Kvantitativna ekspresija gena NAC041, NAC084, DREB2A i HSFA2 u klijanaca divljeg kupusa (Brassica incana) izloženih abiotičkom stresu

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Quantification of *NAC041, NAC084, DREB2A,* and *HSFA2* gene expression in wild cabbage (*Brassica incana*) exposed to abiotic stress

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To my wonderful and inspiring parents, brother, and grandmother – I love you. Thank you for always believing in me and giving me the freedom to find my own path. I hope to make you proud.

„vjerujte mi ništa
materijalno na ovome Svijetu ne može zamijeniti
putovanja, susrete, osmijehe
neke leptire koje vidite u prekrasnim vrtovima
zanimljivih hramova i muzeja
šetnje uz neke rijeke
ili neke Vam nepoznate biljke“

M. K.

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Quantification of *NAC041*, *NAC084*, *DREB2A*, and *HSFA2* gene expression in wild cabbage (*Brassica incana*) exposed to abiotic stress

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As a result of an ongoing climate crisis, abiotic stress factors such as elevated temperature, drought, and increased soil salinity negatively affect plant growth and development, causing damage to the yield and quality of agricultural crops. Unraveling the complex stress response mechanisms in plants, developing crop improvement strategies, and undertaking germplasm conservation efforts are key steps to ensure global food security. In an attempt to reintroduce genetic diversity into *Brassica* crops, crop wild relative species are studied for their valuable stress resistance traits. This study presents a quantitative gene expression analysis of the stress-responsive *NAC041*, *NAC084*, *DREB2A*, and *HSFA2* genes in wild cabbage (*Brassica incana*) exposed to different abiotic stress treatments, including high-temperature stress, osmotic stress, salt stress, and combined abiotic stress. A significant increase in *DREB2A* and *HSFA2* expression in two accessions confirmed the important role of these transcription factors in *B. incana* response to abiotic stress, while the other two accessions showed more moderate expression changes, implying the possible presence of multiple gene variants. A decrease in *NAC041* expression was observed in one accession under osmotic stress, while a substantial decrease in *NAC084* expression was observed under heat stress and combined stress in all four *B. incana* accessions.

Keywords: Brassicaceae, crop wild relatives, plant stress response, temperature, drought, salinity
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Kvantitativna ekspresija gena *NAC041, NAC084, DREB2A* i *HSFA2* u klijanaca divljeg kupusa (*Brassica incana*) izloženih abiotičkom stresu

Josipa Drmić

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Kao posljedica klimatske krize, čimbenici abiotičkog stresa poput povišene temperature, suše i povišenog saliniteta tla negativno utječu na biljni rast i razvoj, što ostavlja značajne posljedice na prinos i kvalitetu poljoprivrednih kultura. Otkrivanje složenih mehanizama biljnog odgovora na stres, razvoj strategija za povećanje otpornosti poljoprivrednih kultura te očuvanje genetičkih resursa ključni su koraci za osiguranje globalne prehrambene sigurnosti. Mnoge divlje vrste iz roda *Brassica* iznimno su otporne na stresne uvjete okoliša u kojima prirodno rastu, stoga predstavljaju bitan izvor poželjnih svojstava za povećanje genetičke raznolikosti u poljoprivrednim vrstama kupusnjača. U ovom radu predstavljena je analiza kvantitativne ekspresije četiriju gena uključenih u biljni odgovor na abiotički stres *NAC041, NAC084, DREB2A* i *HSFA2* u klijanaca divljeg kupusa (*Brassica incana*) izloženih različitim čimbenicima abiotičkog stresa: povišenoj temperaturi, osmotskom stresu, povišenom salinitetu te kombiniranom abiotičkom stresu. Značajno povišenje ekspresije gena *DREB2A* i *HSFA2* u dvije linije potvrdilo je bitnu ulogu ovih transkripcijskih faktora u odgovoru divljeg kupusa na abiotički stres, dok su druge dvije linije pokazale značajno manju promjenu ekspresije, što ukazuje na mogućnost postojanja više genskih varijanti. Utvrđeno je smanjenje ekspresije gena *NAC041* pod utjecajem osmotskog stresa, dok je značajno smanjenje ekspresije gena *NAC084* utvrđeno pod utjecajem visoke temperature i kombiniranog abiotičkog stresa.

Ključne riječi: Brassicaceae, divlje vrste srodne poljoprivrednim kulturama, biljni odgovor na stres, temperatura, suša, salinitet
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Contents

Abbreviations

ABA – abscisic acid

Cas – CRISPR associated protein

cDNA – complementary DNA

Cq – quantification cycle

CRISPR – clustered regularly interspaced short palindromic repeats

CWR – crop wild relatives

DRE – drought-responsive element

DREB – dehydration-responsive element binding protein

DTT – dithiothreitol

EtOH – ethanol

FAO – Food and Agriculture Organization

gDNA – genomic DNA

HSF – heat shock transcription factors

HSP – heat shock proteins

JA – jasmonic acid

NAC – NAM, ATAF, and CUC domain protein

PCR – polymerase chain reaction

qPCR – quantitative polymerase chain reaction

RAPD – randomly amplified polymorphic DNA

ROS – reactive oxygen species

RT – reverse transcription

SE – standard error

SINE – short interspersed elements

SSR – simple sequence repeats

Tm – melting temperature

UV – ultraviolet

WGD – whole genome duplication

1. INTRODUCTION

1.1. Abiotic stress in plants

Plants are sessile organisms, which means they cannot simply escape challenging environmental conditions. Instead, to survive and cope with various biotic and abiotic stress factors, they had to develop sophisticated mechanisms of stress response at a physiological, biochemical, and molecular level (Hirayama and Shinozaki 2010). Biotic stress in plants includes diseases and infestations caused by microbial pathogens and pests, as well as herbivore attacks. Abiotic plant stress includes all challenging conditions in the environment caused by abiotic factors that limit plant growth, development, and productivity, such as extreme temperatures, ultraviolet (UV) radiation, drought, salinity, hypoxia caused by flooding, nutrient deprivation, and the presence of heavy metals in soil. It is estimated that about 96.5% of global agricultural land is affected by some form of abiotic stress (Imran et al. 2021). The utilization of molecular biology techniques has offered a broader perspective on abiotic stress response research in plants, specifically the integrated "omics" approach, which has allowed for a much more comprehensive understanding of these complex molecular mechanisms (Hirayama and Shinozaki 2010). Genomics and transcriptomics research have enabled comprehensive genome-wide expression profiling studies in plants exposed to various abiotic stresses that helped identify stress-related genes and precisely characterize their functions in the abiotic stress response (Hirayama and Shinozaki 2010).

Plant response and tolerance to abiotic stress is a complex network of multiple processes with multiple levels of regulation. The primary level of stress response regulation, as in all other molecular mechanisms, is the transcriptional regulation of stress-responsive genes. Epigenetic mechanisms, such as chromatin remodeling and small RNAs, mediate the drastic changes in gene expression profiles in response to abiotic stress (Hirayama and Shinozaki 2010). Several transcription factors are known to be the key regulators of gene expression under stressful conditions, including NAM, ATAF, and CUC domain proteins (NAC), dehydration-responsive element binding proteins (DREB), and heat shock transcription factors (HSFs). Induced by various epigenetic signals under abiotic stress, these transcription factors activate the transcription of stress-related genes that code for stress-responsive proteins with important roles in the survival of plants exposed to abiotic stress (Bauer et al. 2022). NAC proteins are a group of about 100 plant-specific transcription factors that regulate the expression of genes involved in plant

development and stress response. The expression of abiotic stress-responsive NAC proteins is induced by drought, high salinity, and phytohormones abscisic acid (ABA) and jasmonic acid (JA). Plants that express stress-responsive NAC factors show elevated tolerance to drought and salinity stress (Nakashima et al. 2012). The HSF family consists of three conserved evolutionary classes, A, B, and C, which play a key role in the heat shock response. Heat shock-induced factor HSFA2 is the main HSF in thermotolerant cells (Kotak et al. 2007). DREB proteins are a subfamily in the apetala 2/ethylene-responsive factor (AP2/ERF) family of transcription factors, with a known role in abiotic stress response (Lohani et al. 2020). DREB2A plays a key role in drought tolerance, DREB2C induces salt stress tolerance, while DREB1 factors play an important role in cold stress tolerance in *Arabidopsis thaliana* (Lohani et al. 2020, Yoon et al. 2020). Interestingly, DREB2A was also shown to induce HSF expression in *A. thaliana* and *B. rapa*, suggesting a cross talk between the signaling cascades of heat and drought stress response (Kotak et al. 2007, Dong et al. 2015).

Mechanisms of stress response in plants differ based on the type of stress factor a plant is exposed to, as well as the combined effects of exposure to multiple different stress factors at the same time. Various molecular entities partake in stress response mechanisms, including reactive oxygen species (ROS), secondary metabolites, enzymatic and non-enzymatic antioxidants, osmolytes, and phytohormones (Bauer et al. 2022). For the most part, the role of these metabolites is associated with protection against oxidative stress caused by the rapid rise in ROS production known to occur in stress-exposed plant cells (Bose et al. 2014, Linić et al. 2019). Through lipid peroxidation and protein degradation, ROS cause severe oxidative damage to membranes and other structures in plant cells (Dos Santos et al. 2022). Almost all types of abiotic stress cause oxidative damage, so the role of antioxidants in the stress response is essential (Linić et al. 2019). Additionally, the type of stress response highly varies between different plant species (Bauer et al. 2022). For example, phenolic compounds and phytohormones are the key mediators of the abiotic stress response in the mustard plant family, including many cultivated *Brassica* crops (Pavlović et al. 2018, Linić et al. 2019).

1.1.1. Temperature stress

Climate change, characterized by the rise of average global temperatures, is causing severe heat stress in plants and represents a serious threat to global crop production. Extreme temperatures

that exceed the optimum temperature range for normal cellular function cause irreparable damage to plant health. A 10-15 °C rise in temperature above the optimum value causes heat stress in plants, and the level of damage depends on the stress severity and duration of exposure (Imran et al. 2021). Heat stress causes protein denaturation and aggregation which increases membrane fluidity, changes enzyme kinetics, and disturbs cellular homeostasis, with devastating effects on plant growth and development (Kotak et al. 2007). Heat stress response and thermotolerance in plants largely depend on the accumulation of heat shock proteins (HSPs) which are regulated by HSFs (Kotak et al. 2007). HSPs act as molecular chaperones, guiding protein folding and preventing further protein denaturation (Hasanuzzaman 2020).

On the other hand, plant exposure to temperatures significantly lower than the optimal values causes cold stress, which can be divided into chilling stress (< 10 °C) and freezing stress (< 0 °C) (Nurhasanah Ritonga and Chen 2020). The effects of cold stress include a decrease in membrane fluidity and water uptake, an increase in cytoplasmic Ca^{2+} content, and oxidative damage due to elevated ROS production (Imran et al. 2021). The most extreme outcome of freezing stress is the formation of ice crystals in plant cells, which leads to dehydration, cell puncture, cytosol outflow, and plant death (Nurhasanah Ritonga and Chen 2020). Plants from temperate regions can tolerate freezing temperatures if they are first exposed to lower-than-optimal but non-freezing temperatures, while plants growing in tropical and subtropical areas completely lack this ability of cold acclimation. The main objective of cold tolerance in plants is the prevention of ice formation, which is possible by accumulating cryoprotective polypeptides and osmolytes, such as proline and soluble sugars (Nurhasanah Ritonga and Chen 2020).

1.1.2. Drought stress

Drought is a major abiotic stress factor affecting up to 40% of the total land area on Earth, with devastating impacts on global agriculture (Zhang et al. 2014). A consistent increase in soil aridity is predicted for the Mediterranean region, which could significantly impair the production of *Brassica* crops in the near future. Plants sense drought as a decrease in osmotic potential in the root, the first organ exposed to water deficiency in the soil (Pavlović et al. 2018, Imran et al. 2021). The effects of drought stress in plant cells include an imbalance in osmotic homeostasis, oxidative stress, energy depletion, and inhibition of photosynthesis (Pavlović et al. 2018). Drought is known to cause severe reductions in plant biomass, seed yield, and the nutritional content of agricultural

crops (Zhang et al. 2014). The complex drought stress response in plants includes the accumulation of proline and other osmolytes, activation of the antioxidant system, stomatal closure, reduction in transpiration, and growth inhibition (Zhang et al. 2014, Hasanuzzaman 2020). Drought response in Brassicaceae heavily relies on increased endogenous phytohormone levels, including ABA, auxin, cytokinins, indole-3-acetic acid, and salicylic acid (Pavlović et al. 2018). ABA is a key mediator of osmotic stress response in plants, regulating multiple processes including osmoprotectant accumulation, ROS detoxification, ion transport, transpiration, and stomatal opening and closure (Pavlović et al. 2019).

1.1.3. Salt stress

Soil salinity is another important abiotic stress factor that negatively affects plant growth and development. Salt stress hinders several physiological and biochemical processes in plants including water uptake, mineral ion homeostasis, osmolyte accumulation, antioxidant metabolism, and even photosynthetic efficiency (Linić et al. 2019). Reduction in water intake causes osmotic stress, elevated concentrations of Na^+ ions cause ionic stress, and increased ROS production causes severe oxidative stress in plant cells exposed to high concentrations of salt (Pavlović et al. 2019). The increasing salinity of arable land worldwide, especially in the semi-arid and arid Mediterranean regions, could lead to huge losses in crop production (Zhang et al. 2014). Members of the Brassicaceae family, especially wild species that grow in the Mediterranean, are known for producing plenty of secondary metabolites that allow them to tolerate saline habitats. Salt-tolerant species have a rich basal content of carotenoids and polyphenolic compounds, both of which contribute to salt stress resistance by minimizing the effects of oxidative damage due to their ROS-scavenging properties (Bose et al. 2014). Glucosinolates, a class of secondary metabolites found almost exclusively in Brassicaceae, participate in salt stress response by maintaining the water uptake status and reducing the damaging effects of osmotic stress (Martínez-Ballesta et al. 2015). Endogenous phytohormones, mainly ABA, JA, and brassinosteroids, are also known to play crucial roles in salt stress response. High concentrations of Na^+ ions are toxic as they compete with K^+ ions at protein binding sites, which inevitably leads to enzyme inactivation (Pavlović et al. 2019). Therefore, the ability to maintain a low Na^+/K^+ ratio in plant cell cytoplasm by accumulating Na^+ ions in the vacuole is considered a key criterion for salt tolerance in *Brassica* species (Linić et al. 2019).

1.1.4. Combined abiotic stress

In both natural habitats and agricultural fields, plants are often simultaneously exposed to multiple stress factors, making the stress response mechanisms much more complex (**Figure 1**) (Bauer et al. 2022). A combination of different stresses causes more drastic and often unexpected physiological changes in plant cells, and the plant response to combined stress differs from the responses to individual types of stress (Hirayama and Shinozaki 2010, Zhang et al. 2014). Stress combinations that frequently coexist under field conditions include drought and salinity, flooding and salinity, and temperature and drought (Shabbir et al. 2022). Several physiological processes such as respiration, photosynthesis, starch metabolism, and nitrogen fixation are negatively affected under combined abiotic stress, leading to significant losses in crop production (Shabbir et al. 2022).

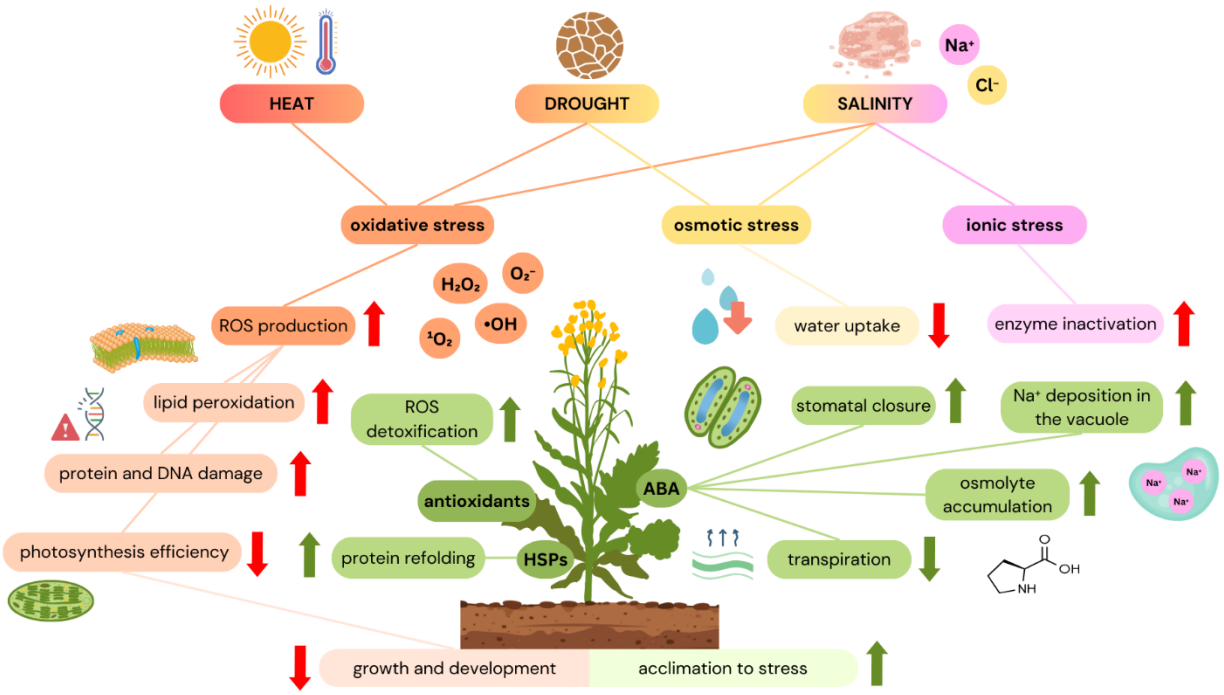

Figure 1. Physiological and biochemical effects of abiotic stress factors (heat, drought, and salinity) on plants, and key mechanisms of abiotic stress response in plants. Arrows pointing up indicate an increase, arrows pointing down indicate a decrease, red arrows indicate negative effects of abiotic stress, and green arrows indicate positive changes that lead to stress tolerance and acclimation in plants. Abbreviations: ABA – abscisic acid, H_2O_2 – hydrogen peroxide, HSPs – heat shock proteins, $^1O_2$ – singlet oxygen, O_2^- – superoxide anion, ·OH – hydroxyl radical, ROS – reactive oxygen species. Image created with Canva online graphic design tool.

1.2. The Brassicaceae family

Brassicaceae, also known as the mustard family and formerly known as Cruciferae, is one of the most agriculturally and economically important plant families. Consisting of mainly 338 genera and about 3709 species, it is globally cultivated and distributed across all continents except Antarctica (Hasanuzzaman 2020). The Brassicaceae family contains mostly herbaceous species which may be annual, biennial, or perennial, and rarely subshrubs. Leaves of the Brassicaceae show alternate arrangements and sometimes form rosettes. The shape of the leaf blade is usually simple and lyrate, with either entire, lobed, or pinnately incised margins, depending on the species. Leaves are exstipulate, usually covered with trichomes, and distinctive for releasing a pungent smell when crushed. Members of the Brassicaceae family generally possess a tap root system which is sometimes modified for storage purposes. Two common root modifications are fusiform in radish (*Raphanus sativus*) and napiform in turnip (*Brassica rapa* subsp. *rapa*). Flowers are strictly tetramerous, hermaphrodite, ebracteate, pedicellate, actinomorphic (rarely zygomorphic), sometimes solitary, but usually in a racemose type of inflorescence. The corolla consists of four petals arranged in the form of a crucifix, which is one of the main distinctive characteristic attributes of this family. Entomophily is the main form of pollination in Brassicaceae. The fruit type is typically a dehiscent bivalve capsule called siliqua if long and slender, or silicula if short and broader (Hasanuzzaman 2020, Aryal and Ojha 2023).

Brassicaceae have been at the focal point of research in the plant kingdom – especially thale cress (*Arabidopsis thaliana*), the main plant model organism that revolutionized genetics and molecular biology. They are interesting because of their genetic diversity, polyploidy, potential for interspecific hybridization, morphological plasticity, and resistance to harsh environmental conditions (Hasanuzzaman 2020). The genome architecture of modern-day Brassicaceae is the result of at least three whole genome duplication (WGD) events named α, β, and γ events. Additionally, a fourth WGD event is thought to have happened about 23 million years ago within the Brassiceae tribe. This complex mesopolyploid nature makes them a great model group for studying the impacts of polyploidy on genome evolution, especially the mechanisms behind multiple gene copy maintenance (Parkin et al. 2014). Various studies have shown a positive indirect effect of polyploidy on species diversity in Brassicaceae. Some research even suggests that polyploidy may facilitate plant adaptation and survival during periods of rapid environmental change (Román-Palacios et al. 2020).

Genetic and phenotypic diversity, as well as the great potential for interspecific hybridization, make the Brassicaceae an essential source of crop cultivars for global agriculture. An especially important agricultural genus is *Brassica*, which includes numerous vegetable, oilseed, and condiment crops, generated through extensive trait selection and interspecific hybridization. Established by the Korean cytogeneticist U Nagaharu in 1935, the Triangle of U shows the evolutionary and chromosomal relationships between six cultivated *Brassica* species (**Figure 2**) (Nagaharu 1935). Some of the most important *Brassica* crops include rapeseed (*Brassica napus*), the world's third main oilseed crop, as well as mustard (*Brassica juncea*), a globally cultivated condiment crop (Hasanuzzaman 2020). By far, the most diverse species in this genus is *Brassica oleracea*, which contains at least 18 cultivated vegetable crop types including cabbage (var. *capitata*), kale (var. *acephala*), broccoli (var. *italica*), cauliflower (var. *botrytis*), Brussels sprouts (var. *gemmifera*), and kohlrabi (var. *gongylodes*) (Mabry et al. 2021). The diversity of crops produced from a single species makes *B. oleracea* a model organism for studying and implementing the power of artificial selection (Mabry et al. 2021).

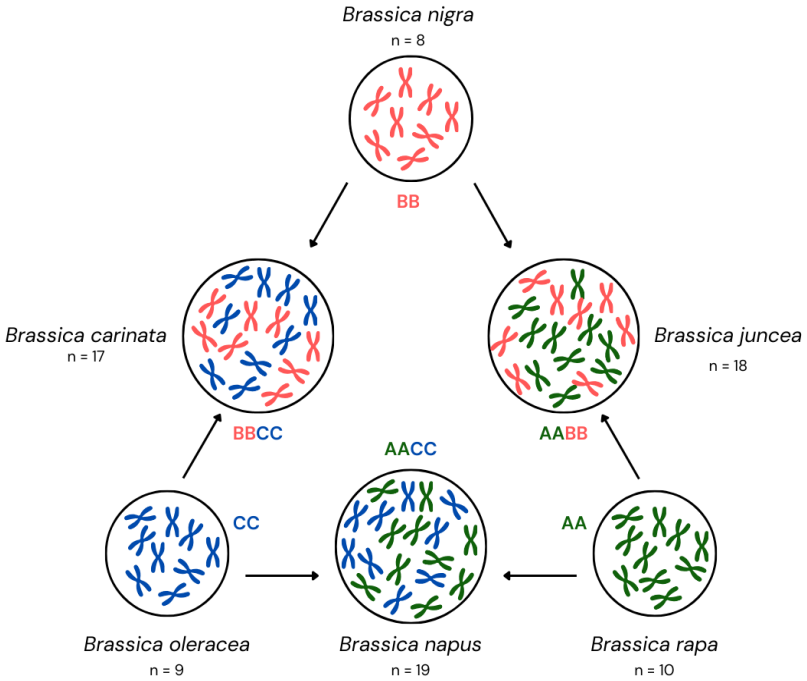

Figure 2. Triangle of U shows the genetic relationships between six cultivated *Brassica* species. Three amphidiploid species, *Brassica carinata* (n = 17, genome BBCC), *Brassica juncea* (n = 18, genome AABB), and *Brassica napus* L. (n = 19, genome AACC) were developed by hybridization and polyploidization of three diploid species, *Brassica nigra* (n = 8, genome BB), *Brassica oleracea* (n = 9, genome CC), and *Brassica rapa* (n = 10, genome AA). Adapted from Nagaharu 1935.

Besides their enormous potential for morphological variation, *Brassica* crops and their wild relatives are highly valued for their rich and diverse phytochemical composition. They are known for accumulating a wide range of secondary metabolites with strong antioxidant properties, such as phenols, thiols, carotenoids, flavonoids, glucosinolates, and ascorbic acid (Miceli et al. 2020, Picchi et al. 2020). Many of these metabolites play an essential role in the natural plant defense against pathogens, pests, herbivores, and other environmental challenges (Picchi et al. 2020). Moreover, the antioxidant, anti-inflammatory, anticarcinogenic, and antimicrobial activity of these compounds make the consumption of *Brassica* crops particularly beneficial for human health, reducing the risk of chronic cardiovascular diseases and cancer (Linić et al. 2019, Miceli et al. 2020). Additionally, they are rich in vitamins A, B1-2, B6, C, E, and K, as well as in various important minerals such as magnesium, iron, and calcium. Members of the Brassicaceae family are also known for their uptake and accumulation of heavy metals from polluted soil (Hasanuzzaman 2020).

1.2.1. Search for the *Brassica oleracea* progenitor species

As opposed to many other crops, there is still a lack of consensus on the progenitor species and true domestication origin of *B. oleracea*, with several challenges adding to the uncertainty of this domestication process. There are many wild *Brassica* species with the same genomic constitution of n = 9 chromosomes, often referred to as the C genome, which are interfertile with domesticated *B. oleracea* crops (Mabry et al. 2021). Many of these cabbage-like species inhabit the Atlantic coastlines of England, France, Spain, and some Portuguese and German islands, as well as the Mediterranean coastlines of Italy and Croatia (Maggioni et al. 2020, Mittell et al. 2020). It remains unclear whether these coastal wild populations represent the progenitor species or are feral descendants of domesticated *Brassica* plants that have escaped cultivation. The complex gene flow between wild and cultivated populations has added to the obscurity of the true evolutionary history of *B. oleracea* (Mabry et al. 2021). The question of the *B. oleracea* center of domestication has divided the scientific community, with two alternative hypotheses proposed so far pointing to either an Atlantic or a Mediterranean origin (Maggioni et al. 2018, Maggioni et al. 2020). Some genetic research has shown that the Atlantic populations intercross with cultivated *B. oleracea* crops with significantly higher fertility rates than the Mediterranean populations, which some authors have taken as a valid indication of an Atlantic origin of *Brassica* domestication (Von

Bothmer et al. 1995, Maggioni et al. 2020). However, multiple studies have shown that the genetic diversity of the Atlantic wild populations is lower than that of the cultivated *B. oleracea* crops, which excludes them from being the possible ancestors of *B. oleracea* and makes them more likely to be ferals escaped from cultivation (Allender et al. 2007, Maggioni et al. 2020). Additionally, various historical linguistic, iconographic, and literary evidence from ancient Greek and Latin literature implies an Eastern Mediterranean origin of cultivation (Maggioni et al. 2018). A recent phylogenetic study points to an endemic Greek wild species *Brassica cretica* as the closest living relative of cultivated *B. oleracea*, further supporting the hypothesis of an Eastern Mediterranean origin of domestication (**Figure 3**) (Mabry et al. 2021).

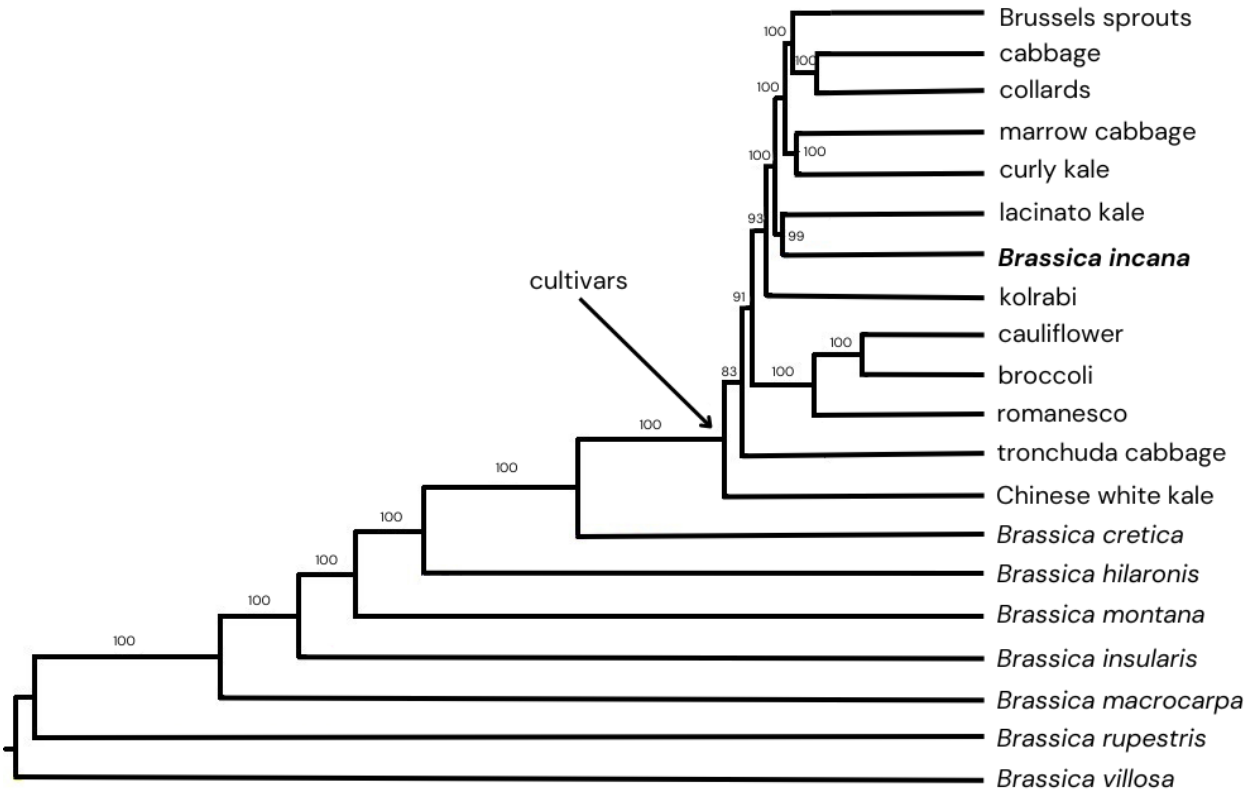

Figure 3. A phylogenetic tree of wild *Brassica* species and *Brassica oleracea* cultivars. Numbers above branches indicate bootstrap support. Adapted from Mabry et al. 2021.

1.2.2. Wild cabbage (*Brassica incana*)

Wild cabbage (*Brassica incana*) is a wild relative of *B. oleracea*, native to the Mediterranean coastlines of Italy, Greece, Croatia, Albania, and Bosnia and Herzegovina. It is a perennial subshrub that grows up to 100 cm in height (**Figure 4**). It has a lignified stem base (Snogerup et al. 1990). The leaf blade is lanceolate and pubescent with complete, lobed, or crenated margins and basal, stem-embracing auricles. The flowers are tetramerous, with yellow petals, and gathered in a long and narrow racemose inflorescence. The fruit is a 40-80 mm long convoluted and cylindrical siliqua (Tripodi et al. 2012). *B. incana* is an edible plant rich in bioactive compounds with antioxidant properties that are thought to be beneficial for human health (Miceli et al. 2020). Populations of *B. incana* mainly occur on calciferous rocky cliffs from sea level up to about 600-800 m altitude (Miceli et al. 2020). This habitat, characterized by harsh environmental conditions, implies strong adaptation of *B. incana* to multiple abiotic stress factors such as high temperature, drought, and salinity (Lučić et al. 2023).

Figure 4. Wild cabbage (*Brassica incana*) located on the island of Palagruža, Croatia. Photo: prof. Nataša Bauer, PhD.

B. incana has been suggested as a possible progenitor species of domesticated *B. oleracea* in the past (Snogerup 1980). Their close phylogenetic relationship and interfertility have been highlighted in multiple genotypic studies using various molecular markers, such as randomly amplified polymorphic DNA (RAPD), short interspersed elements (SINE), and chloroplast simple sequence repeats (SSRs) (Tatout et al. 1999, Allender et al. 2007). However, more recent analyses have shown that it is most likely a feral descendant of a cultivated *B. oleracea* crop, possibly lacinato kale (**Section 1.2.1**, **Figure 3**) (Mabry et al. 2021). Nevertheless, the importance of these feral *B. incana* populations as reservoirs of genetic diversity that contributed to *Brassica* crop diversification during domestication is evident, along with the strong potential for their utilization in agricultural crop improvement (Mabry et al. 2021).

1.3. Climate change and the future of global agriculture

Various devastating impacts of climate change on our planet have been evident and well-documented for the last few decades. Global warming – the rise of average temperatures due to increasing concentrations of industrial greenhouse gases in the atmosphere – is considered the primary effect of climate change, which subsequently causes adverse secondary effects on local climates, such as changes in precipitation patterns and increased frequency of extreme weather events (Leisner 2020, Quezada-Martinez et al. 2021). Anthropogenic carbon footprint has caused a 1 °C increase in average land surface temperature since 1990, and this rise is expected to proceed at a rate of 0.3 °C per decade (Imran et al. 2021).

The impact of climate change on global agriculture is especially concerning, as increasing temperatures and drought cause abiotic stress in plants, which significantly reduces the yield and quality of agricultural crops, including *Brassica* crops (Bauer et al. 2022). Farmers' attempts at lowering the negative effect of droughts on crop yields often include increased irrigation. However, unsuitable irrigation practices often result in increased soil salinity, which is another abiotic stress factor that negatively affects crop yields (Quezada-Martinez et al. 2021). Furthermore, as climate zones start to shift, insect and pest species expand their ecological niches and inhabit new geographical areas, presenting another threat to plants that are already experiencing increased abiotic stress (Quezada-Martinez et al. 2021). While the conditions for global agricultural production are worsening at a speed humans have yet to catch up with, the world population continues to grow, and so do the global food requirements. To prevent this upcoming food security

crisis, an average production increase of 44 million metric tons of food per year is required until 2050 (Tester and Langridge 2010). Considering the scarcity of resources – mainly land, water, and energy – along with the ongoing climate crisis, the tackling of this challenge must come from the perspective of genetic improvement of agricultural crop yield, biomass, and nutritional value (Bohra et al. 2022).

Emphasis on genetic uniformity of agricultural crop cultivars has led to a drastic loss of genetic diversity over the last century, a phenomenon often referred to as the breeding bottleneck (Bohra et al. 2022). During the process of crop domestication, intensive selection for yield-related traits under optimal conditions has resulted in the loss of many valuable traits related to biotic and abiotic stress resilience. However, wild relatives of cultivated crop species (including their possible ancestors), feral populations, and even traditional landrace populations often show significant resistance to biotic and abiotic stresses (Quezada-Martinez et al. 2021). One particularly concerning example of the domestication bottleneck effect in *Brassica* crops is rapeseed (*Brassica napus*), with an extreme decrease in genetic diversity, little to no resistance to insect predation, and no known wild relatives (Quezada-Martinez et al. 2021). Therefore, besides improving crop yield, biomass, and nutritional value, the improvement of crop resistance to biotic and abiotic stresses should be a key priority.

1.3.1. Crop improvement

Crop wild relatives (CWRs) are wild plant species considered the closest living relatives of cultivated crop species, sharing a common ancestry and genetic background (Vincent et al. 2013). They are reservoirs of genetic variation that have survived and adapted to drastic climate variability and harsh environmental conditions in their evolutionary past, therefore possessing many valuable traits that can be used for improving biotic and abiotic stress resistance in high-yielding agricultural crops (Maggioni et al. 2020, Bohra et al. 2022). The strong potential of CWR utilization in crop improvement was first described by Vavilov (1926), but the actual implementation came long after, following advances in genomics and genome editing (Mabry et al. 2021). Originally, attempts at interspecific hybridization between *Brassica* crops and their CWRs mostly aimed to resolve their phylogenetic relationships. Recently, however, the focus has shifted to improving agronomic traits in crop varieties through introgression breeding, a tool for transferring a limited number of specific alleles from one species to another (Katche et al. 2019). Numerous successful gene introgression

attempts have been made so far, with desirable traits such as disease resistance, higher nutritional value of the seed, improved oil quality, and stress resistance being successfully transferred between different *Brassica* cultivars, as well as from CWRs to cultivated *Brassica* species (Katche et al. 2019). Additionally, the use of feral *Brassica* lineages and traditional landrace varieties offers perhaps a more direct way of reintroducing genetic diversity into cultivated populations (Mabry et al. 2021). Another interesting and useful tool for crop improvement is resynthesis – the process of reproducing an already existing crop from its progenitor species to expand the gene pool and subsequently introduce new valuable traits into high-performance cultivars (Katche et al. 2019). Despite the big potential for interspecific hybridization between *Brassica* species, there are many pre-fertilization and post-fertilization barriers that make conventional breeding time-consuming, laborious, and expensive. Some mechanisms of hybrid incompatibility include cytoplasmic male sterility (CMS), hybrid necrosis, and hybrid sterility (Quezada-Martinez et al. 2021). Another big problem in introgression breeding is linkage drag, which occurs when a gene carrying an inferior trait is introduced in the hybrid progeny along with the superior trait of interest. These inferior traits usually cause an undesirable reduction in crop fitness and yield (Bohra et al. 2022). However, numerous different methods have been developed for overcoming these natural reproductive barriers, minimizing linkage drag and successfully increasing the genetic diversity of *Brassica* crops. Embryo rescue is a tissue culture technique where the post-fertilization barrier of seed abortion is crossed by transferring the embryo to a sterile medium and growing the hybrid *in vitro*. Another tissue culture method is somatic fusion, which has the advantage of transferring genes between sexually incompatible species. Various methods of genetic transformation not only cross the reproductive barriers within the *Brassica* genus, but also allow the introduction of foreign genes that are not naturally present within this genus (Katche et al. 2019). Finally, recent advancements in genome editing, specifically the clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR associated protein (CRISPR/Cas) technologies, are the most promising tools for high precision and accuracy molecular breeding of *Brassica* crops in the future (Li et al. 2022).

1.3.2. Germplasm conservation

Besides cultivated crops, many wild plant species, including CWRs, are also experiencing genetic erosion and other adverse effects of anthropogenic environmental changes. The requirement for systematic *in situ* and *ex situ* CWR conservation has been recognized by the Food

and Agriculture Organization (FAO) of the United Nations, as well as in a number of other international treaties (Vincent et al. 2013). *In situ* conservation is a form of CWR conservation that takes place in either agricultural fields or natural wild environments. It is important for the selection and adaptation of CWRs to the changing environmental conditions (Katche et al. 2019). However, *ex situ* conservation, although more expensive and often less successful in management and propagation compared to *in situ* efforts, remains the most common form of germplasm conservation (Katche et al. 2019, Bohra et al. 2022). *Ex situ* conservation of wild *Brassica* species has been a particular priority, with many germplasm banks across the world collecting traditional landrace varieties and wild accessions and conserving their viable seeds under long-term storage conditions for future breeding and research purposes (Branca and Cartea 2011). Together with promising new genome-editing technologies and a growing amount of sequenced and annotated wild genome data, these germplasm banks could revolutionize the use of CWRs for the genetic improvement of *Brassica* crops (**Figure 5**) (Katche et al. 2019).

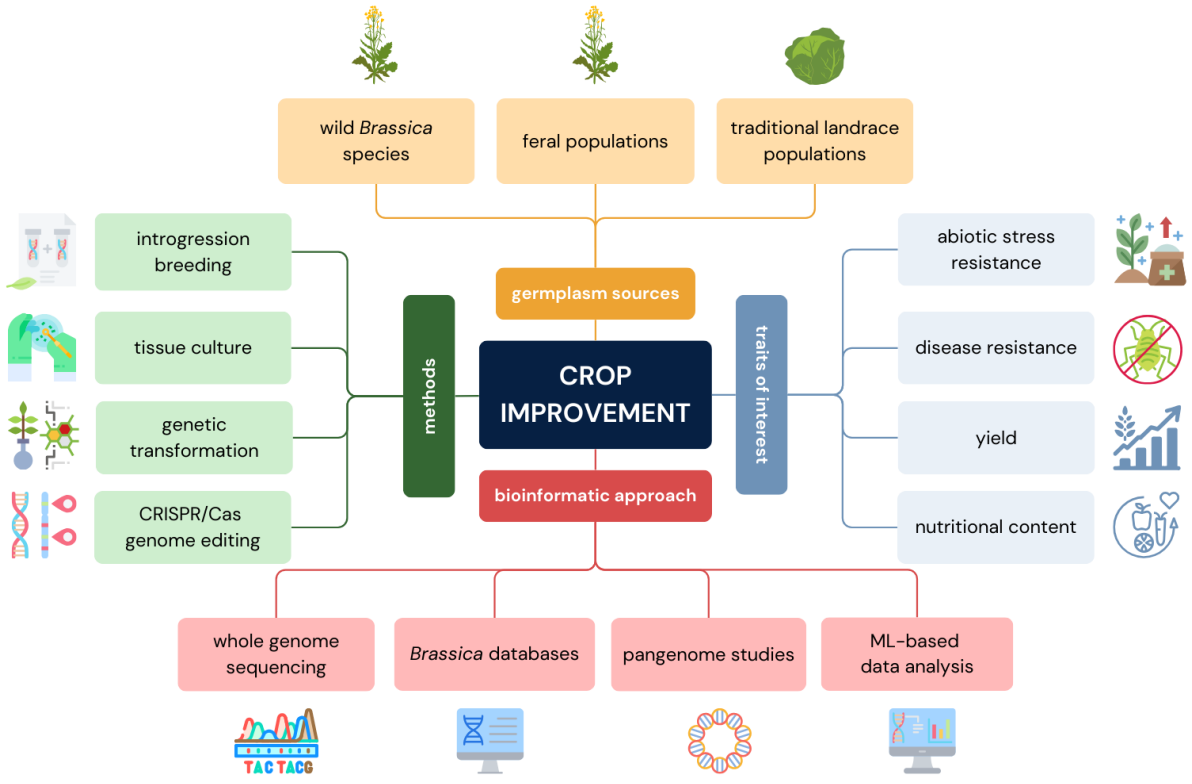

Figure 5. An integrated approach to *Brassica* crop improvement. Abbreviations: CRISPR/Cas – clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR associated protein, ML – machine learning. Image created with Canva online graphic design tool.

2. RESEARCH AIMS

This research aims to establish a method for quantifying the expression of *NAC041*, *NAC084*, *DREB2A*, and *HSFA2* genes associated with plant response to abiotic stress. Further, this research aims to investigate the effects of abiotic stress factors, including elevated temperature, drought, and salinity, as well as the combinations of these stress factors, on the expression of these four stress-related genes in wild cabbage (*Brassica incana*), a CWR of an agriculturally and economically important crop species *Brassica oleracea*, which potentially represents a significant source of germplasm for *Brassica* crop improvement.

3. MATERIALS AND METHODS

3.1. Materials

This section includes all materials that I used in my research, including plant material, chemicals, isolation kits, buffers, reagents, solutions, laboratory devices, and computer programs.

3.1.1. Plant material

Seeds of different wild cabbage accessions (*Brassica incana*) were collected along the Mediterranean and Croatian coast and islands, regenerated, and archived at the Institute of Agriculture and Tourism in Poreč, Croatia (IATPO). In this research, I used 9-day-old seedlings of four different *B. incana* accessions (**Table 1**) grown from the regenerated seeds provided by IATPO. After germination on 1% agar plates (8 days at 4 °C, followed by 24 h at 22 °C and a 16L:8D photoperiod), the seedlings were divided between five treatment groups, which were then exposed to the following abiotic stress treatments: high-temperature stress (1% agar plates exposed to the following temperature cycle: 24 °C 10 h | 30 °C 4 h | 40 °C 4 h 30 min | 30 °C 5 h 30 min), osmotic stress (1% agar plate containing 0.2 M mannitol, incubated at 22 °C), salt stress (1% agar plate containing 0.1 M NaCl, incubated at 22 °C), combined high-temperature and osmotic stress, and combined high-temperature and salt stress, as well as one control group that was not exposed to abiotic stress treatment (1% agar plate incubated at 22 °C). After the 24 h treatment exposure, the seedlings were frozen in liquid nitrogen and stored at -80 °C. Seedling cultivation and abiotic stress treatments were performed by Krunoslav Baotić, as precisely described in Baotić (2023).

Table 1. *B. incana* accessions with their respective collection locations and the number of biological replicates used in this research.

Accession	Location	Number of biological replicates per treatment group
IPT 514	Italy	3
IPT 520	Obljak, Croatia	3
IPT 521	Sušac, Croatia	2
IPT 522	Stupe, Croatia	2

3.1.2. Chemicals

All chemicals, isolation kits, buffers, reagents, and solutions that I used in this research are listed in **Table 2**.

Table 2. Chemicals, isolation kits, buffers, reagents, and solutions, with their respective manufacturers used in this research.

Method	Chemical/isolation kit/buffer/reagent/solution		Manufacturer
RNA isolation	MagMAX™ Plant RNA Isolation Kit		Thermo Scientific
	Ethanol (EtOH)		Kemika
	Dithiothreitol (DTT)		VWR
	DEPC-treated water		Thermo Scientific
Reverse transcription	5× Reaction Buffer		Thermo Scientific
	RiboLock RNAse inhibitor (40 U μl^{-1})		Thermo Scientific
	RevertAid H Minus Reverse Transcriptase (200 U μl^{-1})		Thermo Scientific
	Oligo$(dT)_{18}$ (0.5 μg μl^{-1})		Thermo Scientific
	dNTP mix (10 mM)		Sigma-Aldrich
DNA isolation	Genomic DNA from plant, NucleoSpin® Plant II		Macherey-Nagel
PCR	2× EmeraldAmp® GT PCR Master Mix		Takara Bio Inc.
Agarose-gel electrophoresis	Agarose		Sigma-Aldrich
	TAE Buffer	40 mM Tris	Sigma-Aldrich
		20 mM glacial acetic acid, pH 8.0	Gram-Mol
		1 mM EDTA	Sigma-Aldrich
	GeneRuler 100 bp DNA Ladder		Thermo Scientific
	Ethidium bromide (EtBr)		Sigma-Aldrich
qPCR	2× GoTaq® qPCR Master Mix		Promega

3.1.3. Laboratory instruments

All laboratory instruments that I used in this research are listed in **Table 3**.

Table 3. Laboratory instruments and their respective manufacturers used in this research.

Instrument	Manufacturer
Analytical balance, R 200 D	Sartorius
Centrifuge 5415 C	Eppendorf
GeneAmp® PCR System 2700	Applied Biosystems
Kodak EDAS 290	Kodak
Mic qPCR Cycler	Bio Molecular Systems
NanoVue™ Plus Spectrophotometer	Richmond Scientific
RunOne™ Electrophoresis System	Embi Tec
Thermomixer comfort	Eppendorf

3.1.4. Primers

The sequences of all primers used in standard polymerase chain reactions (PCR) and quantitative real-time PCR reactions (qPCR) are listed in **Table 4**. The primers were designed by Nataša Bauer and Mirta Tokić, based on gene sequences of *B. rapa* ssp. *pekinensis* and *B. oleracea* ssp. *oleracea*, and were previously tested on different *B. oleracea* var. *acephala* and *B. incana* accessions (Bauer et al. 2022, Baotić 2023). The primers were produced by Macrogen.

Table 4. Primer sequences used for standard PCR and qPCR reactions.

Gene	Primer	Sequence 5' - 3'
OGIO **Bra028284**	qB-OGIO-F2	CAGTATCGTAGCTGAGGTAGC
	qB-OGIO-R2	AGAACGGAACACATACTTGACTC
DREB2A **Bra009112**	qB-DREB2A-112-F1	AGCTGCAAAGCCTTGGCTCA
	qB-DREB2A-112-R1	GATCGAAGAAGTCACTACCATCT
NAC041 **Bra021856**	qB-NAC041-F3	CGAAGACGACAACAAGAGTGC
	qB-NAC041-R3	GAGTCACATTCAAATCGCAGC
NAC084 **Bra006229**	qB-NAC084-F2	AGGAAGAAGACAGAGGAAACC
	qB-NAC084-R2	GCTGAGGTAGGAGGAGATG
HSFA2 **Bra000557**	qB-HSFA2-F2	ATGAATGTGATGATGGAAGATGGT
	qB-HSFA2-R2	CTGCCCCAATCCAACGGTG

3.1.5. Computer programs

For quantitative gene expression analysis, I used the Mic qPCR Cycler software (BioMolecular Systems, version 2.12.7). For the statistical data analysis, I used Microsoft® Excel® for Microsoft 365 MSO (Version 2410 Build 16.0.18129.20158) and Statistics Kingdom (https://www.statskingdom.com/).

3.2. Methods

This section contains all experimental laboratory methods and statistical data analysis methods that I used in this research.

3.2.1. RNA isolation

I extracted total RNA from all 60 *B. incana* seedling samples using the MagMAX™ Plant RNA Isolation Kit (**Section 3.1.2**, **Table 2**), following the manufacturer's protocol. First, I homogenized the samples using a pre-cooled mortar and pestle filled with liquid nitrogen. I transferred the homogenized powder into labeled 1.5 ml microtubes and submerged them in liquid nitrogen until further use. I prepared a fresh aliquot of Lysis Buffer supplemented with DTT (50:1 volume ratio, as noted in the protocol). I added 600 µl of Lysis Buffer with DTT to each sample and vortexed the samples for 10 s. I briefly centrifuged the samples to collect liquid at the bottom of the tube, incubated them for 5 min at 56 °C, and centrifuged them for 10 min at 14 000 rpm. Next, I transferred 400 µl of the supernatant to clear 1.5 ml microtubes, added 25 µl of RNA Binding Beads and 400 µl of 96% EtOH in each tube, and vortexed the tubes for 10 s. After briefly centrifuging the tubes, I placed them on a magnetic stand for 2 min. Without removing the samples from the magnetic stand, I carefully removed the supernatant using a micropipette. Next, I added 700 µl of Wash Solution 1 to the beads, vortexed the tubes for 10 s, and briefly centrifuged them to collect liquid at the bottom of the tubes. I placed the tubes on a magnetic stand for another 2 min, after which I carefully removed the supernatant. Next, I incubated the samples on the magnetic strand at room temperature with an open lid to eliminate the remaining EtOH. I prepared the DNAse I Master Mix following the instructions from the manufacturer's protocol, added 200 µl of the DNAse I Master Mix to each tube, and thoroughly resuspended the samples to ensure that the DNAse reaches all DNA molecules in the sample. The only adaptation I made to the original protocol was prolonging the incubation with DNAse at 37 °C from the original 15 min to 1 h, to

ensure enough time for a thorough enzymatic degradation of DNA in the samples. After the incubation, I briefly centrifuged the samples and added 150 µl of Rebinding Buffer and 400 µl of 96% EtOH to each sample. After vortexing for 10 s and briefly centrifuging the tubes, I once again placed the tubes on a magnetic stand for 2 min. After removing the supernatant, I added 700 µl of Wash Solution 1 to the samples, briefly centrifuged, placed the samples on a magnetic stand for 2 min, and removed the supernatant. I repeated this step two more times using Wash Solution 2, after which I incubated the samples with open lids at room temperature for 5 min. I added 100 µl of nuclease-free water to the tubes, vortexed for 10 s, briefly centrifuged, and placed the tubes on a magnetic stand for 2 min. Without removing the tubes from the magnetic stand, I transferred the supernatant to clear 1.5 ml microtubes. I measured RNA concentration (at λ = 260 nm) and purity (absorbance ratios 260/230 and 260/280) in all samples using the NanoVue™ Plus Spectrophotometer (**Section 3.1.3**, **Table 3**). I stored the RNA extracts at -20 °C until further use.

3.2.2. Reverse transcription

Using the spectrometrically measured RNA concentrations, I calculated the volumes of RNA extracts containing 1 µg of RNA. For reverse transcription (RT), I prepared reaction mixtures by adding 1 µg of RNA template, 1 µl of Oligo(dT)$_{18}$ primers, and RNase-free water to bring the total volume of the mixtures to 12.5 µl (**Section 3.1.2**, **Table 2**). I incubated the reaction mixtures for 5 min at 65 °C in the GeneAmp® PCR System 2700 (**Section 3.1.3**, **Table 3**). After that, I cooled the mixtures at 4 °C and prepared the RT master mix by mixing 1× (4 µl) Reaction Buffer, 20 U (0.5 µl) of RiboLock RNAse inhibitor, 2 µl of dNTP mix (10 mM), and 200 U (1 µl) of RevertAid H Minus Reverse Transcriptase per sample (**Section 3.1.2**, **Table 2**). After adding the master mix to the samples, I incubated them for 1 hour at 42 °C for RT, and then for 10 min at 70 °C to inactivate the reverse transcriptase. Finally, I diluted the samples with nuclease-free water to make the approximate cDNA concentration in each sample 10 ng µl^{-1}. To confirm the presence of cDNA and check for possible gDNA contamination, I performed standard PCR analysis as described in **Section 3.2.4**.

3.2.3. Genomic DNA isolation

I extracted genomic DNA from four samples, one from each of the *B. incana* accessions (514, 520, 521, and 522). All four samples were from the control group and were thus not treated

with abiotic stress. For the genomic DNA extraction, I used the NucleoSpin® Plant II kit (**Section 3.1.2**, **Table 2**), following the manufacturer's "Genomic DNA from plant" protocol. I homogenized the plant tissue using a pre-cooled mortar and pestle filled with liquid nitrogen and transferred the homogenized powder into 1.5 ml microtubes. I added 400 µl of Lysis Buffer PL1, vortexed the tubes for 10 s, and added 10 µl of RNAse A. Next, I incubated the samples for 10 min at 65 °C. After incubation, I prepared clear 2 ml collection tubes with NucleoSpin® Filters, loaded the lysates onto the columns, centrifuged them for 2 min at 14 000 rpm, and discarded the filter. For adjusting DNA binding conditions, I added 450 µl of Buffer PC to the collected flowthrough and thoroughly resuspended the samples. I prepared clear 2 ml collection tubes with NucleoSpin® Plant II Columns for DNA binding, loaded 700 µl of the samples onto them, centrifuged for 1 min at 14 000 rpm, and discarded the flowthrough. Next, I added 400 µl Buffer PW1 onto the column, centrifuged for 1 min at 14 000 rpm, and discarded the flowthrough. I repeated this washing step two more times, first with 700 µl Buffer PW2, and then with 200 µl Buffer PW2, after which I centrifuged the samples for 2 min at 14 000 rpm. Finally, I placed the columns into new 1.5 ml microtubes, added 50 µl Buffer PE (pre-warmed at 65 °C) onto the membrane, incubated the samples for 5 min at 65 °C, and centrifuged for 1 min at 14 000 rpm. I repeated the step with Buffer PE one more time. I measured DNA concentration (at λ = 260 nm) and purity (absorbance ratios 260/230 and 260/280) in all samples using the NanoVue™ Plus Spectrophotometer (**Section 3.1.3**, **Table 3**). I stored the genomic DNA extracts at -20 °C until further use.

3.2.4. Standard PCR analysis

For all standard PCR reactions, I prepared mixtures containing 1× (12.5 µl) EmeraldAmp® GT PCR Master Mix (**Section 3.1.2**, **Table 2**), 1 µl of DNA template (~10 ng μl^{-1}), 0.5 µl of forward and reverse primers (0.1 mM) (**Section 3.1.4**, **Table 4**), and 10.5 µl of nuclease-free water in a total volume of 25 µl. All PCR reactions were performed in the GeneAmp® PCR System 2700 (**Section 3.1.3**, **Table 3**), with the initial denaturation step at 98 °C for 3 min, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing at 58 °C for 30 s, and extension at 72 °C for 60 s, and the final extension step at 72 °C for 5 min. After PCR amplification, the reaction mixtures were stored at 4 °C.

3.2.5. Agarose-gel electrophoresis

I prepared 2% and 2.5% agarose gels in TAE Buffer (40 mM Tris base, 20 mM glacial acetic acid, pH 8.0, 1 mM EDTA). I loaded the wells with 3 µl of molecular marker and 10 µl of PCR samples. I used GeneRuler 100 bp DNA Ladder (**Section 3.1.2**, **Table 2**) as a molecular marker (**Figure 6**). Electrophoresis was performed at 25 V for the first 5 min, then at 50 V until the sample stain reached the bottom of the gel, using the RunOne™ Electrophoresis System (**Section 3.1.3**, **Table 3**). I stained the gels in a 10 ng l^{-1} EtBr solution for 5-10 min and photographed them under UV light using the Kodak EDAS 290 camera, with 3 s exposure time and 100% UV strength.

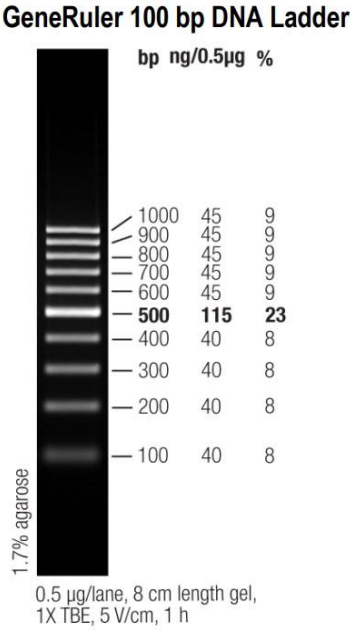

Figure 6. Molecular marker GeneRuler 100 bp DNA Ladder (Thermo Scientific).

3.2.6. Quantitative real-time PCR analysis

For quantifying the expression of stress-related *NAC041*, *NAC084*, *DREB2A*, and *HSFA2* genes in four *B. incana* accessions (514, 520, 521, and 522) under five different forms of abiotic stress, I performed a qPCR analysis. I used a reference gene *OGIO* (Bra028284) as the internal control (Bauer et al. 2022). I prepared the qPCR reaction mixtures by adding 1× (7.5 µl) GoTaq® qPCR Master Mix (**Section 3.1.2**, **Table 2**), 0.2 µl of forward and reverse primers (0.1 mM) (**Section 3.1.4**, **Table 4**), 10 ng (1 µl) of DNA template (or water for no template control), and

6.1 µl of nuclease-free water in a total volume of 15 µl. All qPCR reactions were performed in two technical replicates, using the Mic qPCR Cycler (**Section 3.1.3**, **Table 3**) with the following run profile: initial denaturation at 95 °C for 5 min, followed by 7 pre-cycles of touchdown PCR with denaturation at 95 °C for 5 s and combined annealing and extension step at 66 → 60 °C for 20 s, lowering the temperature by 1 °C in each of the 7 pre-cycles, followed by 30 cycles of 95 °C for 5 s, and 60 °C for 30 s. Melting curves were generated from 70 °C to 92 °C at a ramp speed of 0.1 °C s^{-1}. I calculated the relative gene expression using the ΔΔCt method (Pfaffl 2001).

3.2.7. Statistical data analysis

In the qPCR analysis, I performed three biological replicates for accessions 514 and 520, and two biological replicates for accessions 521 and 522. For accessions 521 and 522, a third biological replicate, taken from Baotić (2023), was included in the downstream gene expression quantification. To test the statistical significance of the differences in gene expression between the control and treatment groups, I performed a Student's t-test. Furthermore, to compare basal gene expression under control conditions between different *B. incana* accessions, I performed a one-way analysis of variance (ANOVA), followed by Tukey's HSD post hoc test. Results were considered significantly different at p-value ≤ 0.05.

4. RESULTS

I performed a quantitative gene expression analysis of four stress-related genes, *NAC041*, *NAC084*, *DREB2A*, and *HSFA2* in four *B. incana* accessions (514, 520, 521, and 522) exposed to five different abiotic stress treatments: high-temperature stress, mannitol-induced osmotic stress, NaCl-induced salt stress, a combination of high-temperature and mannitol-induced osmotic stress, and a combination of high-temperature and NaCl-induced salt stress.

4.1. DNA and RNA isolation

Concentrations and purities of DNA isolated from four *B. incana* accessions under control conditions are listed in **Table 5**. Concentrations and purities of cDNA isolated from all 60 *B. incana* samples are listed in **Table 6**.

Table 5. Concentrations and purities of DNA isolated from four *B. incana* accessions under control conditions.

Accession	DNA (ng µl^{-1})	260/280	260/230
514	11.1	2.274	0.498
520	13.9	2.109	0.724
521	12.2	1.788	0.806
522	86.3	1.425	1.05

Table 6. Concentrations and purities of RNA isolated from four *B. incana* accessions under control conditions and five different abiotic stress treatments. Abbreviations: **C** – control group, **M** – osmotic stress, **S** – salt stress, **T** – high-temperature stress, **TM** – combined high-temperature and osmotic stress, **TS** – combined high-temperature and salt stress.

Accession	Biological replicate	Group	RNA (ng µl^{-1})	260/280	260/230
514	**1**	**C**	24.8	2.255	1.394
		S	51.0	2.257	0.973
		M	76.8	2.087	1.722
		T	96.6	2.148	1.417
		TS	112.2	2.159	1.671
		TM	97.2	2.295	2.025

Table 6. Continued.

Accession	Biological replicate	Group	RNA (ng µl^{-1})	260/280	260/230
514	2	C	115.4	2.098	2.215
		S	94.8	2.116	1.845
		M	71.6	2.132	1.875
		T	59.6	2.114	1.991
		TS	115.5	2.102	1.296
		TM	88.2	2.082	1.800
	3	C	76.4	2.250	1.593
		S	90.8	2.248	1.494
		M	88.0	2.178	1.931
		T	162.8	2.189	1.889
		TS	126.4	2.274	2.122
		TM	98.4	2.268	1.790
520	1	C	181.2	2.051	2.221
		S	111.0	2.042	2.060
		M	94.6	2.092	1.886
		T	109.0	2.034	2.190
		TS	144.6	2.056	2.126
		TM	69.6	1.967	2.486
	2	C	160.2	1.682	1.260
		S	99.8	1.737	1.133
		M	70.4	2.217	1.546
		T	203.4	1.894	1.555
		TS	262.8	1.904	1.613
		TM	124.4	2.102	2.711
	3	C	227.0	1.843	1.455
		S	122.6	1.649	1.182
		M	80.2	2.111	1.630
		T	213.8	1.810	1.578
		TS	144.6	2.146	2.014
		TM	96.2	2.138	1.940
521	1	C	123.8	2.070	1.930
		S	34.2	1.970	21.8
		M	145.8	2.190	1.592
		T	126.6	2.183	1.557
		TS	73.6	2.045	0.866
		TM	22.8	2.683	1.311

Table 6. Continued.

Accession	Biological replicate	Group	RNA (ng µl^{-1})	260/280	260/230
521	**2**	**C**	141.2	2.095	1.990
		S	71.0	2.064	1.942
		M	166.8	2.139	2.317
		T	276.4	2.143	2.097
		TS	351.8	2.122	2.188
		TM	243.4	2.166	2.002
522	**1**	**C**	108.2	1.554	1.120
		S	197.4	1.665	1.378
		M	46.4	2.169	0.737
		T	187.6	1.669	1.370
		TS	87.0	2.104	1.422
		TM	98.6	2.144	1.915
	2	**C**	61.4	2.075	1.264
		S	56.2	2.113	1.861
		M	30.6	2.468	1.866
		T	66.0	2.158	2.143
		TS	55.6	2.079	1.580
		TM	81.0	2.166	1.867

4.2. Primer validation and confirmation of cDNA presence and purity

To validate primer annealing and check for possible gene variation between different *B. incana* accessions, I performed qPCR analysis on *OGIO*, *NAC041*, *NAC084*, *DREB2A*, and *HSFA2* genes using gene-specific primers (**Section 3.1.4**, **Table 4**). As templates, I used one cDNA and one gDNA sample of seedlings exposed to control conditions from each accession. In all four *B. incana* accessions, *OGIO* primers were intron-spanning. The *OGIO* fragment amplified from a gDNA template contained an intronic region and was about 100 bp longer than the fragment amplified from a cDNA template (121 bp). This difference in PCR product lengths between cDNA and gDNA templates is thus an excellent indicator of gDNA presence (contamination) in the cDNA samples. All primers successfully annealed to cDNA and gDNA templates with high specificity, and no gDNA contamination was detected in the cDNA samples (**Figure 7**). There was no difference in *OGIO* band size or intensity between the four *B. incana* accessions. A small but

visible difference in band sizes indicated that the amplified *HSFA2* fragment is somewhat smaller in accessions 521 and 522 than in accessions 514 and 520. Barely visible or completely missing bands indicated very low to non-existent *NAC041* expression in accessions 514, 520, and 521 under control conditions. Similarly, *NAC084* expression was very low to non-existent under control conditions in accessions 520, 521, and 522, while low *HSFA2* expression under control conditions was detected in accessions 514, 520, and 522. Since bands were visible in all gDNA samples and at least one cDNA sample per gene, all the primers were successfully validated.

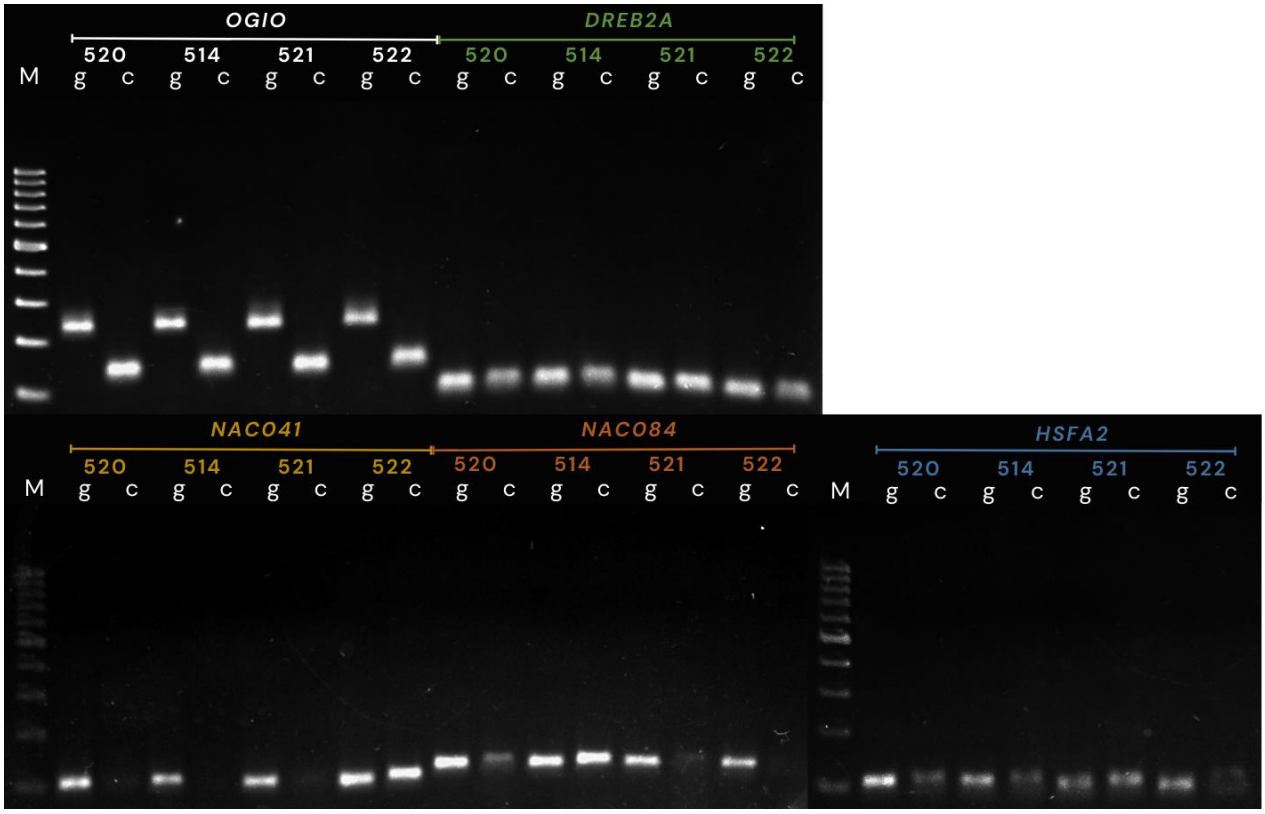

Figure 7. Primer validation and confirmation of cDNA purity in samples of four *B. incana* accessions (514, 520, 521, and 522) under control conditions. The bands represent fragments amplified by qPCR with *OGIO*, *DREB2A*, *NAC041*, *NAC084*, and *HSFA2* primers and either cDNA or gDNA template. Abbreviations: **c** – cDNA, **g** – gDNA, **M** – molecular marker GeneRuler 100 bp DNA Ladder (Thermo Scientific).

To confirm cDNA purity in all 60 samples, I performed standard PCR analysis with *OGIO* primers. All cDNA samples showed successful amplification of the *OGIO* gene fragment with a band at precisely 121 bp, as expected (**Figure 8**). All cDNA samples were free of gDNA contamination and suitable for *NAC041*, *NAC084*, *DREB2A*, and *HSFA2* gene quantification analysis by qPCR.

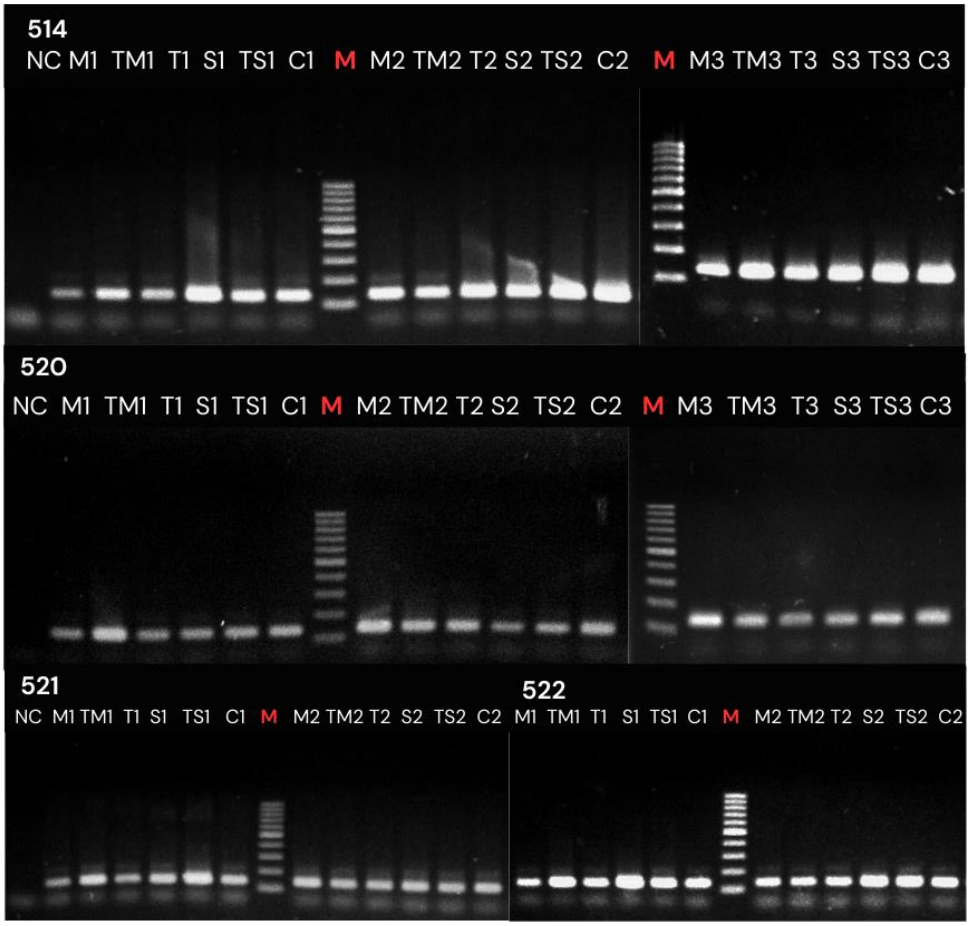

Figure 8. Confirmation of cDNA purity in samples of four *B. incana* accessions (514, 520, 521, and 522). The bands represent 121 bp long fragments amplified by standard PCR with *OGIO* primers and cDNA template. Abbreviations: **C** – control group, **NC –** no template control, **M** – osmotic stress, **S** – salt stress, **T** – high-temperature stress, **TM** – combined high-temperature and osmotic stress, **TS** – combined high-temperature and salt stress, **M** – molecular marker GeneRuler 100 bp DNA Ladder (Thermo Scientific).

4.3. Stress-related gene expression under control conditions

Basal relative gene expression was measured by qPCR analysis, with a reference gene *OGIO* as the internal control. Primer efficiencies used for calculating relative gene expression are listed in **Table 7**. I compared basal *NAC041*, *NAC084*, *DREB2A*, and *HSFA2* gene expression between four *B. incana* accessions (514, 520, 521, and 522) under control conditions (**Figure 9**). The basal expression of *NAC041* did not significantly vary between the four accessions. Accession 514 had a significantly higher basal expression of *NAC084* than the other three accessions, while accession 521 showed a significantly higher expression of *DREB2A* and *HSFA2* genes compared to the other three *B. incana* accessions.

Table 7. Primer efficiencies used for calculating relative gene expression using the ΔΔCt method.

Accession	Biological replicate	E(*OGIO*)	E(*NAC041*)	E(*NAC084*)	E(*DREB2A*)	E(*HSFA2*)
514	**1**	1.830		1.828	1.876	1.833
	2	1.822		1.837	1.842	1.841
	3	1.831	1.856	1.834	1.804	1.843
520	**1**	1.805	1.866	1.833	1.820	1.860
	2	1.829		1.812	1.822	1.856
	3	1.839		1.805	1.845	1.849
521	**1**	1.821	1.853	1.823	1.831	1.837
	2	1.812	1.876	1.821	1.818	1.818
522	**1**	1.812	1.864	1.798	1.833	1.833
	2	1.809	1.880	1.821	1.838	1.829

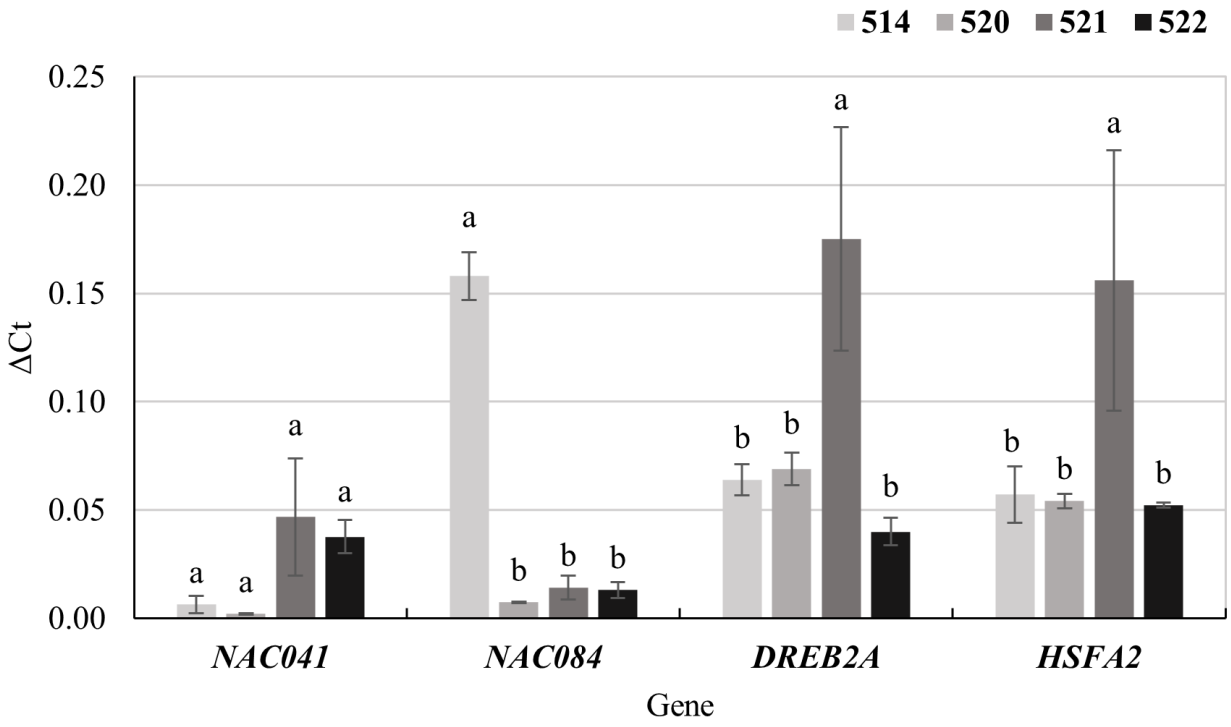

Figure 9. Basal relative gene expression of stress-related genes *NAC041*, *NAC084*, *DREB2A*, and *HSFA2* in four *B. incana* accessions (514, 520, 521, and 522) under control conditions, measured by qPCR compared to a reference gene *OGIO*. The graph shows average ΔCt values of two to three biological replicates ± SE. Different letters represent significant difference between accessions for each individual gene (one-way ANOVA, Tukey's HSD post hoc test, $p \leq 0.05$).

4.4. Stress-related gene expression quantification under abiotic stress

Relative gene expression was measured by qPCR analysis, with a reference gene *OGIO* as the internal control. Changes in transcription levels of gene *NAC041* in *B. incana* accessions 514, 520, 521, and 522 under abiotic stress conditions are shown in **Figure 10**. In accession 520, no *NAC041* expression was detected under any conditions. Low *NAC41* expression detected under control conditions was completely lost under all abiotic stress conditions in accession 514. All abiotic stress conditions caused a decrease in *NAC041* expression in accession 521, although none were statistically significant. Accession 522 exhibited a significant decrease in *NAC041* expression only under osmotic stress and a slight, but statistically non-significant increase in *NAC041* expression under high-temperature and combined high-temperature and salt stress conditions.

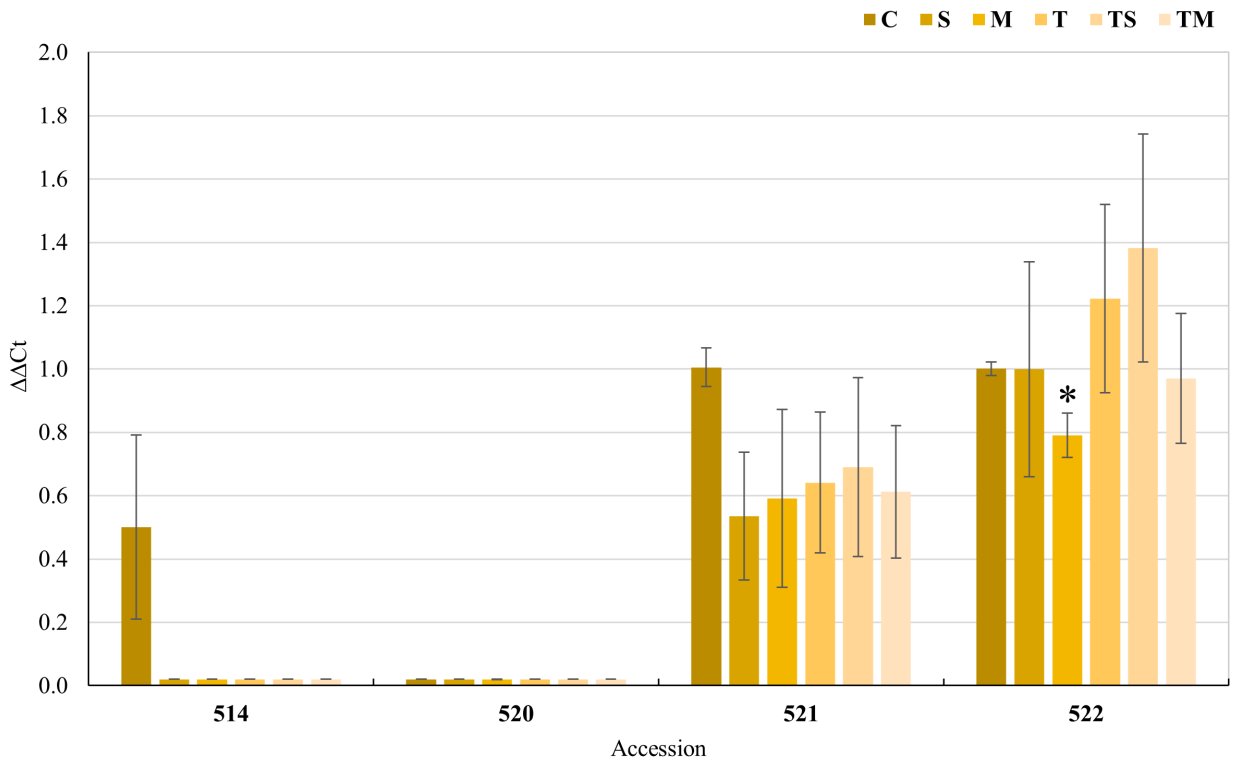

Figure 10. Relative gene expression of stress-related *NAC041* gene in four *B. incana* accessions (514, 520, 521, and 522) under control conditions and five different abiotic stress conditions. Gene expression was measured by qPCR compared to a reference gene *OGIO*, and is presented as fold change compared to the control group. The graph shows average ΔΔCt values of two to three biological replicates ± SE. Asterisk symbol (*) represents significant difference in gene expression under abiotic stress compared to control conditions in each individual accession (Student's t-test, $p \leq 0.05$). Abbreviations: **C** – control group, **M** – osmotic stress, **S** – salt stress, **T** – high-temperature stress, **TM** – combined high-temperature and osmotic stress, **TS** – combined high-temperature and salt stress.

Changes in transcription levels of gene *NAC084* in *B. incana* accessions 514, 520, 521, and 522 under different abiotic stress conditions are shown in **Figure 11**. Accession 514 showed a significant change in *NAC084* expression under all abiotic stress conditions. Under salt and osmotic stress *NAC084* expression had increased, while high-temperature stress and both of the combined stress conditions led to a significant decrease in *NAC084* expression in accession 514. Accession 520 displayed a significant decrease in *NAC084* expression under high-temperature and combined high-temperature and salt stress conditions. Accession 521 displayed a significant decrease in *NAC084* expression under high-temperature stress, while accession 522 displayed a significant decrease in *NAC084* expression under combined high-temperature and osmotic stress conditions.

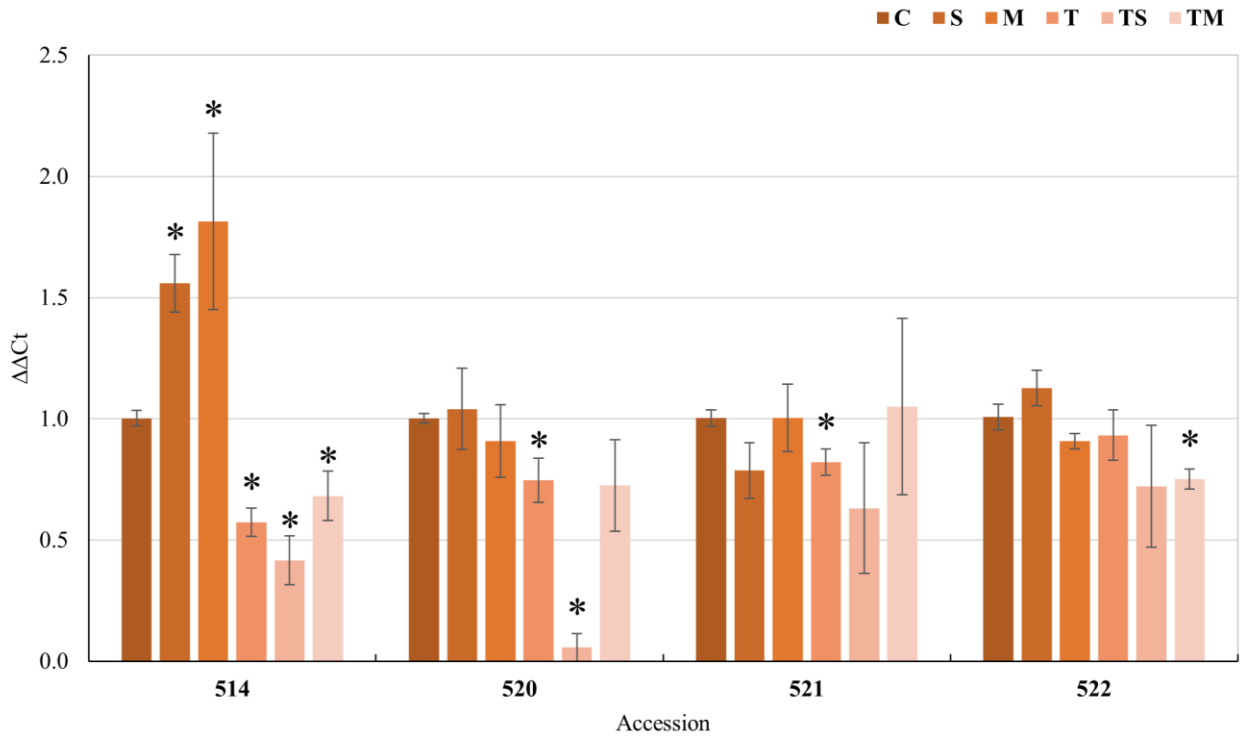

Figure 11. Relative gene expression of stress-related *NAC084* gene in four *B. incana* accessions (514, 520, 521, and 522) under control conditions and five different abiotic stress conditions. Gene expression was measured by qPCR compared to a reference gene *OGIO* and is presented as fold change compared to the control group. The graph shows average ΔΔCt values of three biological replicates ± SE. Asterisk symbol (*) represents significant difference in gene expression under abiotic stress compared to control conditions in each individual accession (Student's t-test, $p \leq 0.05$). Abbreviations: **C** – control group, **M** – osmotic stress, **S** – salt stress, **T** – high-temperature stress, **TM** – combined high-temperature and osmotic stress, **TS** – combined high-temperature and salt stress.

Changes in transcription levels of gene *DREB2A* in *B. incana* accessions 514, 520, 521, and 522 under different abiotic stress conditions are shown in **Figure 12**. Accession 514 exhibited a significant increase in *DREB2A* expression under all five abiotic stress conditions. The fold change was especially large under high-temperature and both of the combined stress conditions. Accession 520 showed a significant increase in *DREB2A* expression under high-temperature and both of the combined stress conditions. Accession 521 showed a significant increase in *DREB2A* expression under salt stress and both of the combined stress conditions. Accession 522 showed a significant increase in *DREB2A* expression under all five abiotic stress conditions, with an especially large fold change detected under osmotic stress and combined high-temperature and salt stress.

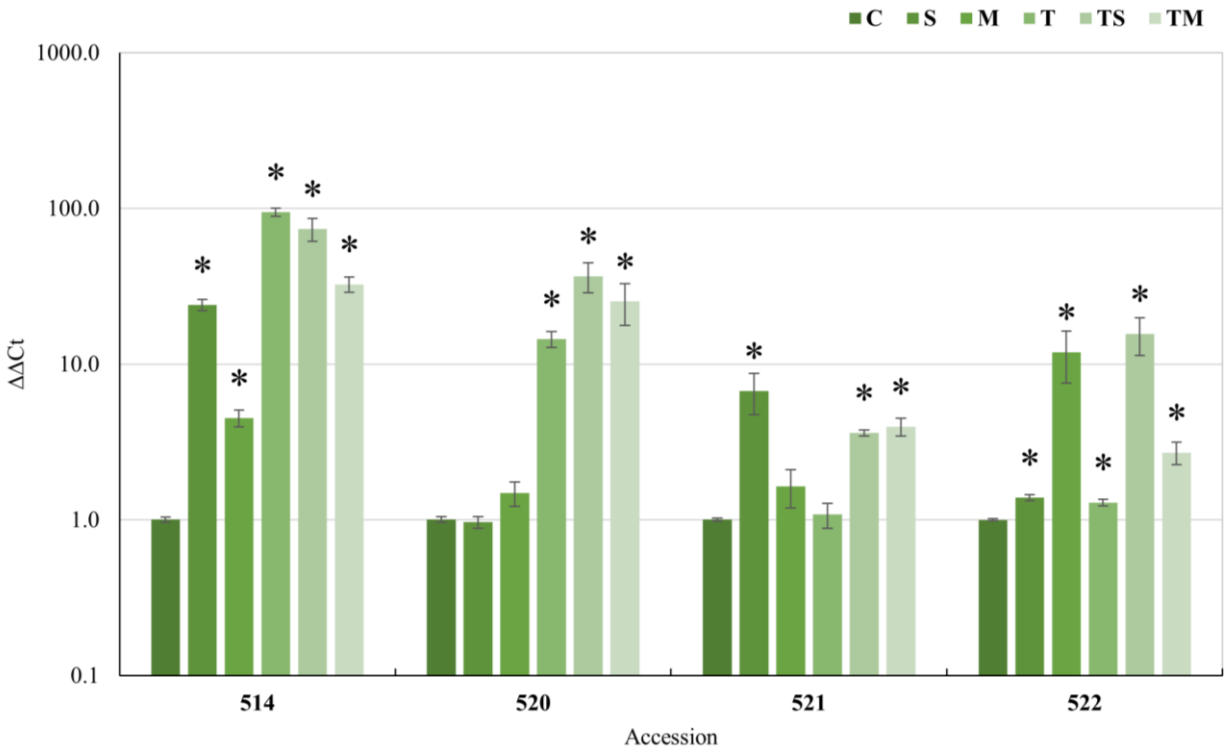

Figure 12. Relative gene expression of stress-related *DREB2A* gene in four *B. incana* accessions (514, 520, 521, and 522) under control conditions and five different abiotic stress conditions. Gene expression was measured by qPCR compared to a reference gene *OGIO*, and is presented as fold change compared to the control group. The graph shows average ΔΔCt values of three biological replicates ± SE. Asterisk symbol (*) represents significant difference in gene expression under abiotic stress compared to control conditions in each individual accession (Student's t-test, $p \leq 0.05$). The *y*-axis is shown in a logarithmic scale. Abbreviations: **C** – control group, **M** – osmotic stress, **S** – salt stress, **T** – high-temperature stress, **TM** – combined high-temperature and osmotic stress, **TS** – combined high-temperature and salt stress.

Changes in transcription levels of gene *HSFA2* in *B. incana* accessions 514, 520, 521, and 522 under different abiotic stress conditions are shown in **Figure 13**. Accession 514 displayed a significant increase in *HSFA2* expression under all five abiotic stress conditions, with an especially large fold change under high-temperature and both of the combined stress treatments. Accession 520 displayed a significant increase in *HSFA2* expression under all abiotic stress conditions except salt stress, with an especially large fold change under high-temperature and both of the combined stress treatments. Accession 521 displayed an increase in *HSFA2* expression under all treatments, especially under combined high-temperature and salt stress, but these changes were not statistically significant due to large variations between different biological replicates.

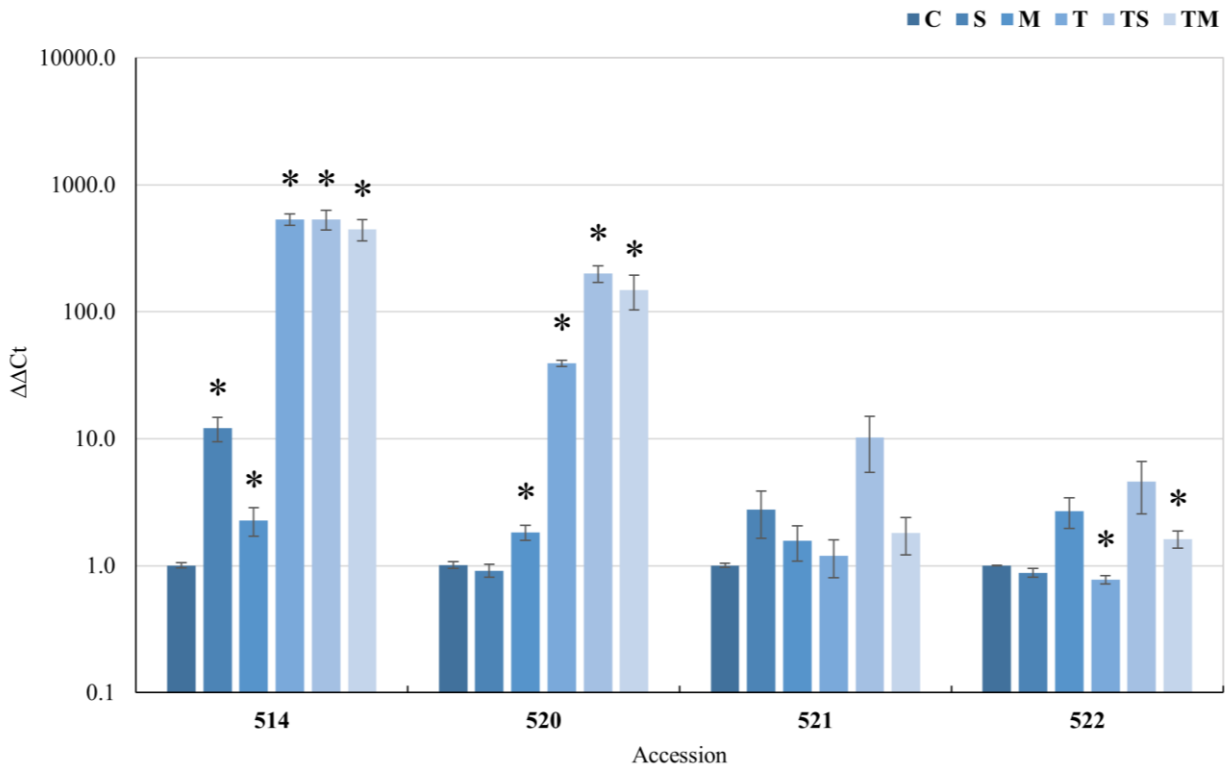

Figure 13. Relative gene expression of stress-related *HSFA2* gene in four *B. incana* accessions (514, 520, 521, and 522) under control conditions and five different abiotic stress conditions. Gene expression was measured by qPCR compared to a reference gene *OGIO*, and is presented as fold change compared to the control group. The graph shows average ΔΔCt values of two to three biological replicates ± SE. Asterisk symbol (*) represents significant difference in gene expression under abiotic stress compared to control conditions in each individual accession (Student's t-test, $p \leq 0.05$). The *y*-axis is shown in a logarithmic scale. Abbreviations: **C** – control group, **M** – osmotic stress, **S** – salt stress, **T** – high-temperature stress, **TM** – combined high-temperature and osmotic stress, **TS** – combined high-temperature and salt stress.

A similar situation was detected in accession 522, where a non-significant increase in *HSFA2* expression was detected under osmotic stress and combined high-temperature and salt stress. Additionally, a significant decrease in *HSFA2* expression was detected under high-temperature stress, and a significant increase in *HSFA2* expression under combined high-temperature and osmotic stress. Quantitative *HSFA2* expression analysis in accessions 521 and 522 was based on only two biological replicates, which could have impacted the statistical significance of these results.

4.5. Melting curves

I analyzed the melting curves generated during qPCR amplification to further evaluate primer annealing and specificity. Most samples showed highly specific primer annealing with a single peak at a specific melting temperature (Tm) point (**Figure 14**).

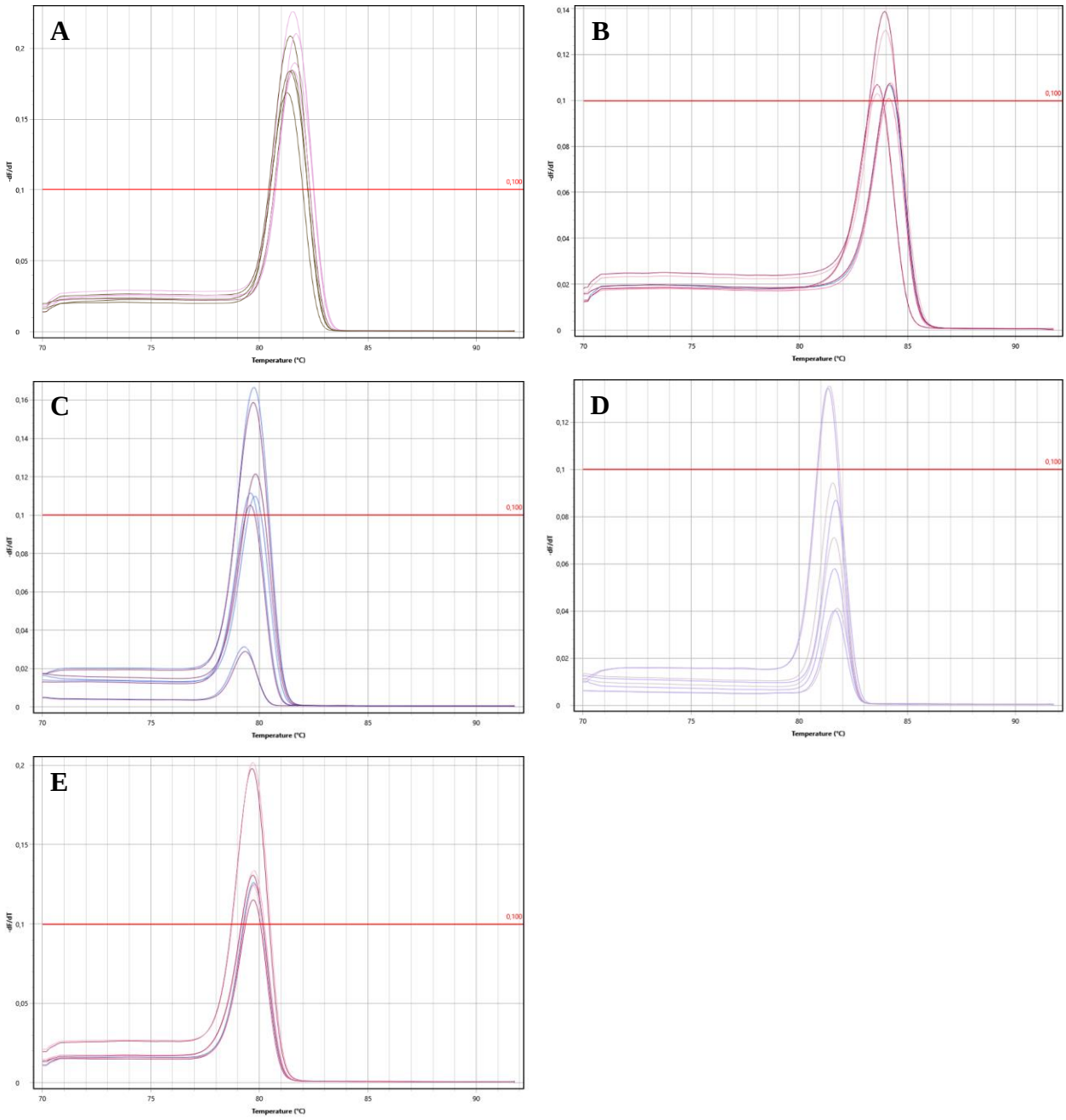

Figure 14. Melting curves of qPCR amplicons from four *B. incana* accessions (514, 520, 521, and 522) under control conditions using the following primers: **A**) *OGIO*, **B**) *DREB2A*, **C**) *NAC041*, **D**) *NAC084*, and **E**) *HSFA2*. Created with Mic qPCR Cycler device and software (Bio Molecular Systems, version 2.12.7).

Interestingly, *DREB2A* melting curves appeared to have a peak in at least two different Tm points, one at about 83.5 °C and another at about 84.2 °C (**Figure 14B**). This could indicate the presence of multiple different *DREB2A* gene or transcript variants. The melting curves of some samples from accession 514 amplified with *HSFA2* primers showed a shoulder peak to the left of the main peak (**Figure 15A**). These *HSFA2* shoulder peaks appeared sporadically within the accession 514, under different abiotic stress treatments. I performed an agarose-gel electrophoresis assay with these samples, and the gels showed only one band of the expected size (around 100 bp) for the *HSFA2* amplicon, which excluded the possibility of non-specific amplification (**Figure 15B**). The shoulder peak could have appeared due to the *HSFA2* amplicon melting in a complex manner, or perhaps due to the amplification of alternative cDNA fragments or pseudogenes of the same size as the expected *HSFA2* fragment.

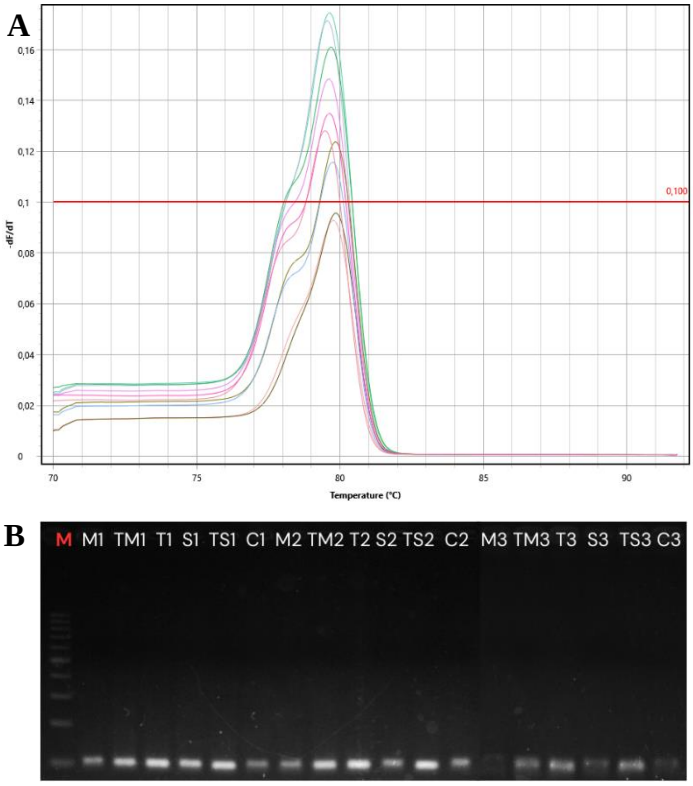

Figure 15. A) Melting curves of qPCR amplicons in samples of *B. incana* accession 514 under different abiotic stress treatments amplified with *HSFA2* primers, showing a shoulder peak to the left of the main peak. Created with Mic qPCR Cycler device and software (Bio Molecular Systems, version 2.12.7). **B**) *HSFA2* fragment on gel in samples of *B. incana* accession 514 under different abiotic stress treatments. Abbreviations: **C** – control group, **NC –** no template control, **M** – osmotic stress, **S** – salt stress, **T** – high-temperature stress, **TM** – combined high-temperature and osmotic stress, **TS** – combined high-temperature and salt stress, **M** – molecular marker GeneRuler 100 bp DNA Ladder (Thermo Scientific).

Further, some samples amplified with *NAC084* primers showed a smaller second peak to the left of the main peak (**Figure 16**). This pattern appeared sporadically across all four *B. incana* accessions, but only in those samples where the amplification didn't exceed the threshold value, indicating a very low or undetectable expression of *NAC084*. Thus, the second peak could indicate primer-dimer formation due to the lack of cDNA template in the sample. Additionally, *NAC084* melting curves exhibited a shift from a singular main peak, which was detected sporadically across all accessions and abiotic stress treatments. The Tm points ranged between 81.0 °C and 81.9 °C (**Figure 14D**, **Figure 16**).

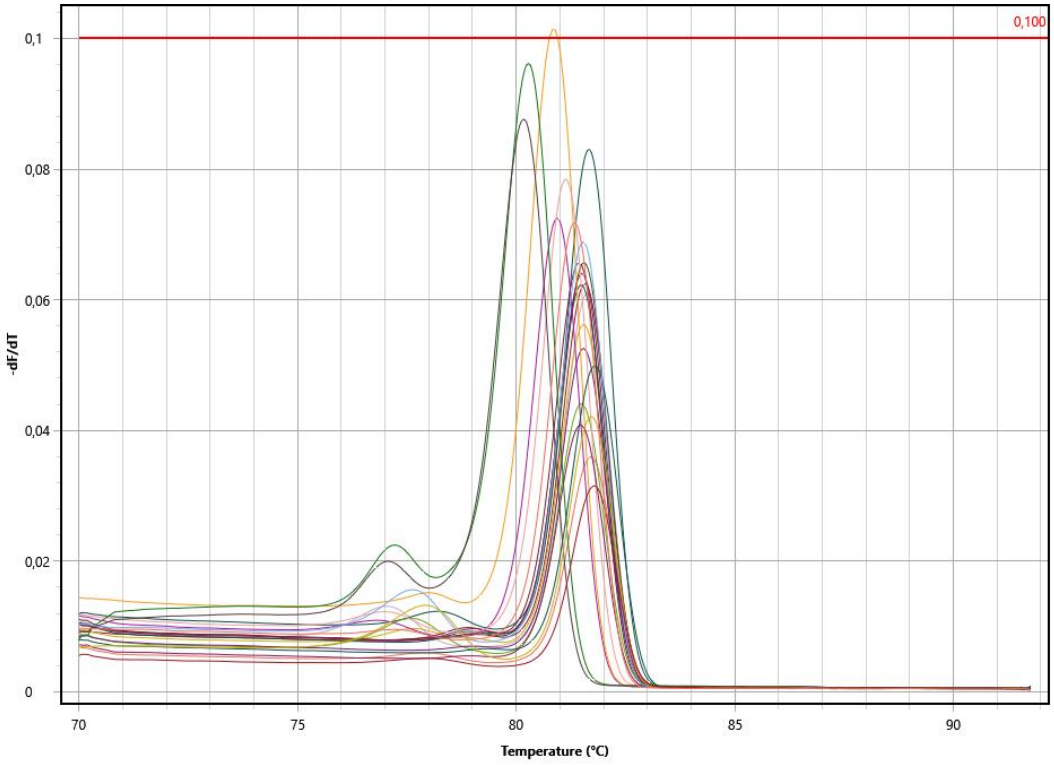

Figure 16. Melting curves of qPCR amplicons in samples of four *B. incana* accessions (514, 520, 521, and 522) amplified with *NAC084* primers, showing a smaller second peak to the left of the main peak. Created with Mic qPCR Cycler device and software (Bio Molecular Systems, version 2.12.7).

5. DISCUSSION

As a devastating consequence of global climate change, wild plant species and agricultural crops are facing extreme environmental conditions. Abiotic stress factors, mainly elevated temperature, drought, and salinity, negatively affect plant growth and development, leading to devastating losses in agricultural crop yield and quality. In field conditions, plants are usually exposed to multiple abiotic stress factors simultaneously. This makes the complex response mechanisms of plants to combined abiotic stresses a research topic of critical importance. Along with germplasm conservation and the development of new crop improvement strategies, selection of valuable resistance traits is the key to ensuring global food security in the face of the ongoing climate crisis. *Brassica* crop wild relatives are especially interesting for studying plant resistance mechanisms since many of them live in harsh environmental conditions and possess abiotic stress resistance traits valuable for the improvement of *Brassica* crops. To study the effects of abiotic stress on the expression of stress-related *NAC041*, *NAC084*, *DREB2A*, and *HSFA2* genes in four wild cabbage (*Brassica incana*) accessions (514, 520, 521, and 522), I performed a quantitative gene expression analysis.

As the internal control in qPCR analysis, I used a reference gene *OGIO* because of its previously confirmed stable expression and reliability in *A. thaliana* (Škiljaica et al. 2022), *B. oleracea* var. *acephala* (Bauer et al. 2022), and *B. incana* (Baotić 2023). The *OGIO* primers I used are intron-spanning, resulting in a 100 bp longer fragment when amplifying from a gDNA template compared to the 121 bp fragment amplified from a cDNA template (Talanga Vasari 2022). This makes them ideal for detecting unwanted gDNA residue in cDNA samples. In this research, I further confirmed stable *OGIO* expression in all four *B. incana* accessions, which makes it a suitable reference gene in quantitative gene expression analysis.

NAC041 and NAC084 are transcription factors from the NAC family of plant-specific transcription factors that are known to have important roles in plant growth, development, and abiotic stress tolerance. The expression of different stress-responsive NAC factors is induced by drought and salinity in *A. thaliana* and rice (*Oryza sativa*) (Nakashima et al. 2012). Multiple studies have shed light on the role of NAC factors in the regulation of ROS machinery, with some suggesting that they induce cell death due to ROS accumulation in *B. napus* and tobacco (*Nicotiana tabacum*), while others suggest that the overexpression of NAC factors leads to enhanced abiotic stress tolerance due to ROS detoxification (Fang et al. 2015, Wang et al. 2015, Lohani et al. 2020).

Many *NAC* genes are known to be downregulated under exposure to abiotic stress. Out of 204 *NAC* genes identified in *B. rapa*, nearly half are repressed under heat and drought stress (Liu et al. 2014). *NAC041* is downregulated under mannitol-induced osmotic stress, heat stress, and combined stress in multiple *B. oleracea* var. *acephala* accessions, along with one accession having a low basal *NAC041* expression (Bauer et al. 2022). This is somewhat consistent with my results on *B. incana*, where accessions 514 and 520 had a very low or no basal *NAC041* expression which did not significantly change under any abiotic stress conditions On the other hand, *B. incana* accessions 521 and 522 expressed *NAC041* under control conditions. A reduction in *NAC041* expression was detected under all abiotic stress treatments in accession 521, although it was not statistically significant due to large variations among biological replicates. A significant decrease in *NAC041* expression was detected in accession 522 under osmotic stress. A similar trend is detected by Baotić (2023), where *NAC041* is downregulated under osmotic stress in the same *B. incana* accession 522, although this change is not statistically significant. Further, Baotić (2023) detects a significant reduction in *NAC041* expression under salt and osmotic stress, and a non-significant reduction under high-temperature and combined high-temperature and salt stress in accession 521. However, it is important to highlight that these quantitative gene expression results are based on only one biological replicate and may not represent realistic gene expression patterns in *B. incana* under abiotic stress. Additionally, accessions 521 and 522 show a significant increase in antioxidant activity under osmotic, salt, and high-temperature stress (Baotić 2023). Overall, these results indicate that abiotic stress, and especially osmotic stress, induces downregulation of *NAC041* expression in accessions 521 and 522, which might relate to an increase in antioxidant activity of these *B. incana* accessions under abiotic stress.

NAC084 expression is upregulated under all stress conditions in abiotic stress-tolerant *B. oleracea* var. *acephala* accessions (Bauer et al. 2022). In Baotić (2023), an increased *NAC084* expression is detected in two *B. oleracea* var. *acephala* accessions under heat stress and combined stress treatments, as well as in *B. incana* accession 521 under combined high-temperature and salt stress treatment. In the same study, downregulation of *NAC084* expression is detected under mannitol-induced osmotic stress in accession 521, while my results showed a downregulation under high-temperature stress in accession 521. My results indicated downregulation of *NAC084* expression under combined stress treatments in accession 522, which is consistent with the results from Baotić (2023). The only difference is that my results showed a significant reduction of

expression under combined high-temperature and osmotic stress treatment, while in Baotić (2023) a significant change is detected under combined high-temperature and salt stress treatment. My results showed a significantly higher *NAC084* expression under control conditions in accession 514 compared to the other three *B. incana* accessions. This was also the only accession that exhibited a significant differential *NAC084* expression under all abiotic stress treatments compared to control conditions, with more than 1.5-fold upregulation under salt and osmotic stress, and significant downregulation under high-temperature and combined stress treatments. Similarly, accession 520 showed significant *NAC084* downregulation under high-temperature and combined high-temperature and salt stress. Some samples with very low or non-existent *NAC084* expression, indicated by very high or undetectable Cq values, had melting curves with a double peak. This could be an indication of primer-dimer formation, which can occur when the sample lacks cDNA template due to low gene expression (Saeed and Ahmad 2013). Overall, my results indicated a downregulation of *NAC084* expression under high-temperature and combined stress treatments across all four *B. incana* accessions.

Members of the DREB family of transcription factors are known to induce plant response to various abiotic stresses (Lohani et al. 2020). DREB2 transcription factors induce the expression of drought-responsive genes by interacting with a conserved drought-responsive element (DRE) in their promoter regions (Haak et al. 2017). DREB2A has a key role in drought tolerance in *A. thaliana* and maize (*Zea mays*) (Sakuma et al. 2006, Qin et al. 2007). All three *DREB2A* genes in *B. rapa* are upregulated under heat stress, indicating an important role of DREB2A transcription factor in *Brassica* heat stress response, possibly in the induction of HSF expression (Dong et al. 2015). *DREB2A* expression is significantly increased under high-temperature stress and combined stress, and a non-significant increase trend is seen under mannitol-induced osmotic stress in multiple *B. oleracea* var. *acephala* accessions (Bauer et al. 2022). *DREB2A* expression is strongly increased under combined high-temperature and salt stress in *B. incana* accessions 521 and 522 (Baotić 2023). Consistent with the aforementioned studies, my results showed a notable increase in *DREB2A* expression under most abiotic stress treatments in all four *B. incana* accessions. The upregulation was the largest under high-temperature and combined stress treatments in accessions 514 and 520. Accessions 514 and 522 showed a significant *DREB2A* upregulation under all five abiotic stress treatments, while accession 520 showed a significant upregulation under all treatments that included high-temperature stress, and accession 521 under salt stress and both of

the combined stress treatments. There was no *DREB2A* downregulation detected under any treatments. Interestingly, the upregulation fold change was the lowest in accession 521, which had a significantly higher basal *DREB2A* expression out of the four *B. incana* accessions. Overall, my results indicate an important regulatory role of DREB2A in *B. incana* response to various abiotic stresses. Additionally, at least two different Tm points were detected among the *DREB2A* melting curves across different *B. incana* accessions, which could indicate the presence of more than one *DREB2A* gene or transcript variant in *B. incana*. These results shed light on the potential for future research on *DREB2* variants in these *B. incana* accessions.

The heat-inducible HSFA2 is a transcription factor with a well-described role in acquired plant thermotolerance and overall heat stress response (Dong et al. 2015). Induction of HSFs under drought stress and salt stress in *B. napus* suggests that HSFs play an important role in response to multiple abiotic stresses (Zhu et al. 2017). Expression of *HSFA2* and *HSFA7* genes is significantly increased under heat stress, osmotic stress, and combined stress treatments in multiple *B. oleracea* var. *acephala* accessions (Bauer et al. 2022). According to the same study, *B. oleracea* var. *acephala* accessions that are more tolerant to drought and heat stress show a modest upregulation of *HSFA* genes and a stronger upregulation of *DREB2A*, compared to the more sensitive accessions. My results confirmed the role of *HSFA2* in heat response, as well as the positive cross-talk between *HSF* and *DREB2A* stress response pathways. This cross-talk was particularly present in accessions 514 and 520, where upregulation of *DREB2A* and *HSFA2* expression was detected under most abiotic stress conditions. Accession 514 showed a significant increase in *HSFA2* expression under all five abiotic stress treatments, with close to a 1000-fold change under high-temperature and combined stress treatments. Accession 520 showed a significant increase in *HSFA2* expression under all abiotic stress treatments except salt stress, with around a 100-fold change under high-temperature and combined stress treatments. Accession 521 was the one with the significantly higher basal *HSFA2* expression than the other three accessions but, interestingly, showed no significant differential *HSFA2* expression under any abiotic stress treatments. Furthermore, accession 522 showed a small, but significant decrease in *HSFA2* expression under high-temperature treatment alone, as well as an increase in both of the combined treatments which included exposure to high temperature along with either mannitol-induced osmotic stress or salt stress. Since the results for *HSFA2* expression in accessions 521 and 522 were based on only two biological replicates instead of the usual three, the phenomenon discovered here needs to be further

examined. After qPCR amplification, some 514 samples displayed a shoulder peak on their melting curves. There are a few possible explanations for this melting trend, such as that the *HSFA2* fragment might melt in a complex manner, perhaps due to a rich GC content or some other sequence-specific characteristic that could hinder uniform melting (Dwight et al. 2011). Another possibility is the HSFA2 gene duplication, which could result in multiple transcripts of slightly different sizes and nucleotide compositions. This could also explain the slight difference in *HSFA2* fragment sizes detected in a gel electrophoresis assay, where a smaller *HSFA2* fragment was detected in accessions 521 and 522 compared to accessions 514 and 520. Overall, my results confirmed a strong upregulation of *HSFA2* expression in *B. incana* exposed to high-temperature and combined stress in *B. incana* accessions 514 and 520. However, accessions 521 and 522 displayed a more modest upregulation than expected, and even a downregulation under combined stress in accession 522, which is another indication of the possible presence of different *HSFA* variants among different *B. incana* accessions. My results call for further investigation of *HSFA* and *DREB2* gene and transcript variants, as well as their respective promoter and regulatory regions, in these *B. incana* accessions.

6. CONCLUSION

This research presents a successful method for quantifying stress-responsive gene expression in a *Brassica* crop wild relative species *B. incana*.

Accessions 514 and 520 did not express *NAC041* under any conditions, including the control conditions. While basal *NAC041* expression was present in accessions 521 and 522, there was no significant differential expression of this gene under abiotic stress, except a downregulation under osmotic stress in accession 522.

Significant downregulation of *NAC084* expression was detected in response to elevated temperature and combined stress treatments across all four *B. incana* accessions.

A significant increase in *DREB2A* expression detected under a majority of abiotic stress treatments implies an important regulatory role of the DREB2A transcription factor in *B. incana* response to various abiotic stresses, including elevated temperature, drought, salinity, as well as different combinations of these abiotic stresses.

A strong increase in *HSFA2* expression under high-temperature treatments in accessions 514 and 520 confirmed the important role of this transcription factor in heat stress response. The lack of upregulation in accession 521, and even an unexpected downregulation detected under heat stress in accession 522, imply a possible presence of a different *HSFA2* variant in these *B. incana* accessions, which gives an interesting perspective for future research.

Accession 514 was the most responsive to abiotic stress based on the differential expression of stress-responsive genes. This accession showed a significant increase in *DREB2A* (up to 100-fold) and *HSFA2* (up to 1000-fold) expression under all abiotic stress treatments and a significant change in *NAC084* expression under all treatments, with an increase under salt and drought stress, and a decrease under high-temperature and combined stresses.

Based on the differential expression of stress-responsive genes, accession 521 was the least responsive to abiotic stress, with no significant changes in *NAC041* and *HSFA2* expression under any abiotic stress treatments. This accession showed a decrease in *NAC084* expression in response to elevated temperature, and an increase in *DREB2A* expression in response to salt stress and combined stress treatments.

7. LITERATURE

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8. BIOGRAPHY

I was born in Đakovo, Croatia. I completed my elementary school education at OŠ „Ivan Goran Kovačić“ Đakovo in 2015. I completed my high school education at Gimnazija Antuna Gustava Matoša in the natural sciences and mathematics programme in 2019. I was awarded the „Student of the generation“ title as the highest-performing student of my generation.

In 2019, I enrolled in the undergraduate university programme of Molecular biology at the Department of Biology, Faculty of Science, University of Zagreb. During my undergraduate studies, I was an active member of the Biology Students Association – BIUS. I was a team leader of the Botany section from 2022 to 2023. I volunteered at the Biology night manifestation at the Department of Biology in 2021 and 2022, and at the Symposium of Biology Students (SiSB 2022). I completed a two-month internship within the BOLDer project headed by prof. Sofia Ana Blažević, PhD at the Division of Animal Physiology within the Faculty of Science, University of Zagreb. I graduated cum laude in 2022.

In 2022, I enrolled in the graduate university programme of Molecular Biology at the Department of Biology, Faculty of Science, University of Zagreb. During my graduate studies, I completed a ten-month internship within the PathoPhyto project headed by prof. Martina Šeruga Musić, PhD at the Division of Microbiology within the Faculty of Science, University of Zagreb. I was a co-author on a review paper “Connecting the dots: Epigenetics, ABA, and plant stress tolerance” (https://doi.org/10.37427/botcro-2025-004). I was a member of the Supervisory Board of the Biology Students Association – BIUS in 2023, and a member of the Management Board in 2024. I presented a poster of the Botany section's research "Inventory of the orchid flora in the region of Hrvatsko zagorje" on the Symposium of Biology Students (SiSB 2024).