced by *B. cinerea.*
    - Predicting new genes with a function that might respond to stresses and especially to *B. cinerea*.
3. Generating *in vitro* approach.
    - Determination of the subcellular localization through a transient expression for the *VvNAC36* gene in a model tobacco plant, *N. benthamiana* L.
    - Comparing the transient expression results with the computational results to validate or find a link between both approaches.
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2. LITERATURE REVIEW

2.1. Plant defence mechanisms

Plants, being sessile organisms, have evolved complex defence mechanisms to protect themselves against a wide array of biotic and abiotic stresses in their environment. These defence mechanisms are the result of millions of years of co-evolution with various stress factors, and they play a critical role in the survival and adaptation of plants (Hönig et al., 2023).

2.1.1. Biotic Stress defence mechanisms

Biotic stresses in plants arise from living organisms, including pathogens, herbivores, and pests. To combat these threats, plants have developed an arsenal of defence mechanisms. One of the most well-known and immediate responses to pathogen attack is the hypersensitive response (HR), characterized by rapid cell death at the infection site. This localized cell death helps to restrict pathogen spread (Pruitt et al., 2021).

HR is often associated with the production of reactive oxygen species (ROS), which can have direct antimicrobial effects. Plant hormones like salicylic acid (SA) play a pivotal role in activating defence responses against pathogens. For instance, SA induces the expression of defence-related genes, such as pathogenesis-related (PR) proteins.

Systemic acquired resistance (SAR) is another defence strategy where the entire plant becomes more resistant to subsequent pathogen attacks following a localized infection, a phenomenon partly mediated by SA. In addition to SA, jasmonic acid (JA) and ethylene (ET) are key players in plant defence against herbivores and necrotrophic pathogens (Yildiz et al., 2021).

These hormones trigger the expression of genes associated with defence against herbivores, such as proteinase inhibitors and volatile organic compounds that attract predators of herbivores. The intricate crosstalk between SA, JA, and ET signalling pathways allows plants to fine-tune their responses based on the nature of the biotic stress.

Furthermore, plants engage in a form of "priming," wherein they pre-activate defence responses in anticipation of future attacks. This priming involves epigenetic modifications and the accumulation of signalling molecules. Once primed, plants can mount faster and stronger defences when challenged by pathogens or herbivores (Hönig et al., 2023).

2.1.2. Abiotic stress defence mechanisms

Abiotic stresses, including drought, salinity, extreme temperatures, and heavy metal toxicity, pose significant challenges to plant growth and productivity. To withstand these stresses, plants have evolved various strategies at the cellular and metabolic levels.

Drought tolerance is a crucial aspect of plant adaptation to arid environments. Plants employ a range of mechanisms to conserve water and cope with dehydration. These mechanisms include stomatal closure, which reduces water loss through transpiration, and the synthesis of compatible solutes like proline and trehalose, which help maintain cellular turgor (Sako et al., 2021).

Dehydration also triggers the production of abscisic acid (ABA), a hormone that induces stomatal closure and activates stress-responsive genes. Salinity stress disrupts cellular ion homeostasis.

To counteract this, plants employ ion transporters and channels to regulate the uptake and compartmentalization of ions, such as sodium and potassium. Additionally, they accumulate osmoprotectants like glycine betaine and mannitol to maintain osmotic balance. In some halophytic plants, specialized salt glands secrete excess salt through trichomes, preventing salt build-up in the plant tissues (Rhaman et al., 2021).

Extreme temperatures, both cold and heat, can damage plant cells and disrupt physiological processes. Plants respond to temperature stress by producing heat shock proteins (HSPs), which assist in protein folding and prevent damage to cellular structures. In cold stress, the production of antifreeze proteins and changes in membrane lipid composition help plants avoid freezing-induced cell damage.

Heavy metal toxicity is a concern in contaminated soils. To mitigate heavy metal stress, plants employ mechanisms such as chelation, where metal-binding molecules like phytochelatins sequester toxic ions, and efflux pumps, which transport metals out of the cell (Kerchev et al., 2020).

2.1.3. Crosstalk between biotic and abiotic stress responses

It's essential to note that plant responses to biotic and abiotic stresses are interconnected. For example, the activation of SA-mediated defence responses against pathogens can lead to increased susceptibility to abiotic stresses like drought. Conversely, JA-mediated defences against herbivores can enhance plant tolerance to some abiotic stresses. Understanding this crosstalk is crucial for developing crop varieties with enhanced stress resilience (Koley et al., 2022).

2.2. Cis-acting regulatory elements of plants

In plants the gene expression is controlled by the existing link between the cis-acting regulatory elements and transcription factors (Ijaz et al., 2020). Thus, the TFs by interacting with the regulatory elements can mediate the target gene activation or repression in a specific tissue or any organ type.

Therefore, recognizing and characterizing these regulatory elements in the genome is important in order to understand more the levels of gene expression (Levine, 2010; Biłas et al., 2016). The CAREs are short linear fragments of non-coding DNA with a variable length, that contain a specific binding site for TFs in the regions of gene promoter (Biłas et al., 2016).

The promoter sequences of a gene are located upstream of the coding region. Through a specific sites in these promoters, the RNA Pol II (RNA polymerase II) enzyme binds to initiate the transcription. Normally, the eukaryotic gene promoter in divided into two regions: core promoter and distal promoter region.

The core promoter is important for initiation the transcription and it consists of: TATA box (present upstream of transcription start site or TSS), TFIIB recognition element (BRE), initiator (Inr) and downstream promoter element (DPE) (Kutach and Kadonaga, 2000; Pandey et al., 2019). The distal promoter or the regulatory region that contains enhancers and silencers, is responsible for gene expression regulation under different conditions (Biłas et al., 2016).

2.2.1. Abiotic stress and CAREs

Plant growth and development are affected by different abiotic stresses such as, drought, temperature and salinity (Zhu, 2002). Thus in order to survive during abiotic stress or any other stresses, plants have made various biochemical and physiological changes to develop a mechanism that detects any growth modifications which may occur through promoting a cascade of signaling pathways controlled by TFs and CAREs, activating several genes for stress tolerance (Gao et al., 2007; Gujjar et al., 2014).

Various cis-acting regulatory elements that showed responded to abiotic stress have been indentified. For example in *Arabidopsis*, dehydration responsive binding element (DREB1, DREB2) regulons and MYB (myeloblastosis)/MYC (myelocytomatosis) regulons plus ABA-responsive element (AREB) and ABA binding factor (ABF) transcription factors (Lenka and Bansal, 2019). The transcription factor NAC was also reported to play a crucial role in abiotic stress. Various transcriptomic studies have identified *NAC* genes responded to drought and salt stresses in the case of soybean, *Oryza sativa* and *Arabidopsis* (Yuan et al., 2019).

2.2.2. Biotic stress and CAREs

In the case of biotic stress, the defense system is complicated whereas different genes are prepared to be expressed to resist any foreign organism. Started by the hypersensitive response (HR) as primary infection fights and followed up by systemic acquired resistance (SAR) as a general protection mechanism for the uninfected parts of plants (Cantu et al., 2013).

The detection of pathogen invasion activates the plant cell defense, followed by an accumulation of salicylic acid (SA) in the cell to change the cytosolic cellular redox potential (Garretón et al., 2002). With these changes the natriuretic peptide receptor 1 (NPR1) protein that is involved in defense mechanisms becomes active and launch the onset of SAR after many steps (Fan and Dong, 2002; Mou et al., 2003).

The gene expression of NPR1 is regulated by TFs through an interaction with W box present in its promoter (Pandey and Somssich, 2009). Similarly, in the case of abiotic stress, TFs also play an important role resisting to biotic stress. For example, in *Arabidopsis* 74 TFs were detected responding to bacterial invasion, in rice with *OsEREBP1* gene that showed an expression in *Magnaporthe grisea* and in tobacco with *SINAC35* gene that improved the resistance to leaf curl virus in transgenic tobacco (Yuan et al., 2019).

2.3. Bioinformatics exploration

2.3.1. Areas of bioinformatics

Bioinformatics can be defined by analyzing and interpreting biological data through the application of a wide range of tools of computation and it is usually an interdisciplinary domain with a mix of computer science, mathematics, and biology that provides different methods in mapping, analyzing and comparing DNA and protein sequences (Luscombe et al., 2001).

Bioinformatics is divided into two complementary subfields: starting with the formation of computational tools and databases, then the application of them in order to produce biological knowledge that helps in better understanding living systems (Xiong, 2009). The first subfield consists of writing software for sequencing and functional analyzing as well as the constructing biological databases.

These developed tools play key roles in three areas of genomic and molecular biology including sequence, structural and functional analysis (Xiong, 2009). Plenty of examples can be listed to explain the diverse roles in each of these three areas: firstly, for the sequence analysis that includes gene and promoter finding, motif and pattern discovery, sequence database searching and alignment.

Secondly, in the case of structural analysis including the prediction, comparison and analysis of protein and nucleic acid structures. Finally with the functional analysis that contains prediction of the subcellular localization of the proteins, determination of the protein-protein interaction and the gene expression profiling (Rao et al., 2008; Xiong, 2009).

The importance of bioinformatics in generating huge databases and biological knowledge leads to a deep understanding of the genetic and molecular basis of all biological processes in plants, helping the biotechnologists engineering new cultivars firstly with high quality and less economic and enviromental costs and resistant to biotic and abiotic stresses (Koltai and Volpin, 2003).

2.3.2. Bioinformatic bases

Bioinformatics encompasses three main elements: databases, computational tools, and software. Databases store vast and complex data, categorized as genome, microarray, and sequence databases (Bourne, 2005). Examples include Xenbase, SEED, ArrayExpress, and Gene Expression Omnibus.

Computational tools play a crucial role in converting these databases into knowledge by utilizing pattern recognition, data mining, and machine learning. Patterns are identified and analyzed through supervised and unsupervised methods, followed by extracting important patterns using techniques like cluster analysis and frequent pattern analysis.

Machine learning, employing statistics and probability theory, helps discover complex patterns. Software provides various programs and tools for database analysis, such as BLAST, SSEARCH, and ENTREZ, which aid in protein and sequence searches, sequence comparison, and retrieving biological information from different databases (Raut et al., 2010).

2.3.3. Bioinformatic applications

As a powerful tool, bioinformatics provide the possibility to study how the cellular activities are altered in different developmental phases, thus help the biologists to increase the understanding of biological processes with computationally intensive techniques including sequence alignment, gene finding, genome and gene expression analysis.

In sequence alignment, the word sequence is defined by the nucleotide sequence present in DNA, RNA and amino acids in protein. In addition, alignment indicates the possibility of changes between the two homologous sequences and a common ancestor sequence that could have occurred during evolution.

The aim of the alignment is to generate a comparison between nucleotides sequences in order to find similarity (Mount et al., 2001). Therefore, a similarity that can be explained by the existence of the same functions between the sequences or by verifying homology due to the origin from a common ancestor. The multiple sequence alignments option is frequently used recently in different sequence analysis areas because it is more accurate because of the combination process between three-dimensional structure information with primary sequences (Wallace et al., 2005). Once the sequence has been obtained, gene finding through databases is a crucial step in understanding the genome of a species (Korf, 2004).

Therefore, starting by identification genes and predicting their functions is important in any study in order to analyze a genome of an organism. Followed by comparison of the identified genes with genome of other organisms to find if they share similarity in different functions such as resistance against various diseases or stresses (Raut et al., 2010).

The gene functions and expressions have always been a target for biotechnologists to analyze, due to their importance in which the gene information is used for synthesis of proteins. For this purpose they created various techniques and tools such as, DNA Microarray, SAGE and Tilling array (Raut et al., 2010).

The synthesis of proteins or the process from DNA to RNA and then to protein, is called the central dogma of molecular biology. The process is started in the nucleus with transcription when the genes are active they produce RNA after copying coding and non-coding sequences. Followed in the cytoplasm with the translation to produce protein from the RNA after many steps. Thousands of messenger RNAs are produced when a single gene is regulated, encoding different proteins and each one of them has a specific role to play (Raut et al., 2010).

In the case of the microarray, thousands of DNA molecules that have been taken from the cells in different conditions are spotted on a glass slide as array followed by pouring a solutions of fluorescently labeled DNA or RNA over the array and each molecule in the solution searches for matching partner on the surface (Raut et al., 2010).

2.3.4. Bioinformatics and stresses

Bioinformatics plays an indispensable role in elucidating the molecular intricacies of plant responses to both biotic and abiotic stresses, providing valuable insights into stress tolerance mechanisms and enhancing crop resilience (Jiang & Tyler, 2012).

In the realm of biotic stresses, bioinformatics aids in the identification and characterization of pathogen effectors and resistance genes within plant genomes, facilitating the development of disease-resistant crop varieties. High-throughput omics technologies, such as RNA-seq and mass spectrometry, enable comprehensive analyses of transcriptomes and proteomes during plant-pathogen interactions, with bioinformatics pipelines essential for the interpretation of large-scale data (Bigeard et al., 2015).

Additionally, metabolomics studies, guided by bioinformatics tools, shed light on the metabolic adjustments that bolster plant defence. In the context of abiotic stresses, bioinformatics leverages genome-wide association studies (GWAS) to identify genetic loci associated with stress tolerance, facilitating the selection of resilient crop genotypes. Functional genomics studies, driven by bioinformatics, unravel gene functions under abiotic stress conditions, including the role of transcription factors (Shinozaki et al., 2022).

Machine learning and predictive models, integrating multi-omics data, enable the forecasting of stress responses and aid in the selection of stress-tolerant cultivars. Notably, bioinformatics promotes the integration of biotic and abiotic stress response data, unveiling shared regulatory networks and crosstalk between these pathways (Kole et al., 2015). As technology advances, bioinformatics remains pivotal for unravelling the intricate web of plant stress responses, contributing to sustainable agriculture and global food security.

2.3.5. Bioinformatics examples

Bioinformatics web tools and databases are crucial components of modern molecular biology and genomics research. PLANT CARE, for instance, significantly contributes to the understanding of gene expression and genome characterization by enabling the identification of cis-acting regulatory elements (CAREs) in plant genes (Lescot et al., 2002).

These CAREs are essential components of gene regulation, and their discovery via PLANT CARE informs researchers about the mechanisms controlling gene expression (Lescot et al., 2002). For example, a researcher studying stress responses in a particular plant species may use PLANT CARE to identify specific CAREs associated with stress-related genes.

MEME, on the other hand, plays a pivotal role in elucidating conserved sequence motifs within DNA, RNA, and protein sequences (Bailey et al., 2009). These motifs often serve as binding sites for transcription factors or other regulatory elements, providing insights into gene expression regulation (Bailey et al., 2009). For instance, MEME can be employed to analyze a set of co-regulated genes and discover common motifs, such as promoter elements shared among genes activated during a stress response.

Moreover, Expression Atlas databases, like the one hosted at EBI, are invaluable resources for studying gene expression and genome characterization (Papatheodorou et al., 2020). These databases compile high-throughput gene expression data from various experiments and organisms, allowing researchers to explore how genes are expressed under different conditions, tissues, or stressors (Papatheodorou et al., 2020).

For instance, a scientist investigating the response of a particular set of genes to a specific pathogen could leverage Expression Atlas to examine the expression patterns of these genes across various infection time points or in different tissues, thereby gaining insights into their regulatory networks and functions.

Additionally, NCBI Gene Expression Omnibus (GEO) and ArrayExpress serve as expansive repositories of high-throughput gene expression data, permitting comparisons of experimental results with publicly available datasets.

Ensembl contributes not only genomic sequences but also RNA-seq data, enabling researchers to explore gene expression across diverse tissues and developmental stages. Focused on developmental biology, the Gene Expression Database (GXD) meticulously catalogues spatial and temporal gene expression patterns during mouse embryo development (Bult et al., 2019).

The STRING Database predicts protein-protein interactions, offering insights into gene expression networks and functional relationships (Szklarczyk et al., 2019). DAVID (Database for Annotation, Visualization, and Integrated Discovery) aids in interpreting experimental outcomes by performing gene ontology enrichment and pathway analysis (Huang et al., 2009).

Furthermore, Genevestigator provides a comprehensive platform for exploring gene expression across various plant and animal species, facilitating the study of tissue-specific expression and responses to different treatments or stressors (Hruz et al., 2008).

In conclusion, these bioinformatics tools and databases play pivotal roles in advancing our understanding of gene expression and genome characterization, and their applications are diverse, ranging from uncovering regulatory elements to discovering conserved motifs and elucidating gene expression patterns under various conditions.

2.4. NAC transcription factors

The NAC is one of the largest TF families in plants that associated with different biological and developmental processes like senescence (Kjaersgaard et al., 2011; Yang et al., 2011), formation of secondary walls (Zhong et al., 2010) and biotic (Christianson et al., 2010) and abiotic stress response (Tran et al., 2010). Plant biotechnologists have succedeed to identify plenty of *NAC* genome sequences in plant species (Tran et al., 2004; Fujita et al., 2004). For example, they found 117 NAC genes in *Arabidopsis*, 151 in rice 163 in poplar, 152 in tobacco (*Nicotiana tabacum*) and 74 in grape (*Vitis vinifera* L.) (Nuruzzaman et al., 2010; Rushton et al.,2008).

2.4.1. Structure of NAC proteins

Normally, the structure of NAC protein consists of two terminals: N terminal NAC domain with almost 150 amino acids and C terminal. The NAC domain in his turn is divided into 5 subdomains (A to E). The subdomains C and D are highly conserved positively charged that bind to DNA, while for the subdomain A that might be involved in the formation of functional dimer and for subdomains B and E with their different roles in *NAC* genes functional diversity (Chen et al., 2011; Ooka et al., 2003).

The C terminal contains the transcription regulatory regions (TRRs) that can activate or repress the transcription. The TRR has different specific motifs that are rich in repeats of proline-glutamine, serine-threonine or acidic residues. For example, ten C terminal motifs were found in the rice NAC proteins (Fang et al., 2008), but it should be taken in consideration that these motifs may vary from subfamily to another one (Shen et al., 2009).

Due to the high degree of intrinsic disorder (ID) of TRRs, they are capable to interact with different target proteins (Jensen et al., 2010. Thus, some NAC proteins have ability for protein binding in their TRRs (Kim et al., 2007; Kleinow et al., 2009), like the case of the NTLs; NAC proteins with a helical transmembrane (TM) motif the responsible for plasma membrane anchoring that may have also regulatory roles under different environmental conditions (Seo et al., 2008).

2.4.2. NAC regulation

The gene is firstly regulated in transcription after binding of TFs to its promoter regulator region (Nakashima et al., 2012). Moreover, the cis-acting regulatory elements (CAREs) present in the promoter region including LTREs (Low-temperature responsive elements), MYB (Myeloblastosis) and MYC (Myelocytomatosis) binding sites, ABREs (ABA-responsive elements), DREs (Dehydration responsive elements) and W-box etc., are responsible for the regulation of the NACs stress responsive genes (Tran et al., 2010).

For example, different studies have reported that *OsNAC3* gene is expressed under abiotic stress due to the presence of DREB (DRE binding) regulon (Takasaki et al., 2010), while the expression of *ONAC045* gene under same conditions was linked to the presence of MYB and MYC core binding sites in the promoter (Zheng et al., 2009).

Secondly, the genes undergo to a post-transcriptional regulation through micro-RNA (miRNA)-mediated cleavage. Therefore, the most recognized miRNA is miRNA 164 that showed high affinity in developmental and stress regulation, it regulates the post-transcriptional RNA processing of *NAC* TF genes (Khraiwesh et al., 2011).

Finally, the NAC TF genes are subjected to a post-translational regulation which includes protein degradation mediated by ubiquitins, dimerization (Hegedus et al., 2003) and interaction with other non-NAC proteins (Greve et al., 2003).

2.4.3.NAC genes expression

The role of *NAC* TF genes have been reported in many studies, that verified the expression of these genes at least under one type of stress like salinity, drought, fungi etc. (Fang et al., 2008). The analysis of the *NAC* gene expression by usying microaaray has shown for example, in the case of rice more than 45 *NAC* genes were induced in abiotic and 26 in biotic stresses (Nuruzzaman et al., 2010). Moreover, in *Arabidopsis* the majority of the *NAC* genes expressed under salt and high temperature stresses (Zeller et al., 2009).

In addition, the expression level of *NAC* genes also has been reported in response to attacks of different bacteria, viruses and fungi (Ren et al., 2000; Oh et al., 2005) and by the application also of the exogenous phytohormones like absicic acid (ABA), ethylene (Et) and salicylic acid (SA) and jasmonic acid (JA) in different species (Yoshii et al., 2010). The phytohormones are associated with a cascade of signaling response in the case of any stresses by acting in conjunction or opposition to each other to maintain cellular homeostasis (Fujita et al., 2006).

2.4.4. Abiotic and biotic stresses response

Plenty of positive results have demonstrated the role of NAC TFs in response to abiotic stress after overexpression in *Arabidopsis* and rice without showing any growth retardation (Liu et al., 2011). Whereas 38 *NAC* genes were found to be involved in soybean drought response (Le et al., 2011), and 40 *NAC* genes were involved in rice drought response (Le et al., 2011); (Fang et al., 2008). Furthermore, 33 *NAC* genes in *Arabidopsis* responded to salt stress (Jiang and Deyholos 2006).

For example in rice, drought, salt tolerance and higher seed production in dry field showed by the *SNAC1* transgenic lines (Hu et al., 2006) and same in the case of *OsNAC10* (Jeong et al., 2010; Song et al., 2011). In transgenic *Arabidopsis* transformed with the wheat *NAC* transcription factor *TaSNAC4-3A* gene demonstrated drought tolerance (Mei et al., 2021). Overexpression of *ANAC019, ANAC055/AtNAC3,* or *AtRD26* enhanced drought tolerance in *Arabidopsis* (Tran et al., 2004). In soybeans, the role of the *GmNAC8* gene as a drought-stress positive regulator was also observed (Yang et al., 2020).

Conversly, dwarfing, late flowering and lower seed yield in the transgenics have been identified after NACs overexpression in some cases but it might be solved by utilizing stress-inducible or tissue-specific promoters like *OsNAC6* or *RCc3* (Nakashima et al., 2007).

Biological stress arises when a number of viruses and living organisms, such as bacteria, fungi, and harmful insects, invade and damage plants. As a result, the plant will activate a complex defense mechanism in response (Masri and Kiss 2023). Moreover, RNA interference/knockouts and overexpression studies have revealed the function of NAC TFs in various plant–pathogen interactions (Collinge and Boller 2001).

Numerous NAC proteins are known to interact directly with virus-encoded proteins to either enhance or inhibit virus replication (Yoshii et al., 2009). Despite the significance of *NAC* TFs in stress responses, there are few studies indicating *NAC* gene responses to pathogens. The most well-known researches were undertaken in rice, where the value of the *ONAC122* and *ONAC131* genes in *M. grisea* resistance responses was discovered using virus-induced gene silencing (VIGS) (Sun et al., 2013).

Furthermore, the same (VIGS) method was used to show that *TaNAC35* acts as a negative regulator for leaf rust resistance in a compatible relationship between common wheat and *Puccinia triticina* in wheat (Zhang et al., 2021). Additionally, overexpression of *ONAC066* gene in transgenic rice positively regulated blast and bacterial blight resistance by inhibiting ABA signaling pathways (Liu et al. 2018), and with *OsWRKY67* gene it positively regulates the same disease by direct activation of PR genes (Liu et al., 2018).

Finally, in *Arabidopsis*, overexpression of *ATAF1* reduced resistance to the necrotrophic fungi *Botrytis cinerea* and *Alternaria brassicicola*, suggesting that *ATAF1* is a negative regulator of necrotrophic pathogen defense (Wang et al., 2009; Wu et al., 2009).

2.4.5.NAC TFs in multiple processes

A crosstalk signaling between abiotic and biotic stresses have been identified in NAC TFs. Thus, the responses against these stresses might be discrete from the transgenic plants after overexpression of the *NAC* genes. For example, in *Arabidopsis*, the overexpression of *ATAF1* have reported to increase the drought tolerance and at same time the sensitivity to ABA, salinity, necrotrophic fungus *(B. cinerea).*

Thus, it negatively regulates ABA levels for efficient basal defense against *Blumeria graminis f.sp. graminis (Bgh)* (Wu et al., 2009). Moreover, *ANAC019* and *ANAC055* were associated with drought tolerance, but their overproduction also decreased resistance to *B. cinerea.* Finally, with the overexpressing of *ATAF1* in rice, *OsNAC6* that showed a slight increase of the resistance to the blast disease in addition to higher tolerance to drought and high salinity (Fujita et al., 2004).

As plants often encounter multiple stresses concurrently, such multi-functionality becomes significant for survival under extreme stress conditions. Several *NAC* TFs also integrate responses to environmental stresses into modulation of plant development processes, like lateral root development, seed germination (Balazadeh et al., 2010), flowering (Kim et al., 2007) and senescence. Such versatility of *NAC* functions may have evolved to ensure plants' longevity, survival and reproductive success under environmental stresses.

2.5. *Vitis vinifera* L.

2.5.1. Description

Vitis vinifera L. (grapevine), a member of the *Vitaceae* family, is a deciduous, woody vine with a southwestern Asian origin was transported by the European to many places over the world. Normally, they can grow to form long spiral tendrils up to 150 cm long and spread horizontally over low-growing bushes and they will be trimmed much smaller down to 20 cm in the case of plants grown for wine production.

Additionally, they start blooming during the period between May and June with small hermaphroditic flower that form very soft pulpy grapes in different sizes and colors which ripen in summer. *Vitis vinifera* L. is one of the world's most valuable fruit crops, with high economic, nutritional value, and a reputation for producing high-quality wines (Ju et al., 2020).

For instance, Pinot Blanc, Pinot Gris, Sauvignon Blanc and Chardonnay in the case of white wine varieties and Cabernet Sauvignon, Merlot and Pinot Noir for the red wine varieties, but beside these very famous grape cultivars the number of varieties has increased to about 10 000 during the centuries of viticulture.

2.5.2. Abiotic and biotic stresses

As mentioned before considering the grape as one of the largest and most important agricultural fruit crop in the world, and, like other crop, grapevines are permanently challenged by various pathogens and changing environmental conditions that cause significant yield and quality losses (Masri and Kiss 2023). Starting firslty by abiotic stresses, such as light, temperature, and drought. The grapevines undergo chemical, physical, and physiological changes in order to resist these stresses and require intensive growing conditions for producing fruit and wine of high quality.

Grapes are susceptible to many insects, disease pests and viruses particularly in humid summer climates including black rot, powdery mildew, gray mold, crown gall, botrytis bunch rot, phylloxera, grape berry moth and Japanese beetle. Several grapevines recognize the pathogen components, transduce the stress signal, and induce a defense response through hormones regulation.

Therefore, many studies have reported the role of *V. vinifera* genes regarding the response to abiotic and biotic stresses (Fang et al., 2016). For example, *VvNAC1 (VvNAC60)* overexpression increased the osmotic, salt, and cold stress resistance in *A. thaliana* (Le Henanff et al., 2013). Overexpression of *VvNAC17* gene isolated from grapevine improved resistance to salinity, freezing, drought and also regulates an ABA-mediated pathway in *A. thaliana* (Ju et al., 2020) and the overexpression of grapevine *VvNAC08* in transgenic *Arabidopsis* increased drought tolerance (Ju et al., 2020).

Additionally, the public microarray data showed that the expression of *VvNAC26* was highly induced under water deficit, cold temperature, and high salinity stresses (Wang *et al.*, 2013). A recent study proved a remarkable regulation of the *VvNAC36* gene in *A. thaliana* in response to powdery mildew colonization, the up-regulation of *VvNAC36* 1.5-fold in the case of powdery mildew (PM) while no response was detected for salicylic acid (SA) (Tóth et al., 2016). Finally, the overexpression of *VvNAC1 (VvNAC60)* increased the resistance to *B. cinerea* and *Hyaloperonospora arabidopsidis* (Le Henanff et al., 2013).

2.5.3. Botrytis cinerea

Botrytis cinerea (B.cinerea) is a one of the most crucial necrotrophic fungal pathogens in grapevine and in more than 200 plant species in different temperate or subtropical regions causing gray mold disease, pre and postharvest decay and fruit quality damaging in fields, greenhouses and even during storage (Williamson et al., 2007).

B.cinerea infection may occur slowly to wide range of hosts like the horticultural crops and fruit crops in their flowers, leaves, shoots and fruits. These symptoms are visible where the fungus begins to rot the plant at wound sites (Sutton 1998).

Two different types of mold disease are identified on the infected grapes. The first is noble rot, that occurs in drier condition following by wetter one resulting of accumulation of aroma and sugar concentration (Breia et al., 2021). The second one is grey rot or bunch rot, caused by heavy rainfalls and high humidity conditions leading to severe losses of the infected bunches (Gubler et al., 2013). Conversely to noble rot, the grey rot affects the production of wine badly by negatively modifying the fermentation process thus the sensory properties of the final product (Hornsey, 2007; Morales-Valle et al., 2011).

Regarding the two types, the rot development mechanism in the infected grape leading to the formation of noble or grey rot has not been well clarified yet but it might be influenced by the intrinsic characteristics of the grape cultivar or different enviromental effects (Sipiczki, 2006).

2.5.4. Applied breeding methods and objectives in Vitis vinifera L.

Vitis vinifera L., has been a focal point of applied breeding efforts for centuries due to its economic importance in wine and table grape production. These breeding programs aim to develop grapevine cultivars with improved traits such as disease resistance, yield, fruit quality, and adaptability to changing environmental conditions. Several breeding methods and objectives have been employed to achieve these goals.

Breeding methods:

1. Conventional Breeding: Conventional breeding, also known as classical or traditional breeding, involves the controlled cross-pollination of two grapevine varieties with desirable traits. The resulting progeny are evaluated for specific characteristics, and the best-performing individuals are selected as potential new cultivars (Emanuelli et al., 2013).

2. Marker-Assisted Breeding:With advancements in genomics, marker-assisted breeding has become a powerful tool. DNA markers linked to important traits, such as resistance to pests or diseases, can be identified and used to select promising individuals at an early stage, reducing the time and resources required for breeding (Bettoni et al., 2021).

3. Genome Editing: Emerging technologies like CRISPR-Cas9 have the potential to revolutionize grapevine breeding. Genome editing allows for precise modification of genes responsible for specific traits, offering the opportunity to create customized grapevine varieties with improved characteristics (Ren et al., 2016).

Breeding objectives:

1. Disease Resistance: One of the primary objectives in *Vitis vinifera* L. breeding is enhancing resistance to diseases like downy mildew (*Plasmopara viticola*) and powdery mildew (*Erysiphe*

necator). Developing cultivars with innate resistance reduces the reliance on chemical treatments (Feechan et al., 2013).

2. Abiotic Stress Tolerance: Climate change and shifting environmental conditions pose challenges for grape cultivation. Breeding for abiotic stress tolerance, such as drought resistance, ensures stable grape production in the face of changing climates (Cramer et al., 2007).

3. Improved Fruit Quality: Enhanced fruit quality attributes, including sugar content, flavor compounds, and aroma profiles, are essential for wine and table grape varieties. Breeding programs target these traits to meet consumer preferences and market demands (Ryan et al., 2015).

4. Yield and Productivity: Increasing grapevine yield without compromising fruit quality is a consistent breeding objective. High-yielding varieties contribute to the economic sustainability of vineyards (de Oliveira et al., 2020).

5. Adaptation to New Regions: As grapevine cultivation expands to new regions, breeding objectives include developing cultivars that can thrive in diverse soil types and microclimates, allowing for more extensive grape production (This et al., 2006).

In conclusion, applied breeding methods and objectives in *Vitis vinifera* L. are driven by the need for sustainable and resilient grape production. Through conventional breeding, marker-assisted techniques, and emerging genome editing technologies, breeders work toward creating grapevine cultivars with improved disease resistance, abiotic stress tolerance, fruit quality, yield, and adaptability. These efforts ensure the continued success of grape cultivation in a dynamic and evolving agricultural landscape.

2.6. *In silico* methods: Definition, applications, and objectives

Definition:

In silico methods in plant biotechnology and molecular biology refer to computational techniques and simulations used to analyze, model, and predict biological processes and phenomena in plants. The term "*in silico*" is derived from Latin, meaning "in silicon," signifying that these methods are conducted in a computer-based, virtual environment. In the context of plant science, *in silico* methods involve the application of computer algorithms and data-driven approaches to understand, study, and manipulate various aspects of plant biology

Applications:

1. Genome Analysis: *In silico* methods are widely applied in plant genome analysis. This includes genome sequencing, annotation, and comparative genomics. Researchers can predict genes, identify regulatory elements, and explore genetic diversity within plant genomes using computational tools (Edwards and Batley, 2010).

2. Functional Genomics: These methods are crucial for interpreting high-throughput data generated by techniques like transcriptomics and proteomics. Researchers can identify and characterize genes, pathways, and regulatory networks associated with plant development, responses to stresses, and other biological processes (You, 2018).

3. Protein Structure and Function: *In silico* methods play a significant role in predicting the structure and function of plant proteins. Predictive modeling, molecular docking, and structural

bioinformatics aid in understanding protein interactions, enzymatic activities, and potential drug targets within plants (Kumari et al., 2013).

4. Crop Improvement: In plant breeding and crop improvement programs, *in silico* methods are used to identify candidate genes associated with desirable traits such as disease resistance, yield, and nutritional quality. These methods accelerate the breeding process by narrowing down potential targets (Schreiber et al, 2018).

5. Metabolic Pathway Engineering: Researchers utilize *in silico* approaches to analyze and engineer plant metabolic pathways. Constraint-based modeling and metabolic flux analysis aid in optimizing metabolic networks for the production of biofuels, pharmaceuticals, and other valuable compounds (Araus et al., 2022).

6. Systems Biology: *In silico* modeling is integral to systems biology, where researchers seek to understand the behavior of entire plant systems. These models integrate data from various omics levels (genomics, transcriptomics, metabolomics) to simulate and predict plant responses to changing environmental conditions (Atkin and Macherel, 2009).

Objectives:

1. Hypothesis Testing: *In silico* methods allow researchers to test hypotheses and generate predictions. For example, they can predict the effects of specific genetic modifications on plant phenotypes or assess the impact of environmental factors on gene expression (Tutar, 2014).

2. Data Integration: Given the wealth of biological data generated in plant research, one objective of *in silico* methods is to integrate and make sense of diverse data types. This integration helps researchers gain a holistic view of plant biology (Walls et al., 2012).

3. Model Construction: Researchers aim to construct accurate mathematical and computational models of plant processes. These models help simulate and understand the dynamic behavior of plants, from growth and development to responses to biotic and abiotic stresses (Gonzalez-Meler et al., 2014).

4. Crop Enhancement: In agriculture and plant biotechnology, the objective is to use in silico methods to identify genes and traits that can enhance crop productivity, nutritional content, and stress tolerance. This contributes to global food security and sustainable agriculture (Cai et al., 2018).

5. Drug Discovery: In the context of medicinal plants, *in silico* methods are employed to discover bioactive compounds and understand their interactions with human receptors. This objective supports the development of plant-based medicines (wan et al., 2019).

6. Environmental Impact Assessment: Researchers use *in silico* methods to assess the ecological and environmental impact of genetically modified plants or novel agricultural practices. This contributes to responsible biotechnology adoption (Denby et al., 2004).

In conclusion, *in silico* methods are indispensable tools in plant biotechnology and molecular biology. Their applications span from fundamental research to practical applications in crop improvement, drug discovery, and environmental sustainability. The objectives of *in silico* methods encompass hypothesis testing, data integration, model construction, and the enhancement of plant traits, ultimately contributing to our understanding and manipulation of plant biology for diverse purposes

3. MATERIALS AND METHODS

3.1. *In silico* part

3.1.1. Detection and data analyses of NAC family genes

A local blast analysis was conducted to locate *NAC* genes within the grapevine genome. The NAM domain and 26,346 predicted genes from the 12X assembled *Vitis vinifera* Pinot Noir PN40024 genome were utilized as search queries and databases. As a result, 74 genes were successfully identified and designated from *VvNAC01* to *VvNAC74*, each associated with a unique gene locus number (Wang et al., 2013).

In this study, we utilized as input the gene loci of the 74 *Vitis vinifera NAC* genes to retrieve the protein sequences from genoscope, the grapevine genomic database available at (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/).

The NAC conserved domain sequences of all the genes were extracted through the Pfam web database (https://pfam.xfam.org) (Finn et al., 2016). Subsequently, a BLASTp analysis was performed using the phytozome v13 database accessible at https://phytozome-next.jgi.doe.gov/ to validate all VvNAC protein sequences employed in this approach (Goodstein et al., 2012).

The amino acid composition, isoelectric point, and molecular weight of the NAC proteins were determined using ExPASy; http://ca.expasy.org/tools/pi_tool.html (Ison et al., 2013). CELLO v.2.5 webtool; http://cello.life.nctu.edu.tw was utilized to estimate the subcellular localization of the proteins (Yu et al., 2004).

TMHMM v2.0 (http://www.cbs.dtu.dk/services/TMHMM/) was used to determine the transmembrane helices (TMHs) of the identified *VvNAC* family proteins (Krzywinski et al., 2009).

TMHs are regions within a protein that span the lipid bilayer of a cell membrane. By analyzing the amino acid sequence of the *VvNAC* genes, TMHMM v2.0 uses computational algorithms and statistical models to identify potential TMHs within the protein. The prediction is based on specific features such as hydrophobicity and amphipathicity, which are characteristic of transmembrane regions.

The nucleotide substitution rates (Ka and Ks) and their ratios (Ka/Ks) for duplicated *VvNAC* genes were computed using the straightforward Ka/Ks calculator feature within the TBtools software, which can be found at https://bio.tools/tbtools (Chen et al., 2020). To facilitate this analysis, the coding sequence (CDS) sequences of the genes were sourced from the phytozome v13 database.

3.1.2. Conserved motifs and cis-acting regulatory elements (CAREs) of VvNAC genes

The protein motifs of *Vitis vinifera NAC* were analyzed using the web database program MEME 5.5.2, which can be accessed at https://meme-suite.org/meme/tools/meme. This program utilizes various statistical modeling techniques to identify prevalent patterns, 10 Motifs were selected based on the criteria of a minimum width of 6 pixels and a maximum width of 50 pixels (Bailey et al., 2009).

The cis-acting regulatory elements of the *VvNAC* genes were identified using the Plant CARE database, which is accessible at http://bioinformatics.psb.ugent.be/webtools/plantcare/html/ (Lescot et al., 2002) (Appendix - Table 7.1).

3.1.3. Protein–protein interaction analysis

In this study, we utilized the web-based STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database version 11.0 https://string-db.org to predict and construct protein-protein interaction networks involving VvNAC proteins, with *Arabidopsis* homologous proteins as references (Szklarczyk et al., 2023).

To configure the STRING analysis, we adjusted specific parameters as follows: the network type was defined as the full STRING network, the meaning of network edges was set to evidence, the minimum required interaction score was established as a medium confidence parameter (0.4), and the maximum number of interactions displayed for each protein did not exceed 10 interactors.

3.1.4. In silico expression profiles of VvNAC genes and statistical analysis

The microarray dataset of grapevine *NAC* gene expression in response to *Botrytis cinerea* stress was obtained from the open science resource Expression Atlas, which can be accessed at https://www.ebi.ac.uk/gxa/experiments/E-GEOD-67932/Results (Blanco-Ulate et al., 2015) (Appendix - Table 7.4). Expression patterns of these *NAC* genes were represented as a heat map viewed in TBtools https://bio.tools/tbtools.

Additionally, by using Bioinformatics.com.cn a statistical analysis for gene expression through hierarchical clustering data was generated.

3.2. *In vitro* part

3.2.1. Construction of plant expression vectors

VvNAC36 gene construct

Based on previous work started in the institute, the *VvNAC36* has been chosen to check how much accurate the *in silico* results might be to compare to the laboratory work. Thus, genomic DNA was extracted from young grapevine leaves using the CTAB (Cetyltrimethylammonium bromide) method by following these steps:

1. Sample collection: We begin by collecting plant material, such as leaves, young shoots, or other tissues of interest, ensuring that the material is fresh and free from contamination.

2. Homogenization: We grind the collected plant material into a fine powder, using either a mortar and pestle or specialized homogenization equipment. Our goal is to break open the cells and release their contents.

3. Cell lysis: We transfer the powdered plant material into a tube or container and add a CTAB-based extraction buffer. The extraction buffer typically contains CTAB, EDTA (Ethylene Diamine Tetraacetic Acid), and other reagents to disrupt cell membranes and stabilize DNA. We then heat the mixture to facilitate cell lysis.

4. Incubation: After adding the extraction buffer, we incubate the mixture at an elevated temperature, usually around 65°C, for a specified period. This step helps break down cell membranes and release DNA into the solution

5. Phenol-Chloroform extraction: Following incubation, we add an equal volume of phenol-chloroform-isoamyl alcohol (PCI) to the mixture. Phenol-chloroform helps to separate DNA from

proteins, lipids, and other cellular components. We mix the solution thoroughly by inverting the tube or using a vortex mixer.

6. Centrifugation: We then centrifuge the mixture at high speed. This separates it into three phases: a lower organic phase (containing proteins and lipids), an interphase, and an upper aqueous phase (containing DNA). Carefully, we transfer the upper aqueous phase to a new tube.

7. Precipitation: To precipitate DNA, we add cold isopropanol or ethanol to the aqueous phase. DNA is not soluble in these alcohols, causing it to precipitate out of solution. We gently invert the tube to encourage DNA precipitation.

8. Centrifugation: After precipitation, we centrifuge the tube again to pellet the DNA. We then remove the alcohol-containing supernatant, taking care not to disturb the DNA pellet.

9. Washing: We wash the DNA pellet with cold ethanol to remove any residual contaminants, which further purifies the DNA

10. Drying: We allow the DNA pellet to air dry for a brief period, ensuring it's completely dry to avoid ethanol contamination in downstream applications

11. Rehydration: To dissolve the DNA, we rehydrate the DNA pellet in an appropriate buffer, such as TE buffer (Tris-EDTA). This buffer helps protect the DNA from degradation.

12. Storage: Finally, we store the extracted DNA at -20°C or -80°C for long-term preservation or at 4°C for short-term use.

Moreover, the *VvNAC36* gene was then amplified by PCR using Phusion High Fidelity Taq polymerase with forward and reverse primers, which contained *HindIII* and *SacI* restriction sites, respectively (Appendix- Table 7.6, 7.7).

The PCR amplification was performed for 34 cycles under the following conditions: 98°C for 30 s, 94°C for 10 s, 60°C for 90 s, and 72°C for 5 min, followed by a final extension step at 72°C for 10 min. The purified PCR product was cloned into the pENTR/D-TOPO vector from Invitrogen, further digested, and subcloned at the *HindIII* and *SacI* restriction sites into the pGWB605 binary vector, resulting in the recombinant plasmid pGWB605::*VvNAC36* (Appendix- Figure 7.1).

The pGWB605 vector contained a CaMV35S promoter and the synthetic green fluorescent protein (sGFP) reporter gene, as shown in a schematic structure using Benchling software (Figure 3.2.1).

The control vector 35S-sGFP (containing green fluorescent protein alone) and the recombinant plasmid pGWB605::*VvNAC36* were introduced into *Agrobacterium tumefaciens* strain GV3101 by direct transformation.

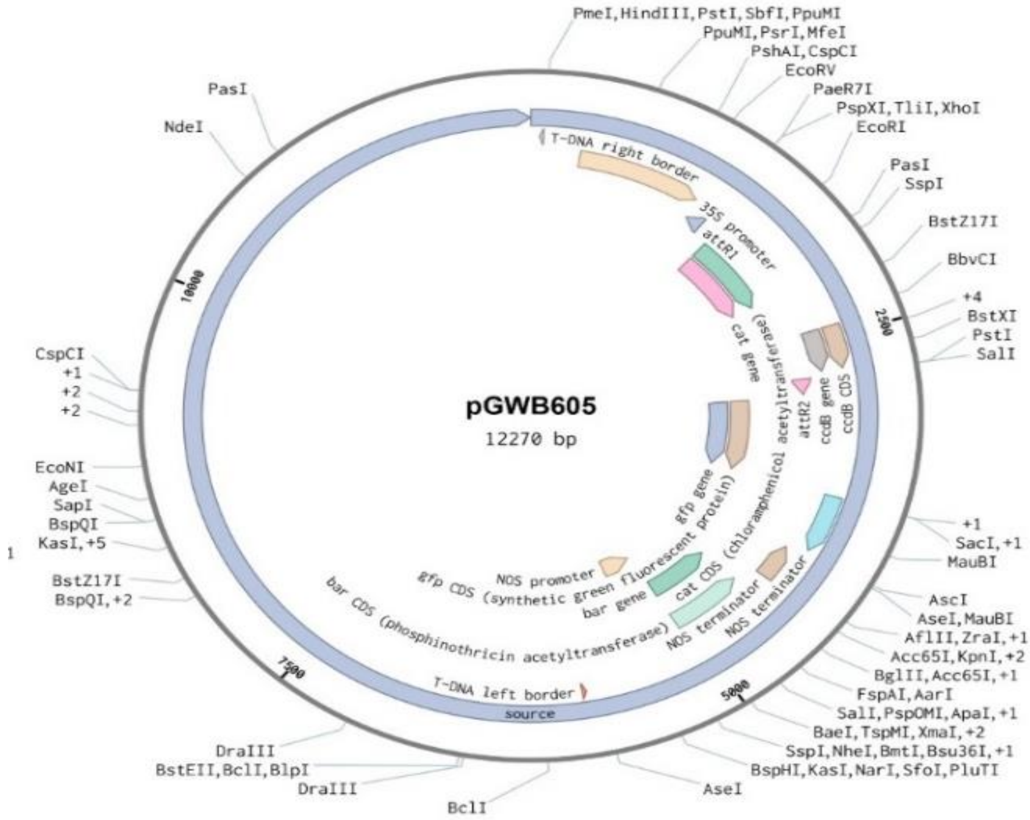

Figure 3.2.1. Schematic structure of the vector pGWB605 using Benchling software.

3.2.2. Transient Expression

For the pre-culture, *Agrobacterium tumefaciens* (*A. tumefaciens*) GV3101 carrying the binary vector pGWB605::*VvNAC36* and 35S-sGFP were grown overnight in 2–3 mL Luria-Bertani medium (LB medium) supplemented with 100 mg/L spectinomycin and 30 mg/L rifampicin at 28 °C (Gusain et al., 2021) . Subsequently, 1 mL of the pre-cultures was inoculated into fresh 25 mL LB medium containing the same antibiotics for the main cultures.

On the following day, culture solutions were centrifuged at 5,000 rpm for 15 minutes at room temperature, followed by resuspension of the *A. tumefaciens* pellet in a buffer consisting of 1 M $MgCl_2$, 0.5 M 2-(N-morpholino)ethanesulfonic acid (MES) (pH 5.6), and 10 mM acetosyringone. The buffer volume was adjusted to achieve an OD 600 of approximately 1 (Hoshikawa et al., 2018). The buffer was then incubated at room temperature for 2–3 hours, as shown in Photo B (Figure 3.2.2).

Using a needleless 1-mL syringe, the cells were co-infiltrated at a 1:1 ratio with *A. tumefaciens* containing the p19 gene, a silencing suppressor (Lindbo, 2007), into the abaxial side of the leaves of 6-week-old plants represented by the photos marked as A, C, and D (Figure 3.2.2).

Following infiltration, the plants were left undisturbed for 5 days before examination with the Leica TCS SP8 confocal microscope. This examination involved analyzing pieces of infiltrated leaves taken from the infected region at the National Agricultural Research and Innovation Centre (NARIC) in Hungary.

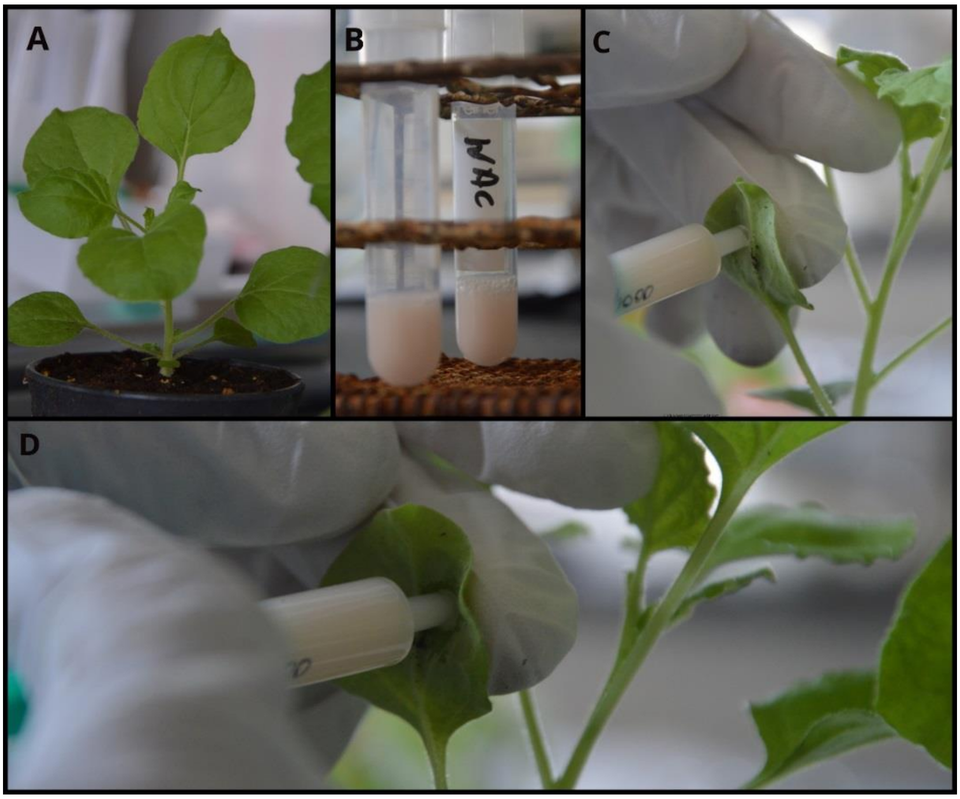

Figure. 3.2.2 Agroinfiltration of tobacco leaves. (A) 6-week-old *N. benthamiana* plant that is appropriate to be infiltrated. (B) *Agrobacterium tumefaciens* containing the binary vector in the infiltration buffer. (C)The suspension was slowly infiltrated into the leaves. (D)The colour of the infiltrated region changed to deeper green.

4. RESULTS

4.1. Identification and genomic distribution of NAC proteins

In this study, different databases were utilized to study and characterize the *Vitis vinifera's NAC* proteins. The genome of *V. vinifera* L. was found to have 74 NAC proteins. Table 7.1 in appendix presents a comprehensive summary of the *VvNAC* genes, including their length, molecular weight (MW), isoelectric point (pI), and cellular location. The length of the proteins produced by *NAC* genes ranged from 128 amino acids (AA) in *VvNAC50* to 994 (AA) in *VvNAC54*. The molecular weight of these proteins varied from 14.76 to 110.67 kDa (*VvNAC50* and *VvNAC54*, respectively). The isoelectric point of the *NAC* proteins ranged from 4.1 in *VvNAC47* to 10.52 in *VvNAC73*.

4.2. TMHs and KaKs value of *VvNAC* genes

Notably, nine out of the 74 *VvNAC* genes identified encoded proteins with transmembrane helices (TMHs). It was found that eight proteins, namely *VvNAC15*, *VvNAC20*, *VvNAC25*, *VvNAC29*, *VvNAC48*, *VvNAC54*, *VvNAC67*, and *VvNAC71* contained only one transmembrane domain while *VvNAC72* contained 6 domains (Table 4.2-A).

Therefore, this information is valuable because the presence and location of transmembrane helices can provide insights into the protein's function and its association with cellular membranes. It suggests that these *VvNAC* proteins are likely to be membrane-associated or have specific interactions with the cell membrane.

Based on the analysis conducted using the KaKs calculator via TBtools, none of the 20 tandem repeat sequences exhibited Ka/Ks values greater than 1 (Table 4.2-B). This outcome implies the absence of positive selection in any of the tandem repeat sequences, which are typically associated with facilitating adaptive genetic variation and potentially influencing species evolution.

Instead, all genes displayed Ka/Ks values below 1, indicating the prevalence of negative selection during the course of evolution. This negative selection process serves to reduce the rate of changes in the amino acid profile. In summary, the findings indicate that the majority of *VvNAC* genes exhibit a slow evolutionary rate, characterized by the absence of positive selection.

Table 4.2. (**A**) The predicted number of transmembrane helices (TMHs) of *VvNAC* genes using TMHMM v2.0.

“Number of predicted TMHs": It refers to the actual count or number of TMHs that have been predicted in the protein sequence; **"Expected number TMHs AAs"**: It refers to the expected number of TMHs predicted throughout the entire length of the protein considering all the amino acids (AAs) in the protein; **"Expected number first 60 AAs"**: It refers to the expected number of TMHs predicted only within the first 60 amino acids of the protein.

Gene ID	**Length Base pair (bp)**	**Number of predicted TMHs**	**Transmembrane sequences (position)**	**Expected number, TMHs AAs**	**Expected number, first 60 AAs**
VvNAC15	572	**1**	541-563	21.57467	0.00038
VvNAC20	559	**1**	530-552	21.15784	0
VvNAC25	632	**1**	600-622	22.00932	0
VvNAC29	214	**1**	190-212	24.22788	0.00015
VvNAC48	560	**1**	526-548	22.59021	0.00015
VvNAC54	994	**1**	537-559	34.49748	0.00212
VvNAC67	375	**1**	341-363	22.82849	0.00112
VvNAC71	578	**1**	551-573	20.45545	0
VvNAC72	659	6	449-471 484-506 526-548 569-591 606-623 636-658	127.28941	0.00023

Table 4.2. (**B**) The Ka/Ks values of *Vitis vinifera* tandem repeat sequences.

Tandem Repeat Sequences	**Ka**	**Ks**	**Ka/Ks**
VvNAC52/VvNAC54	0.156069171	0.226033598	0.690468905
VvNAC50/VvNAC53	0.428950207	0.616323419	0.695982327
VvNAC71/VvNAC25	0.488769856	1.700995236	0.28734346
VvNAC21/VvNAC20	0.428894814	1.341508352	0.319710879
VvNAC64/VvNAC69	0.355003859	2.934806313	0.120963301
VvNAC34/VvNAC37	0.1981519	2.090261815	0.094797646
VvNAC58/VvNAC47	0.219426571	2.011036466	0.109111185
VvNAC01/VvNAC07	0.202803106	1.131456332	0.179240771
VvNAC39/VvNAC08	0.192497524	2.430682324	0.079194851
VvNAC66/VvNAC43	0.281571219	0.652713909	0.43138535
VvNAC03/VvNAC18	0.247258086	1.59634065	0.154890553

VvNAC45VvNAC42	0.351230457	1.161810834	0.302312947
VvNAC11/VvNAC05	0.240144639	2.824820383	0.085012357
VvNAC65/VvNAC16	0.466070377	3.195651915	0.145845164
VvNAC22/VvNAC02	0.131794832	1.188950664	0.110849706
VvNAC49/VvNAC24	0.175166293	1.760241308	0.099512659
VvNAC29/VvNAC28	0.240883303	0.661545539	0.364122028
VvNAC30/VvNAC31	0.045084736	0.074318177	0.606644812
VvNAC10/VvNAC27	0.161614425	1.03734359	0.155796428
VvNAC04/VvNAC41	0.299027751	2.406023034	0.124282996

4.3. Conserved motifs of *VvNAC* genes

The *VvNAC* genes contain a highly conserved DNA binding NAC domain at the N-terminal, consisting of 160 amino acid residues that are divided into five subdomains: A, B, C, D, and E (Figure 4.3-A). However, the C-terminal part is highly variable and lacks any known protein domains.

To further investigate the conserved motifs of NAC proteins from *Vitis vinifera* L., the MEME 5.5.2 web database program was utilized, resulting in the identification of one to ten motifs. A thorough investigation of the conserved motifs present in each *VvNAC* gene was also performed.

The outcomes of the investigation pertaining to the distribution of NAC protein motifs have elucidated significant patterns. Specifically, within the subset of 74 grape NAC proteins, a substantial portion comprising 57 proteins (equivalent to 77% of the total) has been identified as encompassing subdomains A to E. Furthermore, a distinct profile has emerged where six proteins exhibit the absence of a singular NAC sub-domain, namely A, B, or C. In addition, an analogous group of six proteins is characterized by the lack of two sub-domains, manifesting either as B and C or A and C.

A remarkable observation pertains to the presence of two sub-domains, A and B, within a sole protein, VvNAC66. Moreover, a noteworthy subset consists of three proteins, specifically VvNAC35, VvNAC38, and VvNAC50, each harboring only a solitary sub-domain, namely the C sub-domain. Notably, another protein, VvNAC72, distinctly carries the A sub-domain.

Moreover, the range of motifs identified within *Vitis* NAC proteins extended from one to thirteen. Remarkably, six VvNAC proteins namely, VvNAC28, VvNAC30, VvNAC31, VvNAC32, VvNAC36, and VvNAC60 hosted eight motifs each. In parallel, four proteins, specifically VvNAC09, VvNAC35, VvNAC38, and VvNAC66, showcased the presence of three motifs, while VvNAC50 featured two motifs.

Notably, motifs 1 and 7 exhibited a consistent occurrence among the VvNAC proteins. The analysis unveiled six motifs prevalent in 52.7% of the 74 VvNAC proteins, whereas seven motifs were identified in 9.4%, and five motifs presented in 20.2% of the VvNAC proteins. It's noteworthy to mention that VvNAC54 was notable for harboring the highest count of motifs, specifically thirteen, while conversely, VvNAC72 exhibited the lowest, featuring just a solitary motif (Figure 4.3-B) (Table 4.3.).

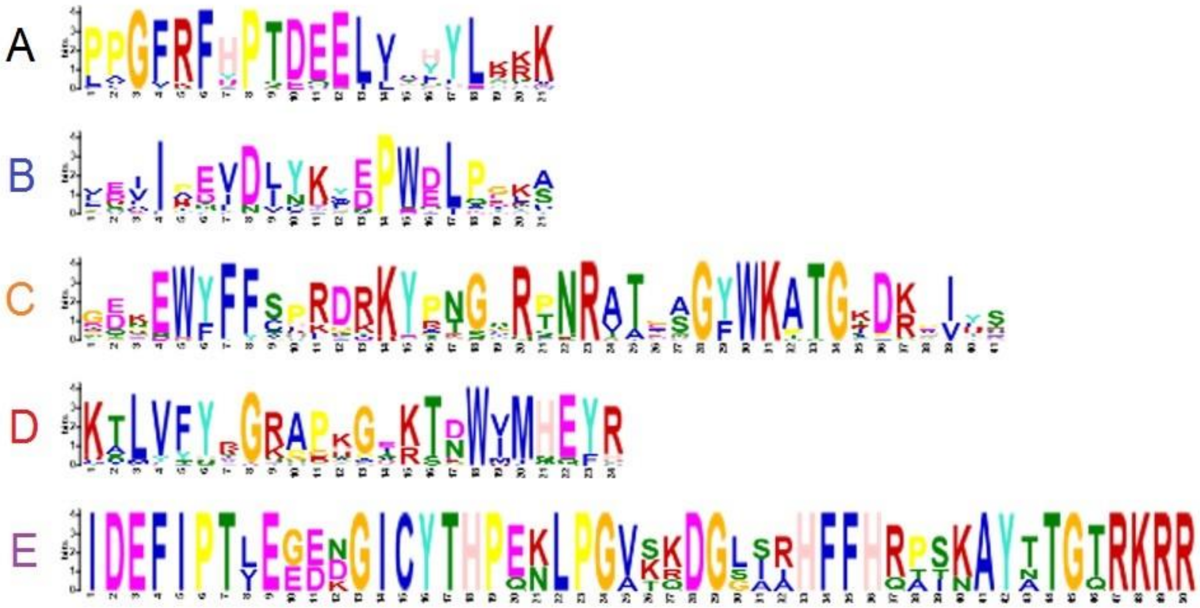

Figure 4.3 (A) Illustration of the predicted subdomains of *VvNAC* genes using MEME 5.5.2. The *VvNAC* genes contain a highly conserved DNA binding NAC domain at the N-terminal, consisting of 160 amino acid residues that are divided into five subdomains: A, B, C, D, and E.

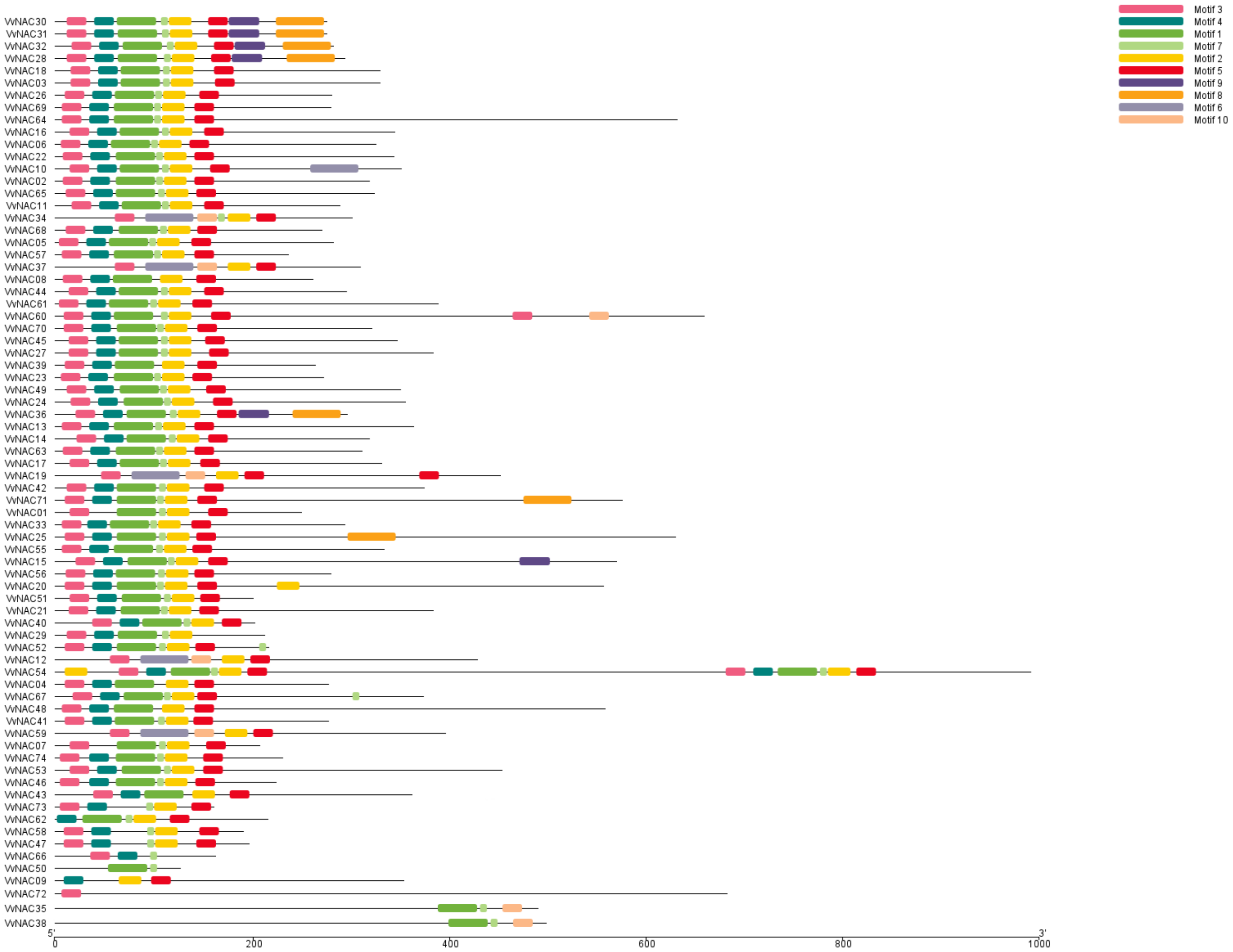

Figure 4.3 (B) Motif analysis of grape *VvNAC* genes using MEME 5.5.2. The distribution of the motifs within the *VvNAC* genes reveals that six motifs are present in 53% of the genes, while seven motifs are found in 9.4% of the genes and five motifs are present in 20.2%. Additionally, *VvNAC54* has the highest number of motifs (13), while *VvNAC72* has the lowest (1).

Table 4.3. Distribution of the identified motifs' number and subdomains of *Vitis vinifera NAC* genes via MEME 5.5.2.

Gene ID	**Number of motifs**	**Sub-domains**
VvNAC01	5	ACDE
VvNAC02	6	ABCDE
VvNAC03	6	ABCDE
VvNAC04	5	ABCDE
VvNAC05	6	ABCDE
VvNAC06	6	ABCDE
VvNAC07	5	ACDE

VvNAC08	5	ABCDE
VvNAC09	3	BDE
VvNAC10	7	ABCDE
VvNAC11	6	ABCDE
VvNAC12	5	ADE
VvNAC13	6	ABCDE
VvNAC14	6	ABCDE
VvNAC15	7	ABCDE
VvNAC16	6	ABCDE
VvNAC17	6	ABCDE
VvNAC18	6	ABCDE
VvNAC19	6	ADE
VvNAC20	7	ABCDE
VvNAC21	6	ABCDE
VvNAC22	6	ABCDE
VvNAC23	6	ABCDE
VvNAC24	6	ABCDE
VvNAC25	7	ABCDE
VvNAC26	6	ABCDE
VvNAC27	6	ABCDE
VvNAC28	8	ABCDE
VvNAC29	5	ABCDE
VvNAC30	8	ABCDE
VvNAC31	8	ABCDE
VvNAC32	8	ABCDE
VvNAC33	6	ABCDE
VvNAC34	6	ADE
VvNAC35	3	C
VvNAC36	8	ABCDE
VvNAC37	5	ADE
VvNAC38	3	C
VvNAC39	5	ABCDE

VvNAC40	6	ABCDE
VvNAC41	6	ABCDE
VvNAC42	6	ABCDE
VvNAC43	5	ABCDE
VvNAC44	6	ABCDE
VvNAC45	6	ABCDE
VvNAC46	6	ABCDE
VvNAC47	5	ABDE
VvNAC48	5	ABCDE
VvNAC49	6	ABCDE
VvNAC50	2	C
VvNAC51	6	ABCDE
VvNAC52	7	ABCDE
VvNAC53	6	ABCDE
VvNAC54	13	ABCDE
VvNAC55	6	ABCDE
VvNAC56	6	ABCDE
VvNAC57	6	ABCDE
VvNAC58	5	ABDE
VvNAC59	5	ABCDE
VvNAC60	8	ABCDE
VvNAC61	6	ABCDE
VvNAC62	5	BCDE
VvNAC63	6	ABCDE
VvNAC64	6	ABCDE
VvNAC65	6	ABCDE
VvNAC66	3	AB
VvNAC67	7	ABCDE
VvNAC68	6	ABCDE
VvNAC69	6	ABCDE
VvNAC70	6	ABCDE
VvNAC71	7	ABCDE

VvNAC72	1	A
VvNAC73	5	ABDE
VvNAC74	6	ABCDE

4.4. Protein–protein interaction of *VvNAC*

The predicted protein-protein interaction map revealed intricate interactions among proteins themselves as well as with several other proteins. To identify optimal matches, NAC protein sequences from *Vitis vinifera* L. were compared to *Arabidopsis thaliana* proteins, and the corresponding proteins were selected for subsequent network analysis.

The *VvNACs* protein interaction network was established based on *Arabidopsis* protein orthologs, and those *VvNAC* proteins exhibiting high similarity to *Arabidopsis* proteins were referred to as STRING proteins (Figures 4.4 and Appendix – Table 7.1, 7.2).

Specifically, VvNAC26 and VvNAC43 displayed homology with NAP, VvNAC67 with NTL8, VvNAC51, VvNAC52, VvNAC53, VvNAC54, VvNAC55, VvNAC69, and VvNAC71 with NTL9, VvNAC64 with NAC028, and VvNAC60 with NAC047. Notably, all of these proteins exhibited significant interactions with the NTL1 protein transporter activity, highlighting their functional relevance.

Furthermore, within a distinct cluster, VvNAC47 and VvNAC58 showed homology with XND1, VvNAC63 with VND7, VvNAC24 and VvNAC49 with NST1, VvNAC01, VvNAC07, and VvNAC73 with NAC083, and VvNAC34 and VvNAC37 with NAC073. These proteins displayed robust associations with various regulatory factors, including MYB46 (involved in regulating secondary wall biosynthesis in fibres and vessels), MYB83 (a key player in the NAC012/SND1-mediated transcriptional network governing secondary wall biosynthesis), MYB80 (critical for the timing of tapetal programmed cell death, a pivotal process in pollen development), MYB85 (a putative transcription factor), AtMYB103 (a putative MYB family transcription factor), and KNAT7 (a KNOTTED-like homeobox protein in *Arabidopsis thaliana* 7, implicated in secondary cell wall biosynthesis).

Moreover, VvNAC21 and VvNAC50 exhibited homology with NAC050, a DNA-binding transcription factor involved in multicellular organismal development and transcription regulation. These proteins demonstrated strong associations with JMJ14, a transcriptional repressor involved in DRM2-mediated maintenance of DNA methylation, as well as with AT1G72460, a leucine-rich repeat protein kinase family protein participating in the transmembrane receptor protein tyrosine kinase signalling pathway and protein amino acid phosphorylation.

Finally, VvNAC08 and VvNAC39, homologous to ATAF1, acted as pivotal genes connecting the two clusters and exhibited strong interactions with VvNAC44 (homologous to NAC032), which positively regulates age-dependent senescence, dark-induced leaf senescence, and stress-induced senescence.

Consequently, based on STRING database, the Gene Ontology (GO) predictions revealed various gene functions within the network, including the nucleus, DNA binding, organic cyclic compound binding, regulation of cellular metabolic processes, regulation of macromolecule metabolic processes, regulation of transcription (DNA-templated), regulation of nucleobase-containing compound metabolism, cell cycle, and DNA repair.

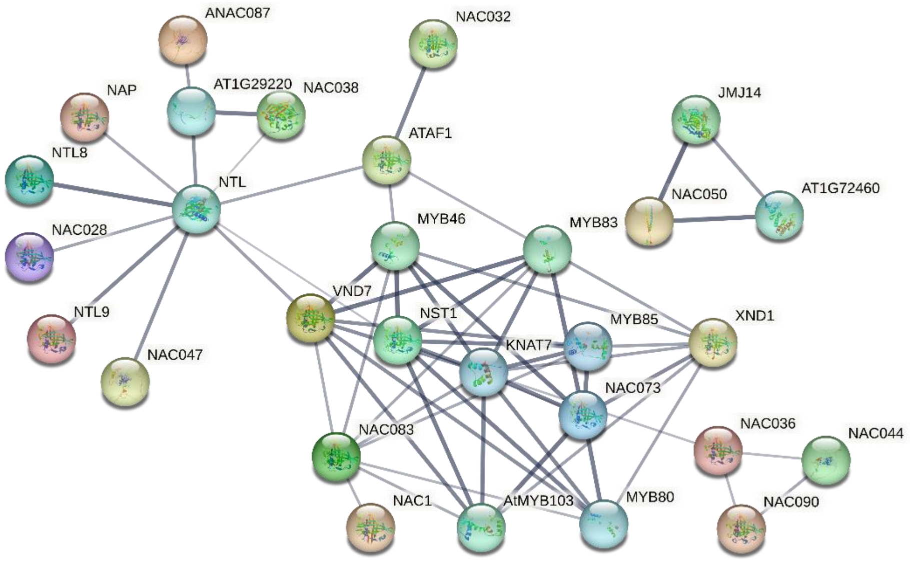

Figure 4.4 Protein–protein interaction network of *Arabidopsis NAC* homologs predicted with STRING 11.5.

4.5. Promoter regions detection and analysis of cis-acting regulatory elements

To identify cis-acting regulatory elements (CAREs) in *Vitis vinifera's NAC* genes, the promoter sequences were analyzed using the Plant Care database. Specifically, the sequences were examined up to 1500 base pairs (bp) upstream of the translation start site. CAREs are small, conserved patterns of nucleotides that are crucial components of genes, commonly found at the 5' ends.

In total, 74 *Vitis vinifera* CAREs were analyzed to explore their response to diverse stress types. The investigation revealed that the *Vitis vinifera NAC* genes contain more than 3000 CAREs. The identified CAREs include crucial elements that respond to both biotic and abiotic stressors, involve in hormonal regulation, assist in the development, and perform various functions in the promoter regions of *VvNAC* genes (Appendix - Table 7.3).

4.5.1. Stress responsive cis-acting regulatory elements

To begin, abiotic stress-related motifs, such as MYC, MYB, and MBS-MYB binding sites, LTR (low-temperature elements), STRE (stress-responsive elements), and DRE (dehydration-responsive elements), were identified. Additionally, various CAREs were discovered in response to biotic stress, including STRE, wound-responsive (WUN motif and WRE3), elicitor-responsive (W box), and TC-rich repeats (defense and stress).

Furthermore, the study demonstrated that all *Vitis NAC* promoters contained motifs specific to light stresses, such as the Sp1, AE-box, Box 4, Chs-CMA1a, GA motif, GT1 motif, GATA-motif, I-box, Box S, G box, Gap box, and Box II.

4.5.2. Cis-acting regulatory elements in hormonal regulation.

The cis-regulatory elements of the *VvNACs* were found to be involved in hormonal regulation, including multiple ligand-responsive elements such as the TGA-box (auxin responsive elements), ERE (ethylene responsive elements), GARE motifs (gibberellin responsive elements), P-box, ABRE (abscisic acid-responsive elements), AuxRR-core, TCA elements (salicylic acid-responsive elements), TATC-box, and TGA-element. These elements enhance the plants' stress resistance capabilities.

4.5.3. Role of cis-acting regulatory elements in cellular development:

Finally, while the number of motifs involved in cellular development is relatively fewer compared to other CAREs, the presence of several elements has been demonstrated. These include the CAT box (for meristem-specific activation), Motif I, GCN4 motif (for endosperm expression), AC-I and AC-II (for xylem-specific expression), O2-site (for zein metabolism regulation), F-box (for reproductive and vegetative development and growth, as well as cell defense and death), ARE (for anaerobic induction), the circadian motif, HD-Zip1 (for leaf development), AP-1 (for inducible flowering), and MBSI (for flavonoid biosynthesis gene regulation).

Given that most *VvNACs* exhibit three distinct types of stress-responsive CAREs, it is plausible to assume that these genes play a crucial role in enhancing tolerance to various biotic and abiotic stresses. Overall, these findings suggest that *Vitis vinifera's NAC* genes contain a diverse range of CAREs that respond to various stressors and are involved in hormonal regulation and cellular development.

4.5.4. In silico analysis of VvNAC gene expression

<u>Biotic expression</u>

The transcription profiling by high throughput sequencing of grapevine *NAC* genes at 3 different stages (early S1, middle S2, and late S3) of noble rot caused by *Botrytis cinerea* was extracted from the Expression Atlas database, as referenced in (Appendix – Table 7.4).

The analysis of the microarray data set showed that several *VvNAC* genes, including *VvNAC39*, *VvNAC44* and *VvNAC73* were highly regulated during stages S2 and S3. Additionally, a slight up-regulation was detected in *VvNAC08*, *VvNAC30*, *VvNAC31*, *VvNAC36*, *VvNAC41*, *VvNAC60*, *VvNAC61* and *VvNAC74.* Conversely, there was a significant down-regulation in *VvNAC13*, *VvNAC51*, *VvNAC52*, *VvNAC53* and *VvNAC54* (Figure 4.5).

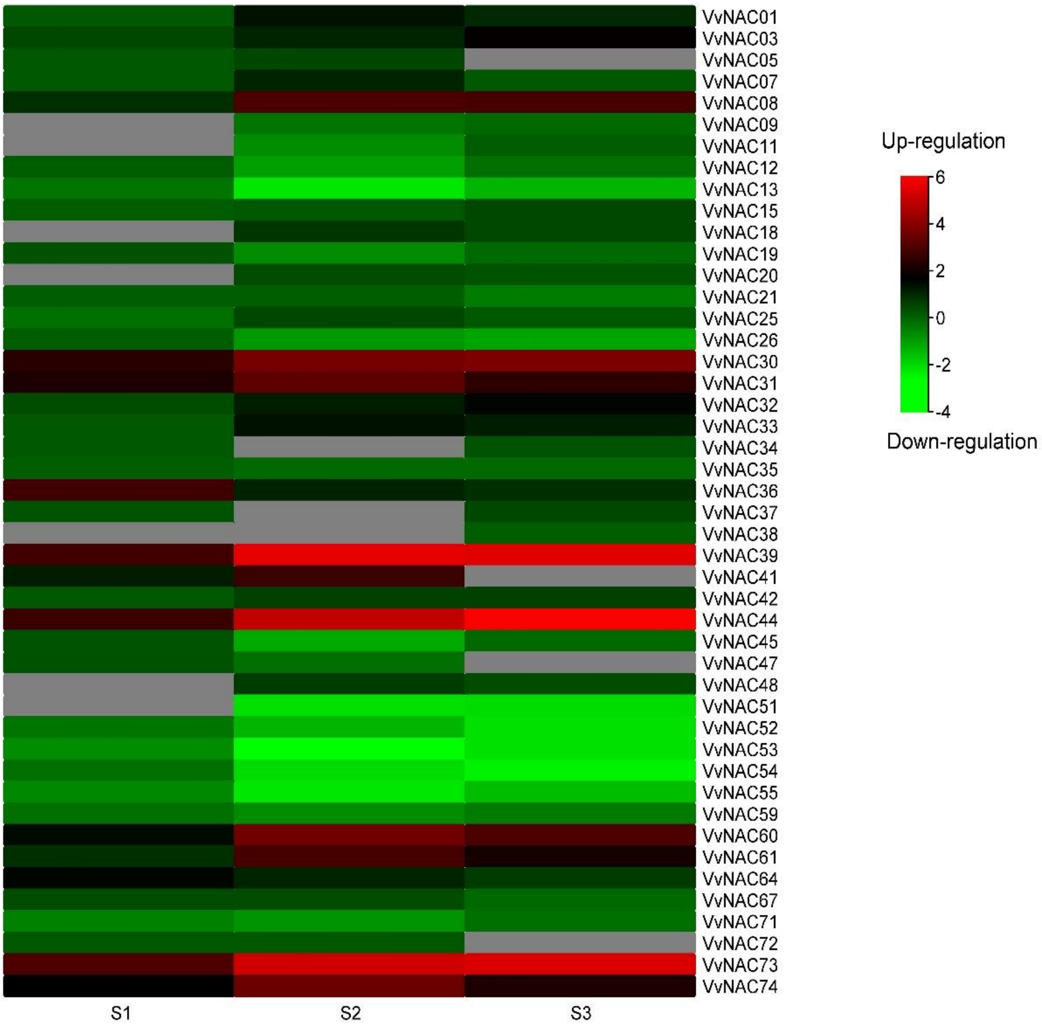

Figure 4.5 Heat map of the microarray data representing the expression analysis of *VvNAC* genes in response to *Botrytis cinerea*. Based on Expression Atlases at three different stages of Noble Rot infection in ripe grapes; early (S1), middle (S2) and late (S3). The expression patterns of *VvNAC* genes were analyzed using the offline program TBtools.

4.5.5. Subcellular localization prediction

The subcellular localization of NAC proteins in *Vitis vinifera* L. was analyzed using the CELLO v.2.5 web database tool. Results revealed that most proteins (73%) were localized in the nucleus, while eleven proteins, namely *VvNAC08, VvNAC15, VvNAC20, VvNAC26, VvNAC30, VvNAC31, VvNAC32, VvNAC36, VvNAC37, VvNAC38,* and *VvNAC64,* were present in both the cytoplasm and nucleus.

Interestingly, *VvNAC72* was highly predicted to be located in the plastid. Moreover, *VvNAC39* and *VvNAC40* were linked to mitochondria, nuclei, and the cytoplasm, while *VvNAC05* was found in the nucleus and mitochondria. In addition, *VvNAC50* was located in the mitochondria and extracellular matrix, while *VvNAC73* was distributed in the cytoplasm and mitochondria. Furthermore, *VvNAC35* and *VvNAC51* were found in the cytoplasm, and *VvNAC52* was located in the cytoplasm and extracellular matrix, as illustrated in Figure 4.6.

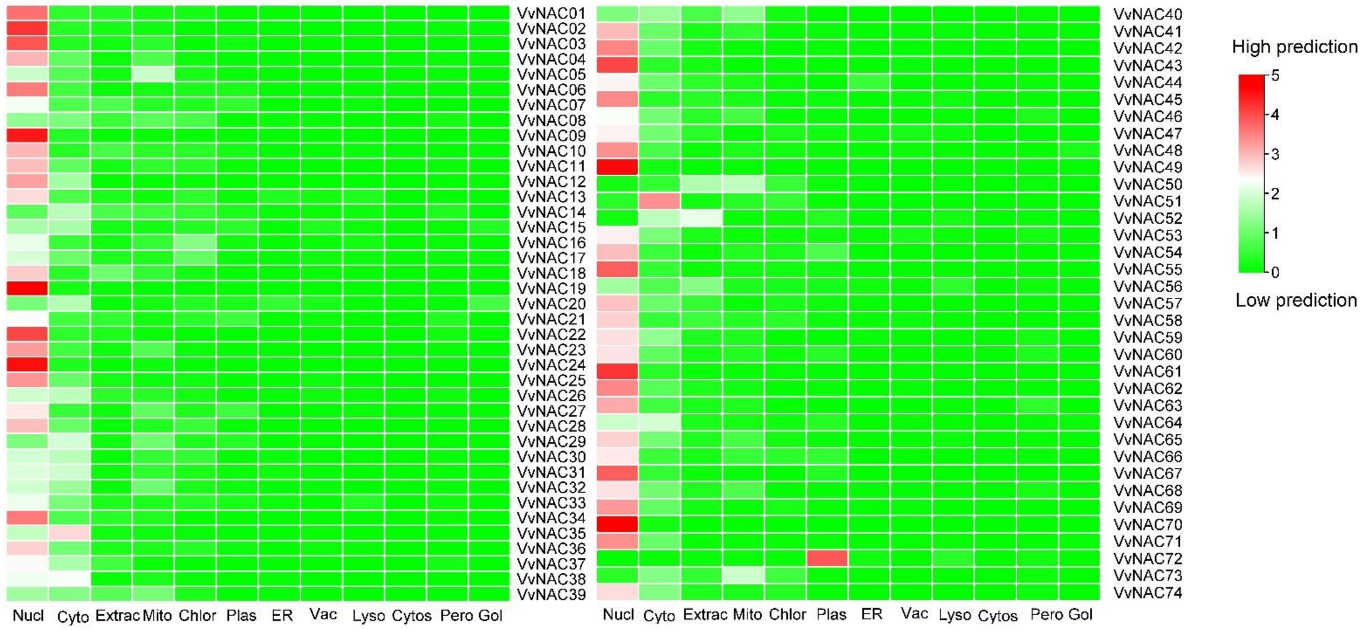

Figure 4.6 Heat map of the subcellular-localization prediction of *VvNAC* genes through web database tool CELLO Life v.2.5 in different organelles. The results were analyzed using the offline program TBtools: Nucl (nucleus), cyto (cytoplasmic), extrac (extracellular matrix), mito (mitochondrial), chlor (chloroplast), plas (plastid), ER (endoplasmic reticulum), vac (vacuolar), lyso (lysosome), cytos (cytosol), pero (peroxisome), gol (Golgi apparatus).

4.5.6. Statistical Analysis

Through hierarchical clustering a statistical analysis for gene expression data was generated, by creating a tree-like structure, known as a dendrogram, the relationships between genes were visually represented.

The dendrogram formed 4 clusters of the *VvNAC* genes with similar expression profiles were grouped together in branches. Cluster 1 and cluster 2 contain low expressed genes while cluster 3 and 4 contain the high expressed genes as shown in Figure 4.7.

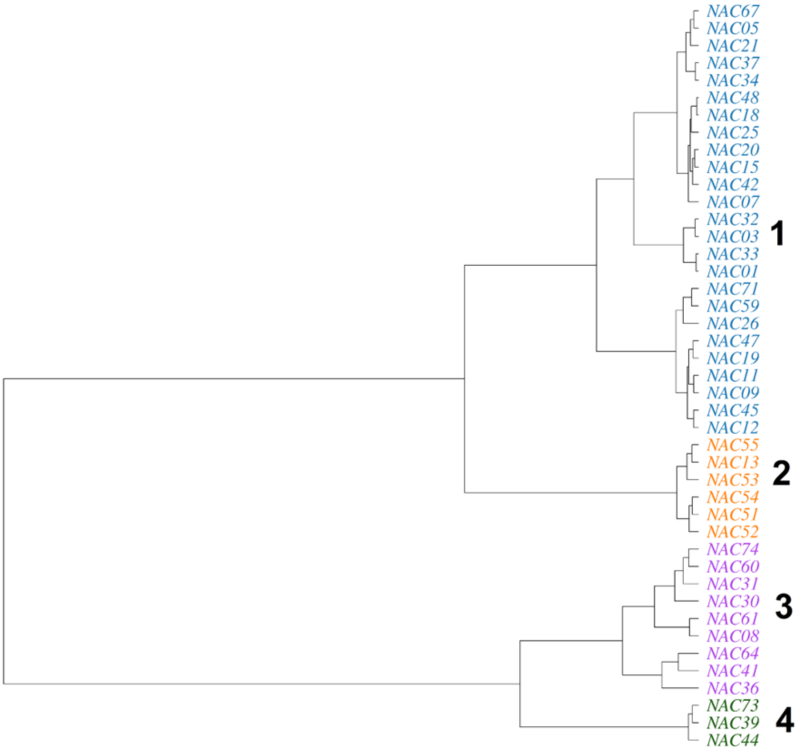

Figure 4.7 The dendrogram of the *VvNAC* genes based on their expression profiles.

The Euclidean distance were used to measure the distance between the clusters to determine their similarities in a quantitative manner based on the Euclidean formula:

$$d_{Uv} = \sqrt{(u_1 - v_1)^2 + (U_2 - v_2)^2 + (U_3 - v_3)^2}$$

As a result, showed in Appendix- Table 7.5, the distance between the clusters 2 & 3 based on the single linkage was 4.2; the distance between the closest pair *VvNAC64&VvNAC52*.

The distance between the clusters 1 & 4 based on the single linkage was: 6; the distance between *VvNAC03&VvNAC73*.

The distance between the clusters 2 & 4 based on the single linkage was: 10.5; the distance between *VvNAC52&VvNAC73*.

While the distance between the clusters 3 & 4 based on the single linkage was: 2.2; the distance between *VvNAC30&VvNAC73*.

Therefore, the proximity of clusters 3 and 4 in terms of gene expression suggests that they may share similar regulatory mechanisms or pathways in response to *Botrytis cinerea*. Genes in these clusters may be involved in common biological processes. While the larger distance between clusters 1&4, 2&3 and 2&4 indicates dissimilarity and the genes in these clusters have distinct expression patterns. This could be due to a different regulatory mechanism or involvement in different biological processes.

4.5.7. In vitro verifications: VvNAC36 model

In *Vitis vinifera* L. a recent study has proved a remarkable regulation of the *VvNAC36* gene in *A. thaliana* in response to powdery mildew colonization. The up-regulation of *VvNAC36* 1.5-fold in the case of powdery mildew (PM) while no response was detected for salicylic acid (SA), has demonstrated the role of *VvNAC36* in response to biotic stress (Tóth et al., 2016).

Transient expression

Continuing Tóth's work, a simple and rapid transient transformation in tobacco leaf was performed to determine the localization of *VvNAC36* protein that might help in better understanding its role in plants.

In plants, yeast, and mammals, the GFP tag is widely used to mark proteins for localization studies. Confocal microscope was used to image GFP fusion proteins that were transiently expressed in leaves. The use of a constitutive cauliflower mosaic virus 35S promoter (CaMV 35S) enabled GFP to be seen in leaf tissues that normally have a lot of autofluorescence. The fusion proteins *VvNAC36::sGFP* and the control *35S::sGFP* were agroinfiltrated into leaves of 3 to 5-week-old *N. benthamiana* plants.

Our investigation of the subcellular localization of the *VvNAC36*::sGFP fusion protein in tobacco epidermal cells indicated that the protein was mainly present in the cytoplasmic area (Figure 4.8; A, B and C).

Furthermore, our analysis suggested that the *VvNAC36* protein might also be present in various organelles, as GFP was detected in different shapes (Figure 4.8; D, E and F). However, this observation could not be confirmed conclusively due to the limited availability of advanced biotechnology methods.

Finally, for the positive control 35S::sGFP, the detection of the green fluorescent protein showed an accumulation in both the cytoplasm and nucleus of the tobacco leaf epidermal cells (Figure 4.8; G, H and I).

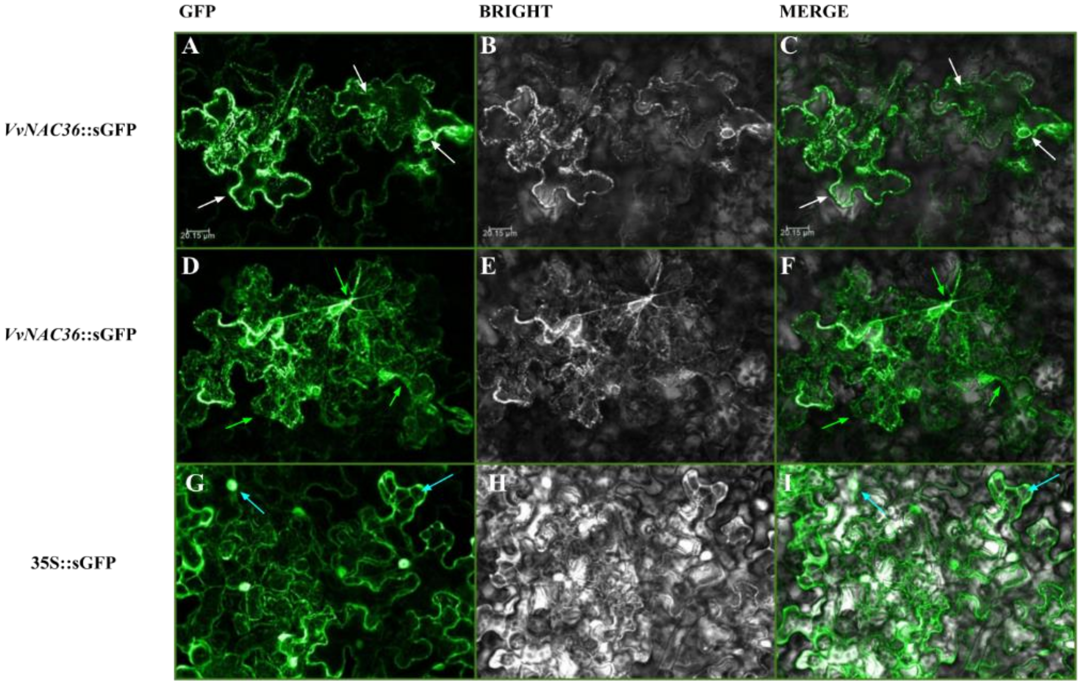

Figure 4.8 Subcellular localization of *VvNAC36* and the control in transiently transformed leaves of *Nicotiana benthamiana* visualized by using confocal microscope, Bars = 20.15 µm. The GFP fluorescence pattern of the fusion protein *VvNAC36*::sGFP was detected in the cytoplasmic area as demonstrated by white arrows in images A, B, and C. Images D, E, and F further depict the visualization of *VvNAC36*::sGFP expression in other regions as indicated by green arrows. For the positive control 35S::sGFP (GFP alone), the GFP fluorescence was observed in the cytoplasm and nucleus, as indicated by blue arrows in images G, H, and I.

5. DISCUSSION AND LIMITATIONS

Botrytis cinerea is a dangerous plant pathogenic fungus with wide host ranges. This aggressive pathogen uses multiple weapons to invade and cause serious damages to the plants, such as grape (*Vitis vinifera* L.); a member of the *Vitaceae* family.

Being one of the largest among plant-specific TFs, the NAC protein family has a role in plant development, abiotic stress and defense responses. In many plant species NAC proteins have been functionally characterized, including e.g. *Vitis vinifera* L., *Arabidopsis thaliana, Oryza sativa, Zea mays, Triticum aestivum, Glycine max, Populus trichocarpa* (Fang et al., 2008; Pinheiro et al., 2009). However, the functions for majority of the *NAC* genes in grape remain unknown.

The current study employed an *in silico* approach to conduct a comprehensive genome wide detection of *NAC* domain transcription factors (TFs) within the *Vitis vinifera* L. genome, aiming to identify and characterize potential NAC proteins that exhibit resistance to *B. cinerea*. Furthermore, to validate the computational findings, an *in vitro* approach was implemented specifically for the *VvNAC36* gene through a transient expression assay.

In this study, a total of 74 *NAC* genes were analyzed from *Vitis vinifera* L.. The identified *NAC* genes varied greatly in protein length from 128 AA (*VvNAC50*) to 994 AA (*VvNAC54*).

Regarding subcellular localization prediction, it was discovered that 54 out of the 74 identified *NAC* genes in *Vitis* (accounting for 73% of the total) were potentially localized within the nucleus. In contrast, the remaining *NAC* genes displayed extracellular localization or were distributed across various organelles. Usually, transcription factors need to be localized to nucleus to execute their function, either independently or by interacting with other partners.

For instance, *ATAF1* is localized to nucleus (Lu et al., 2007), whereas *ONAC020* and *ONAC02*3 completely gets localize to nucleus after interacting with *ONAC26* (Mathew et al., 2016). Similarly, *NTL4* is targeted to the nucleus only upon heat stress after processing (Lee et al., 2014). There are numerous reports which shows the localization of *NAC* genes in different organelles other than nucleus, as in the case of *ONAC023,* which is localized to the cytoplasm (Mathew et al., 2016). Sometimes, TFs gets localized to nucleus after splicing of membrane bound TFs or upon proteolytic cleavage (Ng et al., 2013; Seo, 2014).

Furthermore, analyzing nucleotide substitution rates (Ka and Ks) provides valuable insights into evolutionary processes, genetic diversity, disease mechanisms, and the functional aspects of genomes. The analysis conducted in this study implies the absence of positive selection in any of the tandem repeat sequences, which are typically associated with facilitating adaptive genetic variation and potentially influencing species evolution.

However, all genes displayed Ka/Ks values below 1, indicating the prevalence of negative selection during the course of evolution. This negative selection process serves to reduce the rate of changes in the amino acid profile. In summary, the findings indicate that the majority of *VvNAC* genes exhibit a slow evolutionary rate, characterized by the absence of positive selection.

Thus, this might suggest that these *VvNAC* genes are performing important functions that haven't required rapid changes to their genetic makeup due to advantageous variations. This information can be valuable for understanding the genetic stability and adaptability of the plant species in question.

Additionally, the presence of diverse conserved motifs implies functional divergence among these VvNAC proteins. This divergence underscores their involvement in various critical processes, including meristem development, root development, flowering induction, embryo development, vascular-specific expression, hormone signaling, abiotic stress responses, and defense mechanisms.

The exploration of critical cis-acting regulatory elements (CAREs) highlighted their significance in governing responses to both abiotic and biotic stresses. Among these CAREs, notable examples include 228 Abscisic Acid Response Elements (ABRE), 145 stress-responsive elements (STRE), 17 Dehydration-Responsive Elements (DRE-core), 58 elicitor-responsive motifs (W-box), 78 wound-responsive elements (WRE3 and WUN motifs), and 21 TC-rich repeats, as shown in Figure 5.9.

Besides these, several other promoter elements were also identified which have a role in various plant development processes. CAREs are necessary for stress-responsive transcriptional regulation (Mundy et al., 1990). The existence of different cis-acting regulatory elements indicates the transcription of several stress responsive genes via a variety of TFs. Moreover, the significance of the association between CAREs has already been documented for stress-responsive transcription (Narusaka et al., 2003).

In several reports, various cis-motifs as DNA-binding sites for the NAC TFs have been identified, which include NACRS (NAC-recognition sequence for drought response) (Tran et al., 2004), IDE2 motif (iron deficiency-responsive) (Ogo et al., 2008), SNBE (secondary wall NAC binding element) (Zhong et al., 2007), and calmodulin-binding (CBNAC) (Kim et al., 2007).

Moreover, the *VvNACs* protein-protein interaction network based on their homologous proteins from *Arabidopsis* provides a comprehensive and efficient way to explore molecular interactions, functional annotations, and potential roles of genes within biological systems.

Consequently, as indicated in Figure 4.4, this study's results illuminated robust homology and strong connections between *VvNAC* proteins and established *Arabidopsis* counterparts, including *NAC083, ATAF1, NAC073, NAC050, NAC028, NAC047, NAP, NTL9, NTL8, MYB83, MYB80, XND1, VND7, NST1,* and *NTL1.*

This observation hints at analogous functionalities of VvNAC proteins. Particularly noteworthy was the remarkable homology shared among *VvNAC08*, *VvNAC39*, and *ATAF1*. This is exemplified by prior studies indicating that *ATAF1* plays a pivotal role as a transcriptional activator in response to salt stress, abscisic acid (ABA), and biotic stress (Wu et al., 2009; Liu et al., 2016).

Next, the expression profiles of *VvNAC* genes unveiled up-regulation in response to *B. cinerea* infection for specific genes, including *VvNAC08, VvNAC30, VvNAC31, VvNAC36, VvNAC39, VvNAC41, VvNAC44, VvNAC60, VvNAC61, VvNAC73* and *VvNAC74.*

These genes were found to be enriched with biotic stress-related motifs, thus highlighting their potential involvement in biotic stress responses (Figure 5.10). As the photo showed, *VvNAC39* and *VvNAC44* with TC-rich repeats, STRE and W box; *VvNAC73* with W box and STRE; *VvNAC08* and *VvNAC30* with WRE3, STRE and W box; *VvNAC60* with STRE and WUN-motif; *VvNAC31* with TC-rich repeats and W box; *VvNAC41* with STRE, W box and WUN-motif; finally *VvNAC61* and *VvNAC74* with STRE and WRE3.

Thus, different novel *VvNAC genes* that respond to *B. cinerea* infection were detected using a combination of cis-element and microarray data analysis. As a result, these genes are excellent resources for *B. cinerea* stress in grapes in genetic engineering and molecular breeding.

It is important to mention here that the present study provides very detailed analysis of the identified *Vitis vinifera NAC* genes and their cis-regulatory elements as compared to the previous reports by (Wang et al. 2013; Le Henaff et al. 2013), respectively. We have carried out strict promoter motif analysis, protein-protein interactions, evolutionary rate analysis, and subcellular localization of the stress-related NAC proteins which lacked in previous reports and therefore, provides much deeper understanding of mechanisms involved in stresses and especially in biotic stress tolerance in *Vitis vinifera* L.

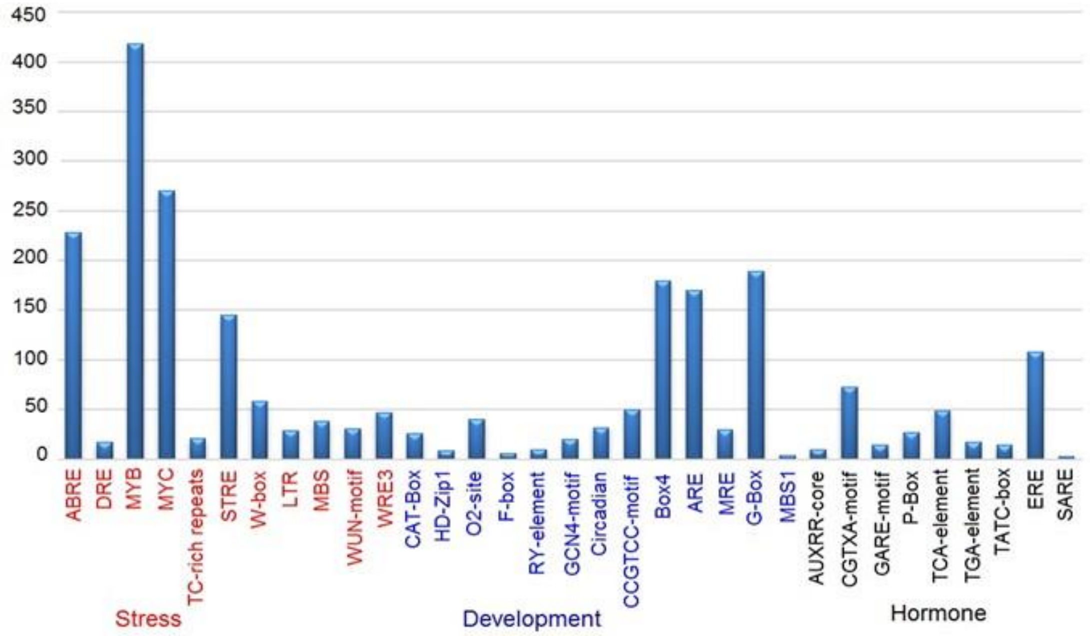

Figure 5.9 Various cis-acting regulatory elements related to stress, hormone and development process in *VvNAC* genes were identified using Plant Care database. The results were analyzed using the offline program TBtools.Among the identified CAREs: 228 Abscisic acid Response Elements (ABREs), 145 stress-responsive elements (STREs), 17 dehydration-responsive elements (DRE-core), 58 elicitor-responsive elements (W box), 78 wound-responsive elements (WRE3 and WUN motifs), and 21 TC-rich repeats. ABRE, DRE, MYB, MYC, TC-rich repeats, STRE, W Box, LTR, MBS, WUN-motif and WRE3 are related to stress; CAT-Box, HD-Zip1, O2 site, F-Box, RY-element, GCN4-motif, Circadian, CCGTCC-motif, Box 4, ARE MRE, G-Box and MbS1 are related to development; AUXRR-core, CGTXA-motif. GARE-motif, P-Box, TCA- element, TGA-element, TATC-Box, ERE and SARE are related to hormone.

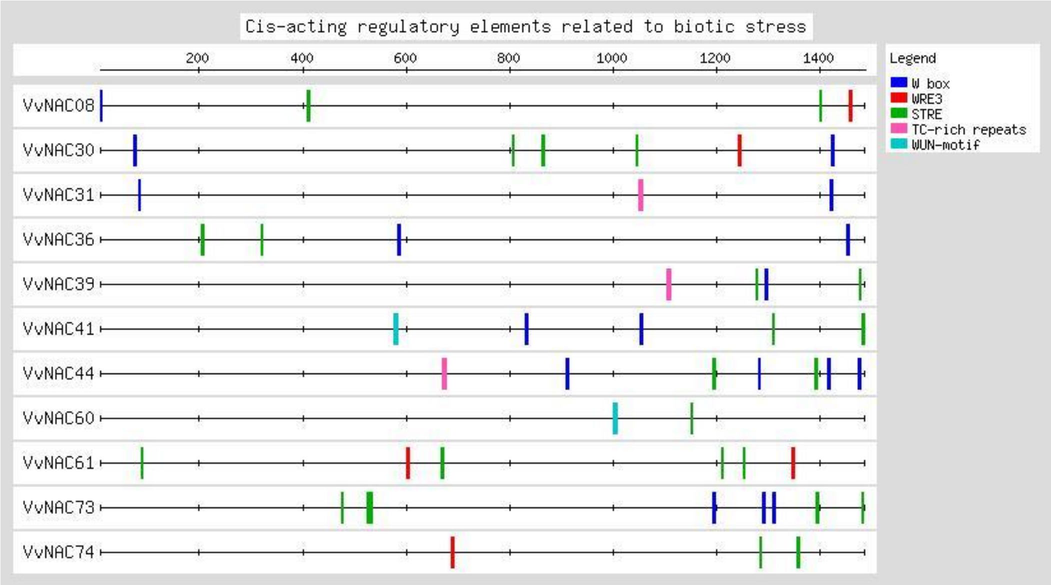

Figure 5.10 The predicted stress responsive *VvNAC* genes with their CAREs related to biotic stress. The results were illustrated using the offline program TBtools. Each gene contains at least two or more CAREs that respond to biotic stress.

5.1. *VvNAC36* protein

Based on CELLO Life, the localization of *VvNAC36* protein is more likely to be in the nucleus and cytoplasm with a percentage of 54.4% and 21.1% respectively.

In order to explain the *in silico* result, a transient expression has been done for *VvNAC36* gene. Thus, as a result in the case of the *VvNAC36* protein, the GFP fluorescence presented in the cytoplasmic area, as shown in the Figure 4.8., while, a localization in the nucleus and the cytoplasmic area showed in the case of the control.

Consequently, comparing to the size of the nucleus of the contol, we confirm the absence of any nuclear localization for the gene *VvNAC36* and just the cytoplasmic one was approved. Despite the good results, but future studies should use more advanced techniques to deeply investigate and confirm the specific localization of this gene.

The expression analysis based on microarray data available has showed a slight up-regulation just in the early stage of infection of the ripe grape (S1), this might be explained by the presence of different CAREs responsible for biotic stress in the promoter of *VvNAC36* such as; STRE and W box as showed in the photo Figure 5.10.

The computational results showed a weak resistance to *B. cinerea* that occurred just in S1 and did not present in the middle or late stages of the infection. Thus, a stable transformation must be done in the future in order to check how *VvNAC36* might respond to *B. cinerea* in different stages.

5.2. Study limitations

The COVID-19 pandemic in 2020 posed significant challenges, hindering my ability to carry out experiments like stable transformation due to lockdown restrictions. In response, I turned to an *in silico* research approach for my theory-focused Ph.D. dissertation. This choice was driven by its efficiency, flexibility, and its potential to offer fresh theoretical insights into complex biological processes. Importantly, it also aligns with ethical research practices and proves cost-effective, making it a practical and responsible solution to the limitations imposed by the pandemic.

6. CONCLUSIONS AND RECOMMENDATIONS

- This study aimed to highlight and interpret the functions of *VvNAC* genes in *Vitis vinifera* L., beyond their known roles in development and stress responses, using both *in vitro* and *in silico* approaches.

- Based on the grapevine genomic databases, we have characterized 74 *VvNAC* genes.

- As a result, the analyzing of the protein's lengths and number of motifs is just one aspect of a gene's potential to respond to biotic stress and it should not be the sole determinant of a gene's function.

- For example: the longest gene *VvNAC54* with a higher number of motifs (13) showed down-regulation in response to *B. cinerea*, while one of the shortest genes, the *VvNAC73* with 5 motifs showed high expression to the disease.

- While it's logical to assume that longer length and more motifs might make a gene more effective in responding to stress, several factors can influence the outcomes, as we have observed in the case of *VvNAC54* and *VvNAC73.*

- Continuing, with the Ka/Ks value, all genes displayed Ka/Ks values below 1. This suggests that the genes in this tandem repeat sequence are likely under strong purifying selection, and they are likely performing important functions without substantial genetic modifications.

- Moreover, in the subcellular localization analysis, it is noteworthy to mention *VvNAC72.* A gene with a specialty to be in the plastid, makes us curious and eager to investigate further.

- In addition, protein-protein interactions analysis is important because understanding these relationships, such in the case of *VvNAC08, VvNAC39* and *VvNAC44* can be valuable in biotechnological applications, such as crop improvement. It may lead to the development of strategies to enhance stress resistance or other desirable traits in grapevines based on the knowledge from *Arabidopsis thaliana.*

- The CAREs in the up-regulated genes promoters might not be unique just to *Vitis vinifera NAC genes* but they represent conserved motifs with potential involvement in biotic stress response.

- Based on the expression profiles analysis, we predicted 10 *VvNAC* genes for *B .cinerea* tolerance. Followed by a statistical analysis through hierarchical clustering for the gene's expression.

- Additional experimental validation, such as quantitative real-time PCR (qRT-PCR) analysis, is required to verify the expression pattern of the predicted *VvNACs* in response to *B. cinerea.*

- In the case of the *in vitro* experiment related to *VvNAC36*, the results of the transient expression encountered with the computational tools' results.

- The stable transformation must be done, and *in vitro* experiments should be performed to the predicted *VvNAC* genes and not just for *VvNAC36.*

- Finally, this study has increased our knowledge of grape *NAC* genes and provided insight into their functions in case of the up-regulated genes.

- Furthermore, our findings have built a robust framework for researchers to select candidates to engineer grape cultivars for enhanced tolerance against biotic stress.

6.1. New scientific results

1. An *in silico* approach was applied for detailed functional characterization of *VvNAC* transcription factor genes in response to *B. cinerea.*
2. We predicted 10 *VvNAC* genes (*VvNAC08, VvNAC30, VvNAC31, VvNAC36, VvNAC39, VvNAC41, VvNAC44, VvNAC61, VvNAC73*, and *VvNAC74*) for *B. cinerea* tolerance, that could be useful as reference for researchers in future grape breeding programs.
3. We have determined through a transient expression the subcellular localization of the *VvNAC36,* in the cytoplasmic area.
4. The feasibility study of *Nicotiana bentamiana* L. in transient transformation with the *VvNAC36* and the correlation between *in silico* and *in vitro* approaches were portrayed.

7. APPENDIXES

A1: Bibliography

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A2: Supplementary data

Table 7.1. Characterization and distribution of the *VvNAC* genes.

Species	**Gene ID**	**Length**	**Transcript Name**	**MW (Da)**	**IP**	**Orthologues in *A. thaliana***
Grape	*VvNAC01*	251	**VIT_01s0146g00280**	28601.2	9.6551	*ANAC083 (AT5G13180.1)*
Grape	*VvNAC02*	320	**VIT_02s0154g00020**	37460.2	7.8983	*ANAC007 (AT1G12260.1)*
Grape	*VvNAC03*	331	**VIT_00s0375g00040**	36744.2	9.7936	*ANAC025 (AT1G61110.1)*
Grape	*VvNAC04*	279	**VIT_04s0008g00150**	32252.8	7.6617	*ANAC036 (AT2G17040.1)*
Grape	*VvNAC05*	284	**VIT_17s0000g06400**	32180.8	10.2287	*ANAC100 (AT5G61430.1)*
Grape	*VvNAC06*	327	**VIT_17s0000g03660**	37083.7	8.4093	*ANAC058 (AT3G18400.1)*
Grape	*VvNAC07*	209	**VIT_17s0000g00770**	23960.8	8.0627	*ANAC083 (AT5G13180.1)*
Grape	*VvNAC08*	263	**VIT_18s0001g02300**	30130.2	7.9471	*ANAC002(AT1G01720.1)*
Grape	*VvNAC09*	355	**VIT_18s0001g11980**	38637	5.4619	*ANAC057(AT3G17730.1)*
Grape	*VvNAC10*	353	**VIT_14s0108g00860**	39332.4	8.2468	*ANAC094(AT5G39820.1)*
Grape	*VvNAC11*	290	**VIT_14s0108g01070**	32887.3	7.8281	*ANAC100(AT5G61430.1)*
Grape	*VvNAC12*	430	**VIT_01s0011g02990**	48383.6	4.7046	*ANAC008(AT1G25580.1)*
Grape	*VvNAC13*	365	**VIT_02s0012g01040**	41109.5	4.5281	*ANAC071(AT4G17980.1)*
Grape	*VvNAC14*	320	**VIT_18s0122g00800**	36190	6.6689	*ANAC031 (AT1G76420.1)*
Grape	*VvNAC15*	572	**VIT_18s0001g01820**	63327.9	4.4857	*ANAC017 (AT1G34190.1)*
Grape	*VvNAC16*	346	**VIT_19s0014g02200**	38342.9	8.002	*ANAC098 (AT5G53950.1)*
Grape	*VvNAC17*	333	**VIT_19s0014g03290**	37190.8	8.8885	*ANAC072 (AT4G27410.2)*
Grape	*VvNAC18*	331	**VIT_19s0014g03300**	36791.5	8.888	*ANAC056 (AT3G15510.1)*
Grape	*VvNAC19*	454	**VIT_11s0016g02880**	51170.8	6.8955	*ANAC075 (AT4G29230.1)*
Grape	*VvNAC20*	559	**VIT_13s0019g05240**	62299.2	4.2277	*ANAC078 (AT5G04410.1)*
Grape	*VvNAC21*	385	**VIT_13s0019g05230**	43682.5	7.6375	*ANAC050 (AT3G10480.1)*
Grape	*VvNAC22*	345	**VIT_16s0022g00690**	40174.2	6.8679	*ANAC007 (AT1G12260.1)*
Grape	*VvNAC23*	274	**VIT_04s0023g03110**	32148.3	9.4087	*ANAC037 (AT2G18060.1)*
Grape	*VvNAC72*	659	**VIT_02s0025g01870**	74672.92	7.26	*ANAC083 (AT5G13180.1)*
Grape	*VvNAC24*	357	**VIT_02s0025g02710**	40855.6	7.1335	*ANAC043 (AT2G46770.1)*
Grape	*VvNAC25*	632	**VIT_02s0025g03020**	70493.1	4.811	*ANAC014 (AT1G33060.1)*
Grape	*VvNAC26*	282	**VIT_01s0026g02710**	32458.7	8.0663	*ANAC029 (AT1G69490.1)*
Grape	*VvNAC27*	385	**VIT_01s0026g02360**	43110.1	8.4525	*ANAC009 (AT1G26870.1)*
Grape	*VvNAC28*	295	**VIT_19s0027g00910**	33681	8.1248	*ANAC042 (AT2G43000.1)*
Grape	*VvNAC29*	214	**VIT_19s0027g00880**	25648.6	10.3268	*ANAC042 (AT2G43000.1)*
Grape	*VvNAC30*	277	**VIT_19s0027g00870**	32287.3	7.8995	*ANAC042 (AT2G43000.1)*
Grape	*VvNAC31*	277	**VIT_19s0027g00860**	32731.8	7.9703	*ANAC042 (AT2G43000.1)*
Grape	*VvNAC32*	284	**VIT_19s0027g00840**	32932	7.53	*ANAC042 (AT2G43000.1)*
Grape	*VvNAC33*	295	**VIT_19s0027g00230**	33364.1	6.767	*ANAC022 (AT1G56010.2)*
Grape	*VvNAC34*	303	**VIT_12s0028g03050**	34457.8	8.6499	*ANAC073 (AT4G28500.1)*
Grape	*VvNAC36*	298	**VIT_12s0028g00860**	34198.3	8.4111	*ANAC042 (AT2G43000.1)*
Grape	*VvNAC35*	480	**VIT_12s0028g02670**	53469.5	5.12	*ANAC062 (AT3G49530.1)*
Grape	*VvNAC37*	311	**VIT_10s0003g00350**	35466.8	6.8317	*ANAC073 (AT4G28500.1)*
Grape	*VvNAC38*	484	**VIT_10s0003g00500**	53537.14	5.58	*ANAC028 (AT1G65910.1)*
Grape	*VvNAC39*	265	**VIT_07s0031g02610**	30286.2	9.1243	*ANAC002 (AT1G01720.1)*

Grape	*VvNAC40*	204	**VIT_12s0035g02020**	24138.6	10.067	*ANAC034 (AT2G02450.1)*
Grape	*VvNAC41*	279	**VIT_03s0038g03410**	31946.5	8.5729	*ANAC036 (AT2G17040.1)*
Grape	*VvNAC42*	376	**VIT_06s0004g03080**	42958.1	8.8622	*ANAC025 (AT1G61110.1)*
Grape	*VvNAC43*	364	**VIT_06s0004g02340**	41280.7	4.9285	*ANAC029 (AT1G69490.1)*
Grape	*VvNAC44*	297	**VIT_06s0004g00020**	34433.6	5.8409	*ANAC032 (AT1G77450.1)*
Grape	*VvNAC45*	349	**VIT_08s0040g02110**	40037	8.5158	*ANAC025 (AT1G61110.1)*
Grape	*VvNAC46*	225	**VIT_18s0041g00700**	26238.6	8.9381	*ANAC090 (AT5G22380.1)*
Grape	*VvNAC47*	198	**VIT_04s0044g01500**	22707.4	4.1292	*ANAC104 (AT5G64530.1)*
Grape	*VvNAC48*	560	**VIT_04s0044g01220**	62640.2	4.8558	*ANAC103 (AT5G64060.1)*
Grape	*VvNAC49*	352	**VIT_15s0048g02660**	40519.1	7.4725	*ANAC043 (AT2G46770.1)*
Grape	*VvNAC50*	128	**VIT_15s0048g02340**	14764	10.0079	*ANAC050 (AT3G10480.1)*
Grape	*VvNAC51*	202	**VIT_15s0048g02320**	22668.8	6.3591	*ANAC116 (AT4G35580.1)*
Grape	*VvNAC52*	218	**VIT_15s0048g02310**	24590.8	6.9826	*ANAC116 (AT4G35580.1)*
Grape	*VvNAC53*	455	**VIT_15s0048g02300**	51587.7	5.3227	*ANAC116 (AT4G35580.1)*
Grape	*VvNAC54*	994	**VIT_15s0048g02280**	110673	7.8715	*ANAC116 (AT4G35580.2)*
Grape	*VvNAC55*	335	**VIT_15s0048g02270**	37092.7	7.2815	*ANAC116 (AT4G35580.1)*
Grape	*VvNAC56*	281	**VIT_07s0005g03610**	31942	6.5797	*ANAC074 (AT4G28530.1)*
Grape	*VvNAC57*	238	**VIT_17s0053g00740**	27340.6	4.622	*ANAC057 (AT3G17730.1)*
Grape	*VvNAC58*	192	**VIT_12s0055g00510**	21978.4	4.9228	*ANAC104 (AT5G64530.1)*
Grape	*VvNAC59*	398	**VIT_14s0066g00360**	44158.1	6.2255	*ANAC044 (AT3G01600.1)*
Grape	*VvNAC60*	661	**VIT_08s0007g07670**	74009	7.0075	*ANAC047 (AT3G04070.1)*
Grape	*VvNAC61*	390	**VIT_08s0007g07640**	44064.2	8.248	*ANAC087 (AT5G18270.1)*
Grape	*VvNAC62*	217	**VIT_08s0007g02940**	24445.9	8.098	*ANAC090 (AT5G22380.1)*
Grape	*VvNAC63*	313	**VIT_18s0072g01060**	36310.7	6.3205	*ANAC030 (AT1G71930.1)*
Grape	*VvNAC64*	634	**VIT_04s0079g00280**	72544.7	5.2116	*ANAC028 (AT1G65910.1)*
Grape	*VvNAC65*	325	**VIT_04s0008g02710**	36575.4	8.6525	*ANAC039 (AT2G24430.2)*
Grape	*VvNAC66*	164	**VIT_04s0008g06550**	18677.4	8.1931	*ANAC025 (AT1G61110.1)*
Grape	*VvNAC67*	375	**VIT_06s0080g00970**	42903.5	6.3296	*ANAC040 (AT2G27300.1)*
Grape	*VvNAC68*	272	**VIT_19s0085g01210**	31198.3	8.0124	*ANAC033 (AT1G79580.1)*
Grape	*VvNAC69*	281	**VIT_19s0085g00950**	32077.6	5.2474	*ANAC116 (AT4G35580.1)*
Grape	*VvNAC70*	323	**VIT_18s0089g01120**	37007.3	8.9677	*ANAC070 (AT4G10350.1)*
Grape	*VvNAC71*	578	**VIT_16s0098g00760**	64685.1	4.6747	*ANAC116 (AT4G35580.1)*
Grape	*VvNAC73*	162	**VIT_14s0068g01490**	18617.7	10.5263	*ANAC083 (AT5G13180.1)*
Grape	*VvNAC74*	232	**VIT_06s0080g00780**	26616.9	7.6584	*ANAC090 (AT5G22380.1)*

Table 7.2. Detailed information of protein-to-protein interaction network in *VvNAC* proteins based on known *Arabidopsis thaliana* proteins

Query Item	Preferred Name	String Id
VvNAC61	***ANAC087***	3702.AT5G18270.1
VvNAC08 and VvNAC39	***ATAF1***	3702.AT1G01720.1
VvNAC03, VvNAC42, VvNAC45 and VvNAC66	***NAC025***	3702.AT1G61110.1
VvNAC64	***NAC028***	3702.AT1G65910.1
VvNAC44	***NAC032***	3702.AT1G77450.1
VvNAC04 and VvNAC41	***NAC036***	3702.AT2G17040.1
VvNAC65	***NAC038***	3702.AT2G24430.2
VvNAC28, VvNAC29, VvNAC30, VvNAC31, VvNAC32 and VvNAC36	***NAC042***	3702.AT2G43000.1
VvNAC59	***NAC044***	3702.AT3G01600.1
VvNAC60	***NAC047***	3702.AT3G04070.1
VvNAC21 and VvNAC50	***NAC050***	3702.AT3G10480.3
VvNAC09 and VvNAC57	***NAC057***	3702.AT3G17730.1
VvNAC70	***NAC070***	3702.AT4G10350.1
VvNAC34 and VvNAC37	***NAC073***	3702.AT4G28500.1
VvNAC56	***NAC074***	3702.AT4G28530.1
VvNAC01, VvNAC07, and VvNAC73	***NAC083***	3702.AT5G13180.1
VvNAC46 and VvNAC62	***NAC090***	3702.AT5G22380.1
VvNAC33	***NAC1***	3702.AT1G56010.2
VvNAC48	***NAC103***	3702.AT5G64060.1
VvNAC26, VvNAC43	***NAP***	3702.AT1G69490.1
VvNAC24 and VvNAC49	***NST1***	3702.AT2G46770.1
VvNAC67	***NTL8***	3702.AT2G27300.1
VvNAC51, VvNAC52, VvNAC53, VvNAC54, VvNAC55, VvNAC69, and VvNAC71	***NTL9***	3702.AT4G35580.2
VvNAC63	***VND7***	3702.AT1G71930.1
VvNAC47 and VvNAC58	***XND1***	3702.AT5G64530.1

Annotation
Involved in chlorophyll catabolic processes, such as NYC1, SGR1, SGR2 and PAO
Transcript level increases in response to wounding and abscisic acid.
May be associated with anther development and pollen production.
Involved in multicellular organismal development, regulation of transcription.
Positively regulates age- dependent senescence, dark-induced leaf senescence and stress-induced senescence.
Involved in leaf and inflorescence stem morphogenesis.
Involved in multicellular organismal development, regulation of transcription.
Negative regulator of leaf senescence and enhances tolerance to various abiotic stresses through the regulation of DREB2A
Involved in multicellular organismal development, regulation of transcription.
Involved in ethylene biosynthesis. Mediates waterlogging- induced hyponastic leaf movement, and cell expansion.

Involved in multicellular organismal development, regulation of transcription.
Involved in multicellular organismal development, regulation of transcription.
Regulates root cap maturation, in a partially redundant fashion.
Plays a regulatory role in the development of secondary cell wall fibers.
Involved in multicellular organismal development, regulation of transcription.
Negatively regulates the xylem vessel formation and mediates signaling crosstalk between salt stress response and leaf aging process.
Involved in multicellular organismal development, regulation of transcription.
involved in shoot apical meristem formation and auxin-mediated lateral root formation.
Involved in multicellular organismal development, regulation of transcription.
Increases levels of the senescence-inducing hormone abscisic acid (ABA). Involved in the control of dehydration in senescing leaves.
Transcription activator of genes involved in biosynthesis of secondary walls.
Regulates gibberellic acid-mediated salt- responsive repression of seed germination and flowering.
Functions synergistically with SNI1 as negative regulator of pathogen induced PR1 expression.
Involved in xylem formation in roots and shoots.
Negatively regulates secondary cell wall fibre synthesis and programmed cell death.

Table 7.3. The description of cis-acting regulatory elements of the *VvNAC* genes associated with plant stress, hormone, and development process.

Element name	Function
TGA-element	auxin-responsive element
TGA-box	part of an auxin-responsive element
TATC-box	*cis*-acting element involved in gibberellin-responsiveness
P-box	gibberellin-responsive element
ABRE	*cis*-acting element involved in the abscisic acid responsiveness
TCA-element	*cis*-acting element involved in salicylic acid responsiveness
CGTCA-motif	*cis*-acting regulatory element involved in the MeJA-responsiveness
TGACG-motif	*cis*-acting regulatory element involved in the MeJA-responsiveness
G-box	*cis*-acting regulatory element involved in light responsiveness
GATA-motif	part of a light responsive element
GT1-motif	light responsive element
circadian	*cis*-acting regulatory element involved in circadian control
LTR	*cis*-acting element involved in low-temperature responsiveness
ARE	*cis*-acting regulatory element essential for the anaerobic induction
MBS	MYB binding site involved in drought-inducibility
Box 4	part of a conserved DNA module involved in light responsiveness
TCT-motif	part of a light responsive element
TCCC-motif	part of a light responsive element
TC-rich repeats	*cis*-acting element involved in defense and stress responsiveness
DRE core	dehydration-responsive element
W box	elicitor responsiveness (disease resistance)
WRE3	wound response element
O2-site	cis-acting regulatory element involved in zein metabolism regulation
F-box	plant vegetative and reproduction growth and development; cell death and defense
as-1	root specific expression
AC-I	vascular-specific expression
STRE	Stress Response Element
HD-Zip1	*cis*-regulatory element for the leaf development
MBSI	involved in flavonoid biosynthesis
MYC	drought responsiveness
MYB	drought responsiveness
ERE	ethylene-responsive element
GARE-motif	*cis*-acting element involved in gibberellin-responsiveness
AuxRR-core	cis-acting regulatory element involved in auxin responsiveness

Table 7.4. Microarrays data profiles related to *Vitis Vinifera NAC* genes in different stages of noble rot caused by *Botrytis cinerea.*

Gene ID	**S1**	**S1; *B. cinerea* vs control. pValue**	**S2**	**S2; *B. cinerea* vs control. pValue**	**S3**	**S3; *B. cinerea* vs control. pValue**
VvNAC01	0.2	0.535123329	1.4	4.05E-13	1	8.31E-07
VvNAC03	0.5	8.97E-04	1.1	1.69E-15	1.8	1.81E-36
VvNAC05	0.2	0.054213407	0.5	0.01939994		-
VvNAC07	0.2	0.784384669	1.1	0.119409044	0.2	0.808673942
VvNAC08	0.9	7.02E-08	3	2.76E-55	2.9	7.35E-108
VvNAC09	-	-	-0.3	0.073799196	-0.1	0.823009682
VvNAC11	-	-	-0.7	4.31E-06	0.1	0.591375038
VvNAC12	0.1	0.579271484	-1	5.20E-06	-0.2	0.495032535
VvNAC13	-0.3	0.371289174	-2.2	1.37E-24	-1.4	7.28E-12
VvNAC15	0.1	0.790590977	0.2	0.070755021	0.5	5.39E-06
VvNAC16	0.5	0.556117243	-	-	-	-
VvNAC18	-	-	0.8	1.86E-10	0.5	2.62E-05
VvNAC19	0.3	0.596523954	-0.7	0.265140926	-0.1	0.885888324
VvNAC20	-	-	0.4	0.003475854	0.3	0.002903393
VvNAC21	0.1	0.866239969	0.1	0.84133058	-0.4	0.537836971
VvNAC25	-0.2	0.552255046	0.5	3.73E-04	0.2	0.215992073
VvNAC26	0.1	0.85741699	-0.9	0.007091799	-1.1	0.003552427
VvNAC30	2.4	1.38E-05	3.7	2.31E-04	3.8	2.71E-04
VvNAC31	2.2	1.16E-04	3.3	0.001521837	2.5	0.036107373
VvNAC32	0.4	0.665733821	1.2	0.36443176	1.6	0.21718526
VvNAC33	0.2	0.753921202	1.4	2.41E-06	1.2	7.67E-04
VvNAC34	0.2	0.86602089	-	-	0.3	0.775124288
VvNAC35	0.1	0.738531823	-0.1	0.768116371	-0.1	0.594757038
VvNAC36	2.8	3.17E-26	1.1	0.015461522	0.9	0.115624957
VvNAC37	0.3	0.389041352	-	-	0.5	0.02987791
VvNAC38		-	-	-	0.1	0.451716644
VvNAC39	2.8	2.59E-34	5.6	0	5.5	0
VvNAC40	0.1	0.971570803	-	-	-	-
VvNAC41	1.2	0.06726654	2.7	0.021384867	-	-
VvNAC42	0.2	0.842798049	0.6	0.444459485	0.6	0.524252737
VvNAC44	2.7	5.97E-08	5	3.16E-22	6	7.89E-49
VvNAC45	0.3	0.818243519	-1.2	0.322247125	-0.1	0.947742753
VvNAC47	0.3	0.804206544	-0.2	0.873140439	-	-
VvNAC48	-	-	0.7	2.53E-09	0.4	2.29E-05
VvNAC51	-	-	-2.1	0.004527507	-2	0.016130879
VvNAC52	-0.3	0.705051056	-1.4	0.17591224	-2.1	0.053624394
VvNAC53	-0.7	0.007015424	-2.6	4.56E-15	-2.1	2.51E-12
VvNAC54	-0.2	0.780162316	-2	4.88E-07	-2.4	1.44E-06
VvNAC55	-0.6	0.007133217	-2.2	6.75E-19	-1.5	9.42E-09
VvNAC56	0.4	0.658823723	-	-	-	-
VvNAC59	-0.2	0.546888979	-0.7	0.025259741	-0.4	0.181143476

VvNAC60	1.5	1.19E-16	3.6	4.03E-73	3	2.74E-81
VvNAC61	0.9	1.98E-14	2.9	1.74E-60	2.1	1.36E-34
VvNAC64	1.6	1.54E-08	1.1	0.019968952	0.7	0.169537728
VvNAC67	0.4	0.381666354	0.4	0.341189888	-0.1	0.874983883
VvNAC71	-0.5	0.009982447	-0.8	9.35E-05	-0.2	0.205413888
VvNAC72	0.2	0.840394162	0.2	0.881535544	-	-
VvNAC73	3	3.26E-17	5.2	3.54E-49	5.4	4.20E-59
VvNAC74	1.7	0.002941632	3.5	6.48E-06	2.2	0.045673339

Table 7.5. Calculation of the Euclidean distance of the clusters. In order to assess the dissimilarity between two clusters, a pairwise comparison of all *VvNAC* genes within each cluster was conducted, wherein the sum of differences in expression across various infection stages was computed.

Clusters	**Dif b/t which *VvNAC* genes**	**D 1^2 (S1)**	**D 2^2 (S2)**	**D 3^2 (S3)**	**Sum of difs**	**ED**
Clusters 2&3	*NAC31 & NAC13*	6.25	30.25	15.21	51.71	7.190967
	NAC31 & NAC51	4.84	29.16	20.25	54.25	7.36546
	NAC31 & NAC52	6.25	22.09	21.16	49.5	7.035624
	NAC31 & NAC53	8.41	34.81	21.16	64.38	8.023715
	NAC31 & NAC54	5.76	28.09	24.01	57.86	7.606576
	NAC31 & NAC55	7.84	30.25	16	54.09	7.35459
	NAC30 & NAC13	7.29	34.81	27.04	69.14	8.315047
	NAC30 & NAC51	5.76	33.64	33.64	73.04	8.546344
	NAC30 & NAC52	7.29	26.01	34.81	68.11	8.252878
	NAC30 & NAC53	9.61	39.69	34.81	84.11	9.17115
	NAC30 & NAC54	6.76	32.49	38.44	77.69	8.814193
	NAC30 & NAC55	9	34.81	28.09	71.9	8.479387
	NAC61 & NAC13	1.44	26.01	12.25	39.7	6.300794
	NAC61 & NAC51	0.81	25	16.81	42.62	6.528399
	NAC61 & NAC52	1.44	18.49	17.64	37.57	6.129437
	NAC61 & NAC53	2.56	30.25	17.64	50.45	7.102816
	NAC61 & NAC54	1.21	24.01	20.25	45.47	6.743145
	NAC61 & NAC55	2.25	26.01	12.96	41.22	6.42028
	NAC08 & NAC13	1.44	27.04	18.49	46.97	6.853466
	NAC08 &NAC51	0.81	26.01	24.01	50.83	7.129516
	NAC08 & NAC52	1.44	19.36	25	45.8	6.76757
	NAC08 & NAC53	2.56	31.36	25	58.92	7.675936
	NAC08 & NAC54	1.21	25	28.09	54.3	7.368853
	NAC08 & NAC55	2.25	27.04	19.36	48.65	6.974955
	NAC64 & NAC13	3.61	10.89	4.41	18.91	4.348563
	NAC64 &NAC51	2.56	10.24	7.29	20.09	4.482187
	NAC64 & NAC52	3.61	6.25	7.84	17.7	**4.207137**
	NAC64 & NAC53	5.29	13.69	7.84	26.82	5.178803
	NAC64& NAC54	3.24	9.61	9.61	22.46	4.739198

	NAC64 & NAC55	4.84	10.89	4.84	20.57	4.535416
	NAC41 & NAC13	2.25	24.01	1.96	28.22	5.31225
	NAC41 &NAC51	1.44	23.04	4	28.48	5.336666
	NAC41 & NAC52	2.25	16.81	4.41	23.47	4.844585
	NAC41 & NAC53	3.61	28.09	4.41	36.11	6.00916
	NAC41&NAC54	1.96	22.09	5.76	29.81	5.459853
	NAC41&NAC55	3.24	24.01	2.25	29.5	5.43139
	NAC36&NAC13	9.61	10.89	5.29	25.79	5.078386
	NAC36&NAC51	7.84	10.24	8.41	26.49	5.146844
	NAC36&NAC52	9.61	6.25	9	24.86	4.98598
	NAC36&NAC53	12.25	13.69	9	34.94	5.911007
	NAC36&NAC54	9	9.61	10.89	29.5	5.43139
	NAC36&NAC55	11.56	10.89	5.76	28.21	5.311309
	NAC74&NAC13	4	32.49	12.96	49.45	7.032069
	NAC74&NAC51	2.89	31.36	17.64	51.89	7.203471
	NAC74&NAC52	4	24.01	18.49	46.5	6.819091
	NAC74&NAC53	5.76	37.21	18.49	61.46	7.839643
	NAC74&NAC54	3.61	30.25	21.16	55.02	7.417547
	NAC74&NAC55	5.29	32.49	13.69	51.47	7.17426
	NAC61&NAC13	1.44	26.01	12.25	39.7	6.300794
	NAC61&NAC51	0.81	25	16.81	42.62	6.528399
	NAC61&NAC52	1.44	18.49	17.64	37.57	6.129437
	NAC61&NAC53	2.56	30.25	17.64	50.45	7.102816
	NAC61&NAC54	1.21	24.01	20.25	45.47	6.743145
	NAC61&NAC55	2.25	26.01	12.96	41.22	6.42028
Clusters 3&4	*NAC36&NAC73*	0.04	16.81	20.25	37.1	6.090977
	NAC36&NAC39	0	20.25	21.16	41.41	6.43506
	NAC36&NAC44	0.01	15.21	26.01	41.23	6.421059
	NAC41&NAC73	3.24	6.25	29.16	38.65	6.216912
	NAC41&NAC39	2.56	8.41	30.25	41.22	6.42028
	NAC41&NAC44	2.25	5.29	36	43.54	6.598485
	NAC64&NAC73	1.96	16.81	22.09	40.86	6.392183
	NAC64&NAC39	1.44	20.25	23.04	44.73	6.688049
	NAC64&NAC44	1.21	15.21	28.09	44.51	6.671582
	NAC08&NAC73	4.41	4.84	6.25	15.5	3.937004
	NAC08&NAC39	3.61	6.76	6.76	17.13	4.13884
	NAC08&NAC44	3.24	4	9.61	16.85	4.104875
	NAC61&NAC73	4.41	5.29	10.89	20.59	4.537621
	NAC61&NAC39	3.61	7.29	11.56	22.46	4.739198
	NAC61&NAC44	3.24	4.41	15.21	22.86	4.781213
	NAC30&NAC73	0.36	2.25	2.56	5.17	**2.273763**
	NAC30&NAC39	0.16	3.61	2.89	6.66	2.580698
	NAC30&NAC44	0.09	1.69	4.84	6.62	2.572936
	NAC31&NAC73	0.64	3.61	8.41	12.66	3.558089
	NAC31&NAC39	0.36	5.29	9	14.65	3.827532
	NAC31&NAC44	0.25	2.89	12.25	15.39	3.923009

	NAC60&NAC73	2.25	2.56	5.76	10.57	3.251154
	NAC60&NAC39	1.69	4	6.25	11.94	3.455431
	NAC60&NAC44	1.44	1.96	9	12.4	3.521363
	NAC74&NAC73	1.69	2.89	10.24	14.82	3.849675
	NAC74&NAC39	1.21	4.41	10.89	16.51	4.06325
	NAC74&NAC44	1	2.25	14.44	17.69	4.205948
Clusters 1&4	*NAC12&NAC73*	8.41	38.44	31.36	78.21	8.843642
	NAC12&NAC39	7.29	43.56	32.49	83.34	9.129074
	NAC12&NAC44	6.76	36	38.44	81.2	9.011104
	NAC45&NAC73	7.29	40.96	30.25	78.5	8.860023
	NAC45&NAC39	6.25	46.24	31.36	83.85	9.156965
	NAC45&NAC44	5.76	38.44	37.21	81.41	9.022749
	NAC09&NAC73	9	30.25	30.25	69.5	8.336666
	NAC09&NAC39	7.84	34.81	31.36	74.01	8.602906
	NAC09&NAC44	7.29	28.09	37.21	72.59	8.519977
	NAC11&NAC73	9	34.81	28.09	71.9	8.479387
	NAC11&NAC39	7.84	39.69	29.16	76.69	8.757283
	NAC11&NAC44	7.29	32.49	34.81	74.59	8.63655
	NAC19&NAC73	7.29	34.81	30.25	72.35	8.50588
	NAC19&NAC39	6.25	39.69	31.36	77.3	8.792042
	NAC19&NAC44	5.76	32.49	37.21	75.46	8.686772
	NAC47&NAC73	7.29	29.16	29.16	65.61	8.1
	NAC47&NAC39	6.25	33.64	30.25	70.14	8.374963
	NAC47&NAC44	5.76	27.04	36	68.8	8.294577
	NAC26&NAC73	8.41	37.21	42.25	87.87	9.3739
	NAC26&NAC39	7.29	42.25	43.56	93.1	9.648834
	NAC26&NAC44	6.76	34.81	50.41	91.98	9.59062
	NAC59&NAC73	10.24	34.81	33.64	78.69	8.870738
	NAC59&NAC39	9	39.69	34.81	83.5	9.137833
	NAC59&NAC44	8.41	32.49	40.96	81.86	9.047652
	NAC71&NAC73	12.25	36	31.36	79.61	8.922444
	NAC71&NAC39	10.89	40.96	32.49	84.34	9.183681
	NAC71&NAC44	10.24	33.64	38.44	82.32	9.073037
	NAC01&NAC73	7.84	14.44	19.36	41.64	6.452906
	NAC01&NAC39	6.76	17.64	20.25	44.65	6.682066
	NAC01&NAC44	6.25	12.96	25	44.21	6.64906
	NAC33&NAC73	7.84	14.44	17.64	39.92	6.318228
	NAC33&NAC39	6.76	17.64	18.49	42.89	6.549046
	NAC33&NAC44	6.25	12.96	23.04	42.25	6.50
	NAC03&NAC73	6.25	16.81	12.96	36.02	**6.001666**
	NAC03&NAC39	5.29	20.25	13.69	39.23	6.263386
	NAC03&NAC44	4.84	15.21	17.64	37.69	6.139218
	NAC32&NAC73	6.76	16	14.44	37.2	6.09918
	NAC32&NAC39	5.76	19.36	15.21	40.33	6.350591
	NAC32&NAC44	5.29	14.44	19.36	39.09	6.2522
	NAC07&NAC73	7.84	16.81	27.04	51.69	7.189576

	NAC07&NAC39	6.76	20.25	28.09	55.1	7.422937
	NAC07&NAC44	6.25	15.21	33.64	55.1	7.422937
	NAC42&NAC73	7.84	21.16	23.04	52.04	7.213876
	NAC42&NAC39	6.76	25	24.01	55.77	7.467931
	NAC42&NAC44	6.25	19.36	29.16	54.77	7.400676
	NAC15&NAC73	8.41	25	24.01	57.42	7.577599
	NAC15&NAC39	7.29	29.16	25	61.45	7.839005
	NAC15&NAC44	6.76	23.04	30.25	60.05	7.749194
	NAC20&NAC73	9	23.04	26.01	58.05	7.619055
	NAC20&NAC39	7.84	27.04	27.04	61.92	7.868926
	NAC20&NAC44	7.29	21.16	32.49	60.94	7.806408
	NAC25&NAC73	10.24	22.09	27.04	59.37	7.705193
	NAC25&NAC39	9	26.01	28.09	63.1	7.943551
	NAC25&NAC44	8.41	20.25	33.64	62.3	7.893035
	NAC18&NAC73	9	19.36	24.01	52.37	7.236712
	NAC18&NAC39	7.84	23.04	25	55.88	7.475293
	NAC18&NAC44	7.29	17.64	30.25	55.18	7.428324
	NAC48&NAC73	9	20.25	25	54.25	7.36546
	NAC48&NAC39	7.84	24.01	26.01	57.86	7.606576
	NAC48&NAC44	7.29	18.49	31.36	57.14	7.5591
	NAC34&NAC73	7.84	27.04	26.01	60.89	7.803204
	NAC34&NAC39	6.76	31.36	27.04	65.16	8.072174
	NAC34&NAC44	6.25	25	32.49	63.74	7.983733
	NAC37&NAC73	7.29	27.04	24.01	58.34	7.638063
	NAC37&NAC39	6.25	31.36	25	62.61	7.912648
	NAC37&NAC44	5.76	25	30.25	61.01	7.81089
	NAC21&NAC73	8.41	26.01	33.64	68.06	8.249848
	NAC21&NAC39	7.29	30.25	34.81	72.35	8.50588
	NAC21&NAC44	6.76	24.01	40.96	71.73	8.469357
	NAC05&NAC73	7.84	22.09	29.16	59.09	7.687002
	NAC05&NAC39	6.76	26.01	30.25	63.02	7.938514
	NAC05&NAC44	6.25	20.25	36	62.5	7.905694
	NAC67&NAC73	6.76	23.04	30.25	60.05	7.749194
	NAC67&NAC39	5.76	27.04	31.36	64.16	8.009994
	NAC67&NAC44	5.29	21.16	37.21	63.66	7.978722
Clusters 2&4	*NAC13&NAC73*	10.89	54.76	46.24	111.89	10.57781
	NAC13&NAC39	9.61	60.84	47.61	118.06	10.86554
	NAC13&NAC44	9	51.84	54.76	115.6	10.75174
	NAC51&NAC73	9	53.29	54.76	117.05	10.81896
	NAC51&NAC39	7.84	59.29	56.25	123.38	11.10766
	NAC51&NAC44	7.29	50.41	64	121.7	11.03177
	NAC52&NAC73	10.89	43.56	56.25	110.7	**10.52141**
	NAC52&NAC39	9.61	49	57.76	116.37	10.78749
	NAC52&NAC44	9	40.96	65.61	115.57	10.75035
	NAC53&NAC73	13.69	60.84	56.25	130.78	11.43591
	NAC53&NAC39	12.25	67.24	57.76	137.25	11.71537

	NAC53&NAC44	11.56	57.76	65.61	134.93	11.61594
	NAC54&NAC73	10.24	51.84	60.84	122.92	11.08693
	NAC54&NAC39	9	57.76	62.41	129.17	11.3653
	NAC54&NAC44	8.41	49	70.56	127.97	11.31238
	NAC55&NAC73	12.96	54.76	47.61	115.33	10.73918
	NAC55&NAC39	11.56	60.84	49	121.4	11.01817
	NAC55&NAC44	10.89	51.84	56.25	118.98	10.9078

Table 7.6. Gene information used for the subcellular localization adapted from Genoscope database.

Species	**Locus Name**	**Gene ID**	**Length**	**Transcript Name**	**Orthologues in *A. thaliana***
Grape	GSVIVT01020834001	*VvNAC36*	298	VIT_12s0028g00860	ANAC042 (AT2G43000.1)

Table 7.7. *VvNAC36* primers used in this study for transient expression vector construction.

Primer Name	**Primer Sequences (5'-3')**	**Annealing Temperature**	**Amplification Length (Bp)**
Forward: For_NAC042_5 gene	CACCACCTAAAGCTTGGACAAACA	60 °C	3000
Reverse: NAC pg_R	TCTACAGTCATAAACATGAGCTCG		

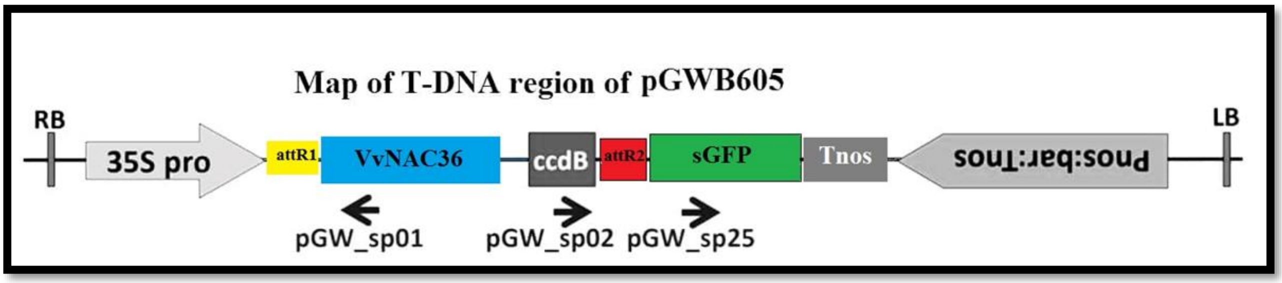

Figure 7.1 T-DNA region map of the construct pGWB605::*VvNAC36*

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