Mutagenesis in wheat:
An approach to make saline green!

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What the mind forgets

…the heart remembers

1. Abstract

The raising salinity in the soils around the world have been widely studied during the last decades due to the massive loss in agricultural land. Today, nearly 8% of the world's arable land can no longer be used for crop cultivation due to salt contaminations, and more than half of the countries in the world are affected. Wheat is the second most grown cereal after corn and covers more growth area on the earth than any other crop. Wheat production therefore needs to be increased to meet the demand of a growing world population. Thus, the possibility to grow wheat on salt-effected soil is crucial to feed the population and avoid economic loss for the countries involved.

In this thesis, the Bangladeshi wheat variety of BARI Gom-25 that is moderately salt tolerant was used as a source to create a mutagenized population with point mutations, and thereafter it acted as a control to the mutated lines. The benefit of point mutations is that a population with high genetic variation can be created. From such a population novel salt tolerant varieties can be developed, as well as many other valuable traits. From a germination screening of approximately 2000 lines on saline filter paper (200 mM NaCl), 70 lines were identified that exhibited a higher germination rate than the BARI Gom-25 control. These lines were further tested in the field in Bangladesh **(Paper I).** Thirty-five of those lines were also analyzed in the Australian Plant Phenomics Facility at Adelaide. In these experiments yield, growth, ion content, and water use were determined **(Paper II).** In **Paper III and IV** bioinformatic tools were used as an approach to look for transcription factor genes in the wheat genome involved in salt tolerance. The focus was on two different transcription factor families; WRKY (**Paper III**) and MYB (**Paper IV**). These studies illustrated the importance of the biological regulation of salt tolerance, and enhanced the understanding behind the mechanisms involved. Moreover, it highlighted putative target genes regulated by WRKY and MYB transcription factors that could be key ones to understand findings from Paper I and II.

This thesis points out the importance of salt tolerant crops in general, and wheat in particular, and shows how mutational breeding can be a great asset in the development of salt tolerant varieties. Specific mutagenized wheat lines with strong salt tolerance are identified based on their performance against various parameters, and the importance of WRKY and MYB transcription factor families in the biological regulation of salt tolerance is shown. Finally, downstream candidate genes encoding the observed phenotypes observed.

2. Populärvetenskaplig sammanfattning

I takt med den ökande globala uppvärmning som följer av klimatförändringarna ökar också översvämningar i stora delar av världen. Ett speciellt utsatt område är kustremsan i södra Bangladesh. Översvämningarna orsakas främst av stora monsunregn som höjer vattenståndet, svämmar över kustlinjen och drar med sig salt vatten in över land. När vattnet sen sjunker tillbaka under torkperioderna blir saltet som fanns i vattnet kvar i jordarna. Detta leder till salina jordar, vilket ändrar odlingsförutsättningarna och helt eller delvis förhindrar odling på de utsatta områdena.

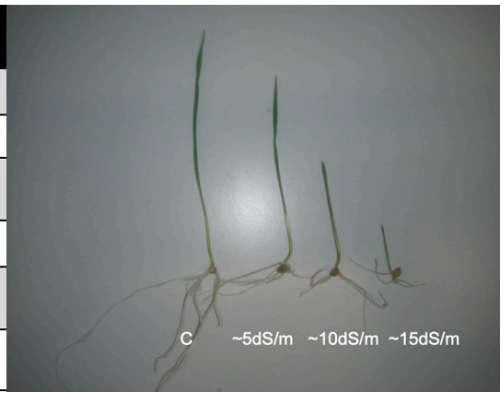
Vad gäller veteodlingen så påverkas den på olika sätt. Höga halter salt leder till en akut stark stress med kraftigt försämrad tillväxt och i princip helt utebliven avkastning. Lägre skadliga stresshalter leder till en försämring av vetets tillväxt och utveckling, och lägre avkastning. När saltet i form av olika saltjoner i jorden överstiger det i plantans rötter är det svårt för plantan att ta upp vatten då vatten naturligt vill jämna ut salthalter och därmed dras till högre salthalter. På så vis minskar saltstress växtens möjlighet att ta upp vatten. Effekten kan liknas vid känslan vi får när vi äter saltade jordnötter och gärna vill dricka vatten då munnen blir torr, det vill säga vi vill jämna ut salthalten.

Det vatten som plantan trots allt tar upp vid saltstress innehåller mycket högre mängder salter än vid normalt upptag. Dessa salthalter kan då ge upphov till skador inuti växten. För att kompensera den högre salthalten inuti växten kan växten aktivt flytta salter till olika lagringsplatser i plantan och på så sätt undvika förgiftningen. Om plantan inte har kapacitet att ta upp vatten pga. höga salthalter så uppstår en akut uttorkning och hela eller delar av plantan dör beroende på hur hög salthalten är i jorden. Om plantan överlever men exempelvis inte klarar av att fullt avlägsna höga halter inuti plantan så skapas en långvarig tillväxtförsämring eftersom gröna vävnader då vissnar och dör. Därmed minskas den totala fotosyntesytan vilket leder till en minskad biomassa och i slutändan en lägre avkastning. Detta kan innebära en ekonomisk kris inte bara för enskilda bönder, men också för hela länder då den totala livsmedelsproduktionen går ner och man kan behöva importera jordbruksprodukter i större utsträckning för att kompensera bortfallet av egenproducerad föda.

Salthalt i jord brukar vanligtvis anges som deci-Siemens per m (dS/m), vilket är ett mått på jordens ledningsförmåga av salter/joner. Avkastningen minskar hos de flesta grödor när salthalten i jorden ligger på ca 5 dS/m (Figur 1.) och när värdet överstiger 8 dS/m är det bara salttoleranta plantor som ger bra utbyte. I södra Bangladesh kan salthalterna bli upp emot 9 dS/m (**Paper I**), vilket skapar stora problem för bönderna.

För att få fram ett vete som kan tolerera salta jordar på ett bättre sätt än nuvarande sorter har vi använt oss av en kemikalie EMS (etylmetansulfonat), ett organiskt mutagen. EMS ger upphov till slumpmässiga byten av basparet G-C till basparet till A-T i DNA kedjan, dvs punktmutationer. I denna avhandling har en stor population (>2000 olika linjer) med en mycket stor genetisk variation tagits fram. Beroende på vilka gener mutationerna ändrar uppstår olika karaktärsdrag i de olika muterade linjerna. Vissa linjer kan t.ex. ha förändringar i proteinproduktionen, andra kan vara salttoleranta och så vidare. I mutationsprocessen utgick vi från en Bangladeshisk vetesort som visat sig tillväxa bättre än andra inhemska vetesorter i Bangladesh på salthaltiga jordar.

Salthalt (dS/m)	Växtrespons	Skördeeffekten
<0.75	Ingen	Ingen
0–2	Lätt	Ingen
2–4	Måttlig	Begränsad skörd av vissa känsliga växter
4–8	Stark	Begränsad skörd av många grödor
8–16	Väldigt stark	Bara toleranta grödor ger tillfredsställande skördar
Över 16	Extrem	Bara väldigt toleranta grödor ger tillfredsställande skördar



Figur 1. Olika salthalters effekt på växtodling
Tabellen till vänster visar typiska tecken på hur växter generellt påverkas av salttress vid olika saltnivåer i jord. Figuren till höger visar en svensk vetesort, Dacke, odlad sju dagar på filterpapper i en petriskål med olika salthalter efter tillsats av NaCl (0 dS/m, 5 dS/m, 10 dS/m och 15 dS/m).

I den här avhandlingen har jag identifierat ett antal salttoleranta linjer (OA-linjer) och med hjälp av dessa studerat hur växtens respons på saltstress kan regleras på molekylär nivå. Jag har studerat så kallade transkriptionsfaktorer som kan slå på och av andra gener som bland annat är involverade i att motverka salt stress. Om vi vet vilka transkriptionsfaktorer som är involverade i salttolerans kan vi studera uttrycket av dessa gener i våra olika OA-linjer och se om uttrycket av gener som kodar för dessa har ändrats p.g.a. de mutationer som vi har introducerat. Vi kan då också studera hur de gener som slås på och av verkligen regleras av transkriptionsfaktorerna under saltstress. På så vis kan vi korrelera transkriptionsfaktoruttryck till hur bra växterna klarar att växa i salt (**Paper I, III)** samt identifiera exakt vilken eller vilka gener som är ansvariga för salttoleransen, dvs som regleras av transkriptionsfaktorerna (**Paper II, IV**).

Om vi lyckas förädla fram ett vete som kan växa på salta jordar i Bangladesh har vi nyckeln till att sedan via traditionell korsning även få fram vetesorter för andra drabbade länder som till exempel Oman, Argentina, Egypten, Indien, Oman, Kenya och Vietnam. Tillsammans finns ca 21 miljoner hektar jordbruksmark i dessa länder som inte kan användas optimalt p.g.a. höga salthalter. För att sätta detta i ett perspektiv har Sverige 2.7 miljoner hektar jordbruksmark, varav vi odlar spannmål på ca. 1 miljon hektar. Sverige med sina drygt 10 miljoner invånare är självförsörjande vad gäller spannmålsprodukter. Så om vi kunde restaurera och återinföra spannmål på hälften av den obrukbara jorden i de sju länderna ovan hade vi alltså potentiellt kunnat producera pasta, bröd etc. till ca 100 miljoner människor!

3. List of publications

Lethin Johanna, Shahriar S. M. Shakil, Hassan Sameer, Sirijovski Nick, Töpel Mats, Olsson Olof, and Aronsson Henrik. 2020. 'Development and characterization of an EMS-mutagenized wheat population and identification of salt-tolerant wheat lines', *BMC Plant Biol*, 20: 18.

Lethin Johanna, Byrt Caitlin, Berger Bettina, Brien Chris, Jewell Nathaniel, Roy Stuart, Mousavi Hesam, Sukumaran Selvakumar, Olsson Olof, Aronsson Henrik. 'Improved salinity tolerance associated variables observed in EMS mutagenized wheat lines'

Hassan, Sameer, **Lethin Johanna**, Blomberg Rasmus, Mousavi Hesam, and Aronsson Henrik. 2019. 'In silico based screening of WRKY genes for identifying functional genes regulated by WRKY under salt stress', *Comput Biol Chem*, 83: 107131.

Sukumaran Selvakumar, **Lethin Johanna**, Pelc Justyna, Zeng Peng, Hassan Sameer, Aronsson Henrik. 'Genome-wide analysis of MYB transcription factors in the wheat genome and their roles in salt stress responses'

4. List of abbreviations

- AGR actual growth rate
- BARI Bangladesh Agriculture Research Institute
- bZIP basic leucine zipper
- CDPK calcium-dependent protein kinases
- CaM calmodulin
- CML calmodulin like
- DAP days after planting
- EC electrical conductivity
- EMS ethyl methanesulfonate
- HKT high affinity K^+ transporter
- mM millimolar
- MYB myeloblastosis
- NHX sodium/hydrogen antiporter
- OA OlsAro wheat lines
- PSA projected shoot area
- RGR relative growth rate
- SOS salt Overly Sensitive
- sPSA smoothed projected shoot area
- TF transcription factor
- WU water use
- WUI water use index
- WRKY protein with a conserved WRKY domain

5. Introduction

5.1 Climate change

Climate change is currently occurring at a faster pace than ever before. One way to monitor changes in climate is by recording the global average temperature, and this has been done since 1900. In the data between 1900 and 1980 a new heat record was seen on average every 13,5 years. In contrast, from 1980 until now, a new heat record has been obtained every third year. The ten warmest years all occurred 2005 or later (Figure 2) (Dahlman 2021). In addition to this, the world population is increasing generating cities covering larger areas, more roads, grazing animals needs to move further out, which in turn causes a great strain on agricultural land needing to produce more food in rapidly limiting areas. The need to breed crops tolerance to both heat, drought, salinity, and with higher yield is therefore a main priority to meet the public demand.

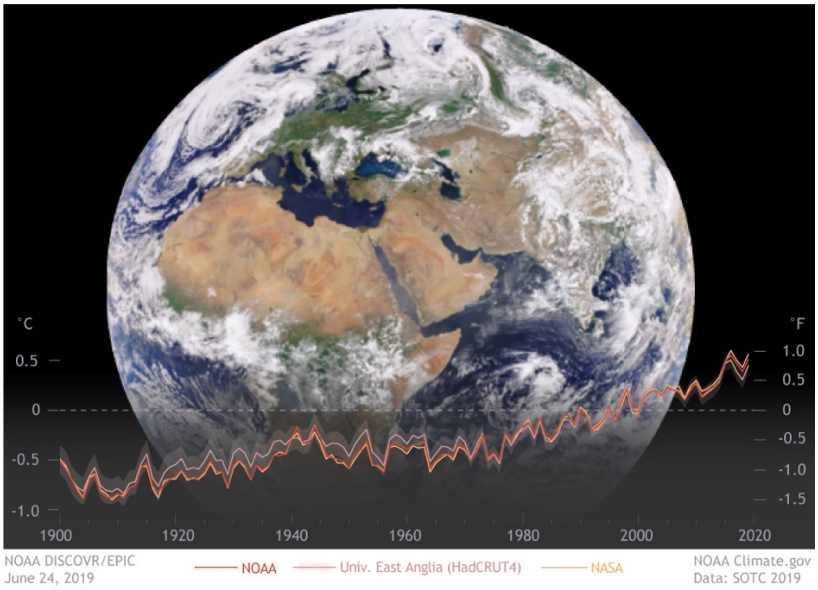


Figure 2. Global temperature increase from 1900-2019.
The years measured are seen on the x-axis while the temperature in °C is seen on the y-axis to the left, and in °F in the y-axis to the right. Background image from NOAA DISCOVR/EPIC. Graph by NOAA Climate.gov based on data from the Bulletin of the American Meteorological Society's State of the Climate 2019.

5.2 Effects of climate change on wheat production

For a crop such as wheat, raising temperatures have a strong negative impact on the total wheat production. Estimations predict a yield decrease of up to 6% for each degree of temperature increase (Asseng, Ewert et al. 2015, Zhao, Liu et al. 2017) Furthermore, wheat stands for a higher global trade than all other crops combined. Due to improving technologies and cultivation practices, particularly in developing countries, wheat yield is expected to increase about 9 % by 2030 (OECD/FAO 2021). Unfortunately, climate change does not only lead to a direct temperature stress on the crop, but there are many other negative effects. In particular, soil salinization is becoming increasingly prevalent. There are many different ways in which soil salinization can occur. Natural causes are flooding, heat waves, contaminated ground water etc., which are occurring more frequently with increasing global temperatures. Soil salinization can also be human caused due to improper water management, i.e., irrigating

agricultural land with saline or brackish water (Clarke, Williams et al. 2015, Shokat and Großkinsky 2019). Salt contaminated soils are one of the major global constraints of agriculture and food production today, affecting more than 8 % of the world's arable soils (FAO 2021). Every day for the past 20 years, around 2000 hectares of land has been lost due to salt induced degradation. This corresponds to an area of more than 62 million hectares equal to the size of France, which cannot be used for sustainable agriculture (M. Qadir 2014).

Figure 3. Dried-up salt patch from the ground in an agricultural area.
Picture taken from one of the fields used for testing OA-lines in Taltoli, Barguna, and the patch pointed to is salt laying on the top of the ground.

In the case of Bangladesh, with a gross cultivable area of ca 8 000 000 hectares (BBS 2019), climate change has proven to be a strong challenge for people and agriculture. This is partially due to its close proximity to the Bay of Bengal. The constant rise in temperature shown in Figure 2 has caused a chain reaction with increased flooding, both in extent and frequency. Rising sea levels, greater monsoon precipitation and increased glacial melt, has made the lowlands were the agriculture takes place very susceptible to flooding (Ayers 2008). In fact, the glaciers in the Himalayas are melting at an exceptional rate, showing a 10 times faster decrease only during last four decades than during the previous seven centuries (Lee, Carrivick et al. 2021). Despite the heavy periods of monsoon rain, on a very dry summer, salt can accumulate up above 5 ppt (freshwater is normally around 0.5 ppt). Heavy rain falls will not be enough to leach the dry soil from the salt deposits. This makes the salt stay in the ground all year, and as a consequence of this agricultural production can be reduced with as much as 50 % threatening the food production and livelihoods for farmers residing in these areas (Clarke, Williams et al. 2015). Different dried up saline patches in fields often exhibits a white or grey crust on the ground (Paul and Lade 2014), which also was observed while visiting one of the field in southern Bangladesh were our OA-lines were grown (Figure 3).

Today, more than 2 million farmers are involved in the Bangladeshi wheat production. During the last 20 years, wheat has increased while rice, potatoes, and legumes have slightly decreased since these crops require more water and maintenance than wheat. Wheat is now

the second most important staple food after rice in Bangladesh (Md. Rezaul Karim 2010, Mottaleb, Rahut et al. 2017).

In line with above, depending on which salinity level is present in the soil, wheat and other crops can be affected to various degrees. In the worst scenario, soil salinity leads to low or no seed germination or premature plant death (Janda, Darko et al. 2016). One of the most important parameters for final yield is germination and increased germination in tolerant plants leads to improved growth and yield. This correlation is clearly shown in the field trial of the mutagenized OA-lines (**Paper I**). However, the mechanisms of salt tolerance are very complex, including other factors such as stress avoidance, salt exclusion, salt dilution, compartmentation of ions, osmotic adjustment, and gene regulation, where the most important effects are the induction of antioxidative defense systems, ion homeostasis and accumulation of compatible solutes, which is explained in detail in section 6. To breed plants with improved salt tolerance is therefore of great importance. The Bangladeshi Agriculture Research Institute (BARI) released BARI Gom-25 during 2010, which is a wheat variety with enhanced qualities like tolerance to leaf rust, leaf spot diseases and improved heat tolerance. In addition, it is moderately salt tolerant. BARI Gom-25 can give a yield of 3600-4600 kg/ha depending on sowing time, place, and year and with a 1000 seed weight normally varying between 54-58 grams (Mia 2017). Due to this positive characters, BARI Gom-25 was chosen as the starting variety to create the mutagenized population in **Paper I** from.

6. Agricultural land- from useless to useful through breeding

6.1 Breeding methods

Bread wheat, which is the most common wheat used today is hexaploid, i.e., it has three different genomes denoted AA, BB and DD. The AA genome originates from *Triticum urartu* which, when crossed with the BB genome *Aegilops speltoides* gave rise to the AABB tetraploid *Triticum turgidum.* Later crosses of *Triticum turgidum* with the DD genome wheat *Triticum tauschii* gave rise to todays domesticated hexaploid variety *Triticum aestivum* (AABBDD) (Kerber 1964, Chrispeels and Sadava 2002).

When it comes to wheat, different ways to improve yields started over 10 000 years ago with the ancient domestication. During this process farmers were, in a similar way as today, pushed to produce more to feed to a growing population. This resulted in more specialized growers focusing on selecting plant individuals where random mutations has added enhanced properties such as more yield, reduced seed shattering, elimination of seed dormancy, bigger seeds, more tillers etc. They also started to adapt plants to different soil conditions, and induced genetic variation through crossing to further increase beneficial properties. Very similar to the problems breeders are working on today (Chrispeels & Sadava, 2002). Presently, breeding procedures are more advanced, involving not only crossing and backcrossing, but also hybrid breeding, mutation breeding (EMS), genetic engineering and gene editing. In addition, selection methods have advanced significantly and genomic selection and marker-assisted selection technologies are now commonly used.

Mutation breeding started with radiation in the 1920' when Stadler used X-rays to create mutations in maize (Stadler 1930) and thereafter in the 1940's (Westergaard 1957) by ethyl methanesulfonate (EMS). EMS is a chemical that alkylates guanine bases which results in a base pair transition from G-C to A-T, i.e., a point mutation (Till, Cooper et al. 2007, Yan, Deng et al. 2021). EMS is a hazardous, carcinogen chemical where an optimal concentration needs

to be titrated to obtain a maximal genomic variation on the seeds treated and at the same time minimize the number of seeds dying. During recent years chemical mutagenesis using EMS has become popular due to the high number of point mutations that can be obtained, i.e., a high genetic variation is created. A large number of seeds can be treated and a large number of mutated lines raised from these, creating a mutagenized population of a very high genetic variation, with a minimum of non-desired genetic alterations like deletions, DNA rearrangements etc. (Sikora, Chawade et al. 2011). Several EMS based plant populations have been developed including oat (Chawade, Sikora et al. 2010, Sunilkumar, Leonova et al. 2017), barley (Caldwell, McCallum et al. 2004), maize (Till, Cooper et al. 2007), and wheat)(Chen, Huang et al. 2012)**(Paper I)** to name only a few. This technique is therefore well studied and proven to be reliable in the identification of new lines and varieties with special properties. Since EMS is creating many mutations in the genome, when producing a large population many different up- or down-regulated genes will be present.

Marker-assisted selection (MAS) is the use of morphological, biochemical, or DNA markers as the selection criteria to identify individual lines in a crossing population. The more precise and the more specific the marker is the better. Therefore, DNA based markers are the best since they can be screened with high precision and at an early development stage long before the actual phenotype has been manifested. Additionally, when a DNA marker is used, the term molecular marker is the mostly used.

Genetic engineering is commonly referred to as Genetic Modification (GM) and is a process often involving recombinant DNA containing genes from other species, plants or animals (foreign DNA). The recombinant gene construct is most commonly transferred to the target organism by direct transformation (e.g., ballistics) or using a biological carrier (e.g., Agrobacterium). The target cells can be callus, protoplasts, pollen, leaf discs etc. In all cases, the process involves *in vitro* modification genes in order to introduce a desired phenotype into the target organism (NHGRI).

A technique based on *gene editing*, known as CRISPR/Cas (Clustered Regularly Interspaced Short Palindromic Repeats), is a system enabling plant breeders to modify target genes at high precision. CRISPR/Cas is based on a virus protection system originally found in bacteria. To perform editing a guide RNA molecule is synthesized that is complementary to the target sequence in the genome that is to be edited. There are many variations of them, but when the gRNA is combined with the Cas protein it will target and modify genes and thus specific plant characteristics with enormous precision (America 2022). Like natural double stranded breaks, the Cas protein also creates a cut in the targeted sequence, which initiates a DNA repair process leading to the introduction of mutation(s) in the target (Khatodia, Bhatotia, Passricha, Khurana, & Tuteja, 2016). Unfortunately, gene editing technologies have been included into the GM legislation in EU, making it less attractive to use for plant breeders since it complicates the marketing of such lines, at least in Europe.

To find the mutations created by EMS mentioned above, a sequenced genome is a great advantage. Unfortunately, full sequencing of the bread wheat genome has long been considered a difficult task due to its high complexity with a large genome size, three different sub genomes (A, B, and D) and >80% repetitive sequences (**see section 6a**). However, with the recent rapid development of new fast DNA sequencing technologies in combination with advanced genome assembling methologies, the International Wheat Genome Sequencing Consortium (IWGSC) managed to sequence 94% of the Chinese Spring wheat genome including 107,891 high-confidence protein coding loci (Appels, Eversole et al. 2018, Guan,

Garcia et al. 2020). This was done by breaking down the genome into smaller pieces, sequence them, and then building the genome back together with help of bioinformatic tools. Since the wheat genome contains about 15 billion base pairs, is hexaploidy and therefore contains about 85% of repetitive sequences, multiple groups sequenced the genome several times to get all the pieces correctly. (Zimin, Puiu et al. 2017)

The mutation frequency is a measure of how many mutations have occurred in the material of interest. By DNA sequencing analysis variations in different genomes at the single nucleotide level can be detected. Such changes (mutations) are commonly referred to as single nucleotide polymorphisms, or SNPs. To achieve an accurate calculation of the mutation frequency in mutated lines, a genomic reference sequence with a comprehensive coverage is required in order to map the SNPs at the resolution required (**Paper I**), (Uauy, Paraiso et al. 2009, King, Bird et al. 2015). Initially Li-Cor was a common method to detect mutations (Sikora et al. 2011), but has more or less been replaced by various DNA sequencing methods.

6.2 Soil remediation

There are a number of different techniques available to decrease soil salinity and enhance plant growth. One example is phytoremediation, or strip cropping, where the farmer rotates different plant species that have the ability to extract salt from the soils on the farmland (Toderich *et al*., 2008). Some halophytes have been shown to be good salt remediators since they reduce the Electric Conductivity (EC) in the soil, with a corresponding increase of NaCl in the plants. Two halophytes (*Suaeda maritima* and *Sesuvium portulacastrum)* were shown to, during a 4-month period, remove 504 kg and 478 kg of NaCl per hectare in a saline soil with an EC value of 4,9 dS/m. Furthermore, these halophytes compartmentalized Na^+ in their vacuoles by regulating cytosolic and ion accumulation to increase the vacuolar volume (Ravindran, Venkatesan et al. 2007).

In addition, physical soil cleaning, (restoration through scraping off the salty top layer prior to planting) can be done (Yuvaraj 2020). However, this method is mostly applicable when the area is very small, and it only improves plant growth temporarily until the salt accumulates again. Also, the salinity levels below surface often still are high enough to inhibit plant growth, which means the method has to be combined with leaching or rainfall to be useful for remediation (Endo and Kang 2014). Soil washing with $CaCl_2$ is another method for removal but is primarily used for the removal of cadmium. In this case Ca is added to the soil to restore the pH levels. (Sastre-Conde, Carmen Lobo et al. 2015). The most common way to remediate the soil is through leaching, where the farmer adds fresh water to the fields in such amounts that the salt will be washed away from the soil surface (Massoud 1988). Unfortunately, not only NaCl are washed away, but also nitrogen and other important nutrients, also fresh water is often limited or not available at all.

7. Salt stress and mechanisms of salt tolerance in plants

7.1 Salt stress

Since the global population is estimated to reach almost 10 billion people by 2050 (Janet Ranganathan 2019), it is vital to secure food for as many as possible Unfortunately, the impacts of global warming are vast, and salt effected soils are already a large problem for many farmers and the problem only increases.

Soil salinity is most commonly referred to, and measured by EC. The soils is considered saline if the EC reaches above 4 dS m^{-1} (Figure 1), being roughly equivalent to 40 mM NaCl, and soil salinization is the term used on salt affected soil and refers to land being affected with high salt concentration, high sodium cation concentration, alkaline soil (high pH), or all of the above (Daliakopoulos, Tsanis et al. 2016).

Salt stress occurs when the salt concentration in the soil is greater than the osmolarity inside the roots, causing an osmotic imbalance. Plants with the highest salt tolerance are referred to as halophytes while less-tolerant plants such as wheat, are referred to as glycophytes. Halophytes tolerate higher salinity levels due to proteins moving the salt into the plant's vacuoles. Halophytes also use osmotic adjustments to sustain growth, whereas glycophytes lack such a system to cope with higher salinity levels (Blumwald, Aharon et al. 2000, Flowers and Colmer 2008, Yuan, Xu et al. 2019).

Lower, non-lethal salt concentrations will cause ion toxicity, resulting in disturbances of the plant's ion homeostasis and growth. This happens when the salt concentration in the soil has reached the plants endogenous salt concentration threshold.

The threshold level is defined as the maximum amount of salt the plant can tolerate at the root zone level without growth impairment (Sajid Hussain 2019). After this, plant growth will keep decreasing with increasing levels of salt in the soil, which is illustrated in Figure 4 and shows the difference in growth between BARI Gom-25 grown under 80 mM NaCl compared to BARI Gom-25 grown under 0 mM NaCl **(Paper II).**

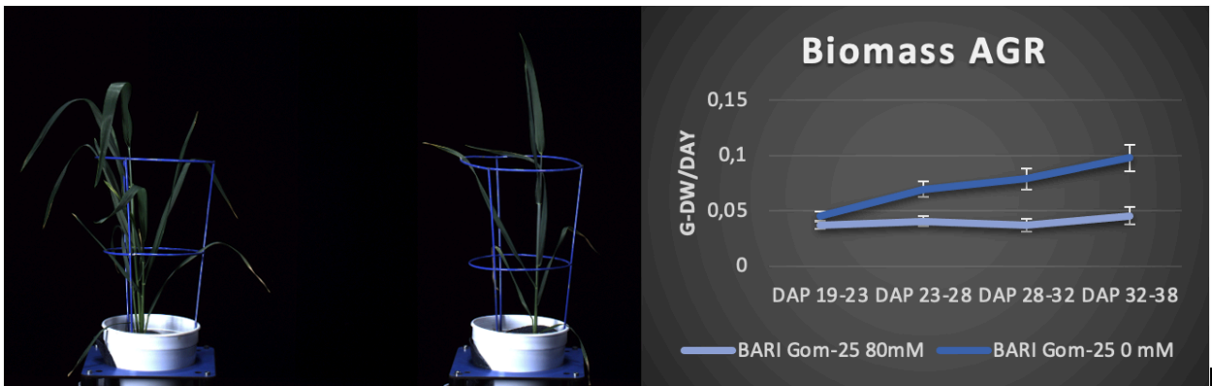


Figure 4. Biomass in g-dw/day for BARI Gom-25.
Panel to the left, BARI Gom-25 from the Plant Phenomics Facility taken on DAP 38 showing the difference in growth at 0 mM (left) and 80 mM (right) NaCl. Panel to the right, BARI Gom-25 biomass in different salinity conditions in grams (g) throughout the measuring period DAP19-38 under 0 mM NaCl and 80 mM NaCl.

If a high salt concentration occurs in the soil after seedling stage, a response will occur within minutes of exposure resulting in leaf reduction and root reduction due to disturbances of the plants ion homeostasis Hours after exposure there is a steadily reduced root and leaf growth rate, continued inhibition of root development, metabolic activities. Homeostasis of both micro (Mn, Zn, Cl etc.) and macronutrients will be disturbed. Days after exposure visible differences between salt sensitive and salt tolerant plants appear. In salt tolerant plants leaf growth is affected to various degrees but in salt sensitive plants leaf death starts to occur. Weeks and months after exposure, a salt sensitive plant can die, while a salt tolerant plant will survive, often with a reduced leaf size and yield. During the whole salt stress process both a sensitive and a tolerant plant will exhibit cell dehydration, stomatal closure and decline in chloroplast pigments, leading to lower photosynthesis, protein synthesis and metabolism of protein and phospholipids. All this leads to reduced plant growth, reduced yield, or plant

death (Munns 2002, Janda, Darko et al. 2016, Wenbo Li and Li 2017, Hussain, Khalid et al. 2018, Liang, Ma et al. 2018). How big the actual yield reduction will be depending on the plants ability to cope with various stresses, including salt stress (Jafari-Shabestari, Corke et al. 1995, Asgari, Cornelis et al. 2011, James, Blake et al. 2013, Kalhoro, Rajpar et al. 2016). Of special importance are the leaves where photosynthesis occurs. When treating a wheat leaf with 500 mM NaCl, a 78% decrease in photosynthesis compared to a control wheat leaf was shown, suggesting that high salt stress inhibits the electron transfer rate at the donor side of Photosystem II (Mehta, Jajoo et al. 2010). Studies have also suggested that a rapid decline in photosynthesis is specific to osmotic stress, while a slower and more long-term decline suggests ionic stress (Zhang and Xing 2008, Mehta, Jajoo et al. 2010). As biomass reduction and photosynthesis go hand in hand, and biomass reduction have previously been seen in salt stressed plants (Khataar, Mohammadi et al. 2018), monitoring photosynthetic activity can be one way to determine if the plants are suffering from salinity stress. An alternative, more direct approach to determine plant biomass is to calculate the actual growth rate (AGR). The AGR is a simple index of plant growth determining the increase in mass per unit of time through imaging (Hunt 1990). AGR was also used, see (Figure 5), to show observed differences in growth in BARI Gom-25 at 0 mM compared to 80 mM NaCl at DAP 32-38 (left panel), and through image analysis during the growth period DAP19-DAP38 (right panel).

7.2 Response genes

Genes are categorized into two different gene groups, structural genes and regulatory genes. Structural genes are involved in the plants structure, and codes for the proteins in the plant cell needed for structure and function, while regulatory genes regulate the expression of other genes, i.e., they act like a turn off to turn or switch and vice versa. Moreover, when a plant undergoes salt stress, a cascade of events are initiated by the primary stimuli, activating a signal transduction chain with many different proteins involved. The final outcome is a different regulation of hundreds of different genes, where the downstream genes encode the end product responsible for the salt stress response. Typical defense mechanisms induced are immobilization of salt, change the osmotic balance, biosynthesis of transporter proteins to move the salt to the vacuole, and many other genes in the group of salt responsive genes.

7.3 Mechanisms of salt tolerance

Key mechanisms that influence Na^+ and K^+ transport in wheat include High affinity K^+ Transport (HKT) encoding genes. For example, the *HKT1;4*, *HKT1;5* and *HKT2;1* type genes influence Na^+ and K^+ transport. *HKT1;5* is expressed in the roots, *HKT1;4* and *HKT2;1* is expressed in roots and leaf sheath tissues (Huang, Spielmeyer et al. 2008, Singh, Malviya et al. 2021) (Huang, Spielmeyer et al. 2008, Singh, Malviya et al. 2021). The *TmHKT1;4-A2* and *TmHKT1;5-A* genes confer sodium exclusion mechanisms named *Nax1* and *Nax2* which originated from *Triticum monococcum*, they are not usually found in modern bread wheat. However, a homologue of *Nax2, Kna1* is found in modern bread wheat and is shown to be associated with the wheat *TaHKT1;5-D* gene (James, Davenport et al. 2006, James, Blake et al. 2011). Previous studies have shown that *Nax1* is expressed in both roots and shoot, whereas *Nax2* and *Kna1* are usually only expressed in the roots, hypothetically one or more of the OA-lines may exhibit an upregulated *Kna1* gene. *Nax1* differs from *Nax2* by removing the Na^+ in both the root and the lower part of the leaf, conferring a function not known to be present in bread wheat, and *Nax1* lowered the flag leaf concentration of Na^+ by 20% compared to *Nax2*.

However, it was also observed that *Nax2* increased the yield of durum wheat by 25% (James, Davenport et al. 2006, Munns 2010). Furthermore, the results from the Kalapara field trials in **Paper I** revealed that all 70 OA-lines showed a higher germination percentage than the BARI Gom-25 controls and this could be linked to differences in Na^+ transport mechanisms in these lines. In addition to this, one of our OA-lines was ranked in the top in both germination % and final yield on the field, but the mechanisms influencing the phenotype are unknown. Future research directions may include investigating if there are differences in *Kna1* or other HKT genes in these lines to see if these key mechanisms are influencing the phenotypes observed.

The Ca^+ regulation pathways in the cell are including CDPK (Calcium Dependent Protein Kinases), CBL (calcineurin B-like proteins), CIPK (CBL-interacting protein kinases), and SOS2,3 pathways. CDPK have since long been connected with the activation and participation of stress signal transduction pathways, where the genes are present and essential for many biotic and abiotic stresses (Sheen 1996, Li, Zhu et al. 2008). The expression patters of CDPK genes from wheat and rice under various stress conditions showed a response suggesting some of the CDPK genes are involved in the crosstalk between different signal transduction pathways such as salt, drought, cold or other factors elicited by environmental stresses (Wan, Lin et al. 2007, Li, Zhu et al. 2008).

Furthermore, the CBL and CIPK are both involved in the Ca^{2+} signaling pathway for plant adaption to different biotic and abiotic factors and an expression of both CBL and CIPK (CBL-CIPK regulatory module) in the plant suggests further the progress of salt tolerance as well as activation of different downstream target proteins(Ma, Cheng et al. 2017, Ma, Li et al. 2020, Tong, Li et al. 2021) In wheat, 24 CBL, respective 79 CIPK families have been found were some of the corresponding genes have been identified to function in the plasma membrane (CBL), and throughout the cell inclusive the plasma membrane (CIPK) (Ma, Li et al. 2020). Furthermore, during the genome wide study of WRKY transcription factor **(Paper III)**, TraesCS3B02G519000 was found as a target gene involved in salt tolerance by enabling the transfer of calcium (Ca) ions from one side of a membrane to the other, suggesting salt tolerance as one of the abiotic stresses involved in the CBL-CIPK pathway, which also confirms previous studies on the subject**.**

The SOS pathways consists of three major components; SOS1, SOS2, and SOS3, consisting of a Na^+/H^+ antiporter, a serine protein kinase, and a Ca^{2+} sensor respectively (Yang, Chen et al. 2009). When the plant responds to high salt stress, the cytosolic Ca^{2+} increases, SOS3 activates the SOS2 kinase, which then connects to the SOS1 pathway connecting the K^+ and Na^+ transporters, suggesting SOS3 operates through SOS2 in order to activate SOS1 (Halfter, Ishitani et al. 2000, Serrano and Rodriguez-Navarro 2001, Qiu, Guo et al. 2002). Additionally, a study using yeast cells showed increased salt tolerance when all three SOS pathway members where present instead of only SOS1, suggesting regulation of the SOS1 activity by the SOS2 and SOS3 functions in the same salt-tolerance pathway (Halfter, Ishitani et al. 2000).

Table 1. Groups of salt responsive genes

Group	Gene Family	Characteristics	Reference
Stress sensing and signaling	SOS2,3	Regulation of calcium in the cell	(Yang, Chen et al. 2009)
	CDPK		(Li, Zhu et al. 2008)
	CBL		(Ma, Cheng et al. 2017, Tong, Li et al. 2021)
	CIPK		(Tong, Li et al. 2021)
Transcription factors	MYB	A protein controlling the rate of transcription from DNA to messenger RNA, by binding to a specific DNA sequence. Thereafter regulates genes by turning them on or off.	(Zhang, Zhao et al. 2012)
	NAC		(Xia, Zhang et al. 2010) (Xu, Gongbuzhaxi et al. 2015)
	WRKY		(Zhou, Zheng et al. 2019)
	bZIP		(Bi, Yu et al. 2021)
	DREB		(Jiang, Sun et al. 2017)
Salt-stress related genes	SOS1	Na+/H+, Na+ and K+ transporters	(Assaha, Ueda et al. 2017, Amirbakhtiar, Ismaili et al. 2019)
	NHX		
	Nax1,2		
	HKT	Cation transporters	(Assaha, Ueda et al. 2017, Amirbakhtiar, Ismaili et al. 2019)
	HAK		
	AKT1		

7.4 Transcription factors (TFs)

Eukaryotic plant transcription factors (TFs) are a group of regulatory proteins controlling gene expression in plants by binding to a unique DNA motif in order to activate or repress transcription initiation The up- or down-regulation of a particular gene is dependent on specific DNA binding motifs (cis acting DNA-elements) in the gene's promoter region, to which a transcription factor can bind. When a plant is exposed to salt stress, a signal is perceived by the sensor protein, which through a series of signal transduction events leads to activation of promoter binding proteins that activate appropriate TF genes. The TFs, in turn, activate many other genes involved in the response, in this case leading to salt tolerance (Agarwal, Shukla et al. 2013, Hong 2016). The same TF can bind to several different promoters, as long as the correct DNA binding site for the particular transcription factor (same cis-element) exists (Siggers and Gordân 2014). The WRKY transcription factor protein has a 60 amino acid domain containing the conserved amino acid sequence WRKYGQK at the N-terminal, coupled to a novel zinc-finger-like motif (Eulgem, Rushton et al. 2000). Genes regulated by the WRKY TF family have been widely studied over the years and have been shown to be involved in regulating tolerance of several biotic and abiotic stresses (Eulgem, Rushton et al. 2000), including salt stress (**Paper III**) (Wu, Ni et al. 2008, Chen, Song et al. 2012, Wang, Zeng et al. 2015, Zhou, Zheng et al. 2019).

By scanning genomic regions for various known TF binding sites, down-stream target genes for a particular TF can be predicted. Using such strategy, genes potentially involved in salt tolerance can be identified (**Paper III**)(Inukai, Kock et al. 2017). We developed a model that identified cis elements similar to known binding motifs for the TF-family WRKY in upstream 5´-region of annotated genes in the wheat genome. This analysis highlighted 47 genes predicted to be WRKY regulated (**Paper III)**. However, to finally determine the specific binding DNA sequence for each TF, experimental verification is necessary. Nevertheless, to determine the binding site motif of a particular TF is a good starting point to predict which downstream genes that, if expressed, would be important in mediating salt stress tolerance (**Paper III, Paper IV**) (Inukai, Kock et al. 2017).

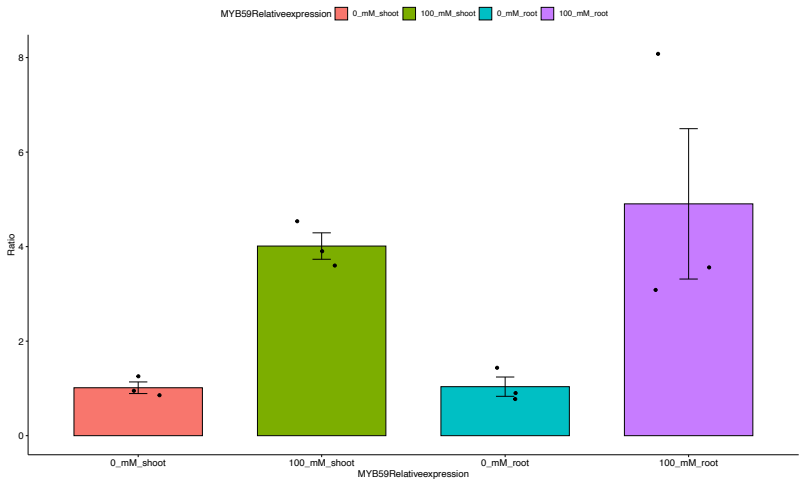


Figure 5. Salt mediated MYB gene expression
Expression of the MYB59 was quantified by qPCR in BARI Gom-25 wheat roots and shoots six days after exposure to 100 mM NaCl. The x-axis shows control and 100 mM NaCl for root and shoot expression, and y-axis represents the ratio.

One of the widest spread TF families among eukaryotes found in both animals, plants, slime molds, and fungi are the MYB TF family. They were first identified in a v-Myb oncogene of an avian myeloblastosus virus (AMV), and was together with c-Myb sequenced and cloned for further studies (Klempnauer, Gonda et al. 1982, Li, Ng et al. 2015). The MYB superfamily is further grouped into four subclasses depending on the number of repeats found in the MYB domain. They are denoted R2R3-MYB, 1R MYB or MYB-related, 3R MYB and 4R MYB. The R2R3-MYB variant is the most abundant in plants (Dubos, Stracke et al. 2010). The MYB-R2R3 includes a DNA binding domain consisting of a C-terminal with a highly variable region, and a N-terminal consisting of MYB domains (Katiyar, Smita et al. 2012). The former is responsible for regulatory activity and the later carrying the MYB-DNA binding domain. MYB TFs are highly involved in many processes controlling plant abiotic stress. Numerous studies have identified genes involved in plant salt tolerance that are regulated by MYB transcription (Zhang, Zhao et al. 2012, Wei, Luo et al. 2017, Song, Yang et al. 2020, Zhang, Wang et al. 2020), factors including wheat TraesCS5D02G411800 and TraesCS7D02G208000 identified as an uncharacterized protein with Myb-like DNA binding domain **(Paper III).** Another example is the TF MYB59, which also is one of the MYBs that in previous studies have shown to be involved in salt tolerance in a halophytic grass species (Vaziriyeganeh, Khan et al. 2021). This expression was also confirmed in our analysis where 60 wheat plants of BARI Gom-25 were treated with 100 mM NaCl for 6 days, then pooled and split into three batches of 20 plants

each, and thereafter measured with qPCR to see gene expression of MYB59. Both roots and shoots were significantly upregulated during qPCR analysis (Figure 5, **Paper IV)**.

The NAC TFs are mainly involved in regulating plant development, react to environmental stimuli, and respond to hormone signaling (Saidi, Mergby et al. 2017). Thus, as has been shown in several studies, NACs are also regulating the expression of many genes involved in salt stress response (Xia, Zhang et al. 2010, Xu, Gongbuzhaxi et al. 2015, Saidi, Mergby et al. 2017)

DREB TFs, that belong to the ethylene response factor (ERF) family, also play important roles in abiotic and biotic stress responses. DREBs are divided into two subclasses, DREB1 and DREB2, which are induced by cold and drought stress regulate genes in the ABA-independent stress pathway (Khan 2014). DREBs have also been found involved in salt stress in plants (Zhang, Tang et al. 2013, Jiang, Sun et al. 2017, Guo, Lu et al. 2019, Hassan, Berk et al. 2021).

The basic region leucine zipper motif (bZIP) family is a large family of ca 75 putative TF-genes. They encode TF proteins carrying a bZIP leucine zipper, which is a leucine dimerization motif and a basic region that binds DNA. bZIP proteins are present in all eukaryotes so far analyzed (Jakoby, Weisshaar et al. 2002). In plants, the bZIP TFs have been reported to be involved in ABA signaling and in salt stress response (Zhang, Zhang et al. 2017, Gai, Ma et al. 2020, Bi, Yu et al. 2021).

8. Bioinformatics as a tool to study and understand salt tolerance

A computer is a very useful tool to elucidate biological interactions in order to investigate, for example, salt related genes. Due to the enormous amount of data being uploaded on the different databases; it is often possible to predict different gene functions by finding a similar sequence that has already been experimentally studied, predict protein structures, or for example to use sequenced data to determine evolutionary relationships of organisms as investigated in Paper II and Paper IV. The different generic databases covers a broad range of species and genomes, which can act as a resource for the understanding and improvements for specific crops (Lai, Lorenc et al. 2012).

For **Paper III** and **Paper IV**, the data analysis conducted was based on the improved version of the Chinese Spring wheat genome, International Wheat Genome Sequencing Consortium (IWGSC RefSeq v1.0) (Consortium IWGES, 2018).

To retrieve the sequences from MYB and WRKY, the HMM file of the respective domain was downloaded from the Protein families (PFAM) database. The HMM profile was used as a query to scan across the wheat proteome with a E-value set to 1e-05 to verify the presence of WRKY or MYB in the collected protein sequences and Cd-hit was used to avoid redundant protein sequences. Only full WRKY or MYB domains were used for further analysis which was confirmed using an in-house python script. For **Paper III,** an numbering of the sequences was made from previous studies by (Ning, Liu et al. 2017) by database search such as UniProt. For both **Paper III and Paper IV,** a multiple sequence alignment was made from the trimmed sequences using Clustal Omega. The HMM profile was used in HMMER to get an accurate alignment. The HMM is using position-specific scoring to give a degree of conservation and frequency specific to the amino acids in in each residue in the multiple sequence alignment (Eddy 1998).

8.1 Phylogenetic analyses

We constructed a phylogenetic tree for **Paper III** using MEGA7 of the MYB and WRKY domains showing the clustering of the different groups. The phylogenetic tree was constructed from the method described by (Le, Lartillot et al. 2008), and a bootstrap value of 100 replications were used. The sequences retrieved was cleaned by using a sequence identity matrix to make sure no identical domains were used. No sequenced obtained from the scaffolds of the chromosomes were used in order to avoid inclusion of uncertain data. In **Paper III** the sequences were assigned to different groups and subgroups based on previous studies by (Ning, Liu et al. 2017). The approach of dividing the sequences into different groups and subgroups based on the amino acid sequences between the groups in the different families, giving insight to functional variation and evolutionary origin (Wilkins, Erdin et al. 2012).

8.2 Sequence comparison

For determination if any residues with a certain physicochemical characteristic are group specific a graphical representation of the multiple sequence alignment was made on **Paper III** and **Paper IV** using WebLogo3. This will show the frequency and conservation of amino acids detected at specific positions. A sequence logo summarizes a data set of aligned binding sequences. Where the binding is unchanged, or conserved, the base is illustrated by the height of the letter. If there is more than one conserved base, a stack of letters is made with corresponding height to the number of bases at that position. This has been used in both MYB and WRKY and the method is a robust way of measure the sequence conservation (Chattoraj and Schneider 1997, Crooks, Hon et al. 2004).

8.3 Principal component analysis (PCA)

PCA was used in **Paper I** and **Paper III** as a tool in interpreting high-dimensional data sets, for example in Paper I where we were working with data with 11 numbers of variables for many different samples (72). In **Paper I** PCA was used to extract the most relevant and useful features from a dataset containing 1320 features. PCA is made of a mathematical algorithm that can sort variables into principal components, retain the features with very high variance in the data, but exclude variables where the samples are showing high similarity, or used for analysis of the structure in order to assess protein function (Mei et al. 2005). For the analyses of **Paper I** and **Paper III**, the data input to PCA was organized as a matrix denoted X, which was composed of N and K dimensions, where N represented the number observations recorded for salt tolerant lines **(Paper I)** and protein sequences **(Paper III),** and K represented the number variables of field observations **(Paper I),** and protein sequence features **(Paper III).** The K dataset is the greatest contributing to each of the original variables to the principal components. The components of the X were eigenvectors of the covariance matrix and the greatest eigenvectors corresponded to the dimension that explained the greater variation in the amount of data.

From this, a principal component 1 (PC1), was calculated describing the highest percentage of variance in the whole dataset (X), and principal component 2 (PC2) explains the second highest percentage of variance in the data set. These two components are uncorrelated to each other meaning one can go on with a PC3, and PC4 etc. depending on how many variance points one will have. Both **Paper I** and **Paper III** consists of PC1 and PC2.

8.4 Gene ontology (GO)

A gene ontology (GO) analysis gives a representation of the knowledge within a given domain. Usually there are a few classes with relations operating between these classes or genes. The gene ontology then describes known knowledges of the biological domain with three different sets:

1. Molecular Function
2. Cellular Component
3. Biological Process

For the functional annotation of the 47 target genes found in the WRKY transcription factor, all three sets of GO where used. The genes involved in biological process were mostly found linked to cellular and metabolic processes, regulation of biological processes, and response to stimuli. In the category molecular functions, over 60% of the genes were involved in binding, and for cellular component most genes were found in cell part, cell, membrane, and catalytic activity **(Paper III)**.

9. Scientific aim

The first aim for this thesis was to investigate the possibility to use mutagenized wheat (*Triticum aestivum* L.) to create different lines with increased salinity tolerance for cultivation in areas that are too saline for present agricultural systems (Paper I and Pape II). The second aim was to better understand the mechanism behind increased salt tolerance in wheat (Paper III and Paper IV).

This was investigation was divided into through four different papers:

a. By creating a mutagenized wheat population through EMS (Paper I)
b. Developmental phases, germination by controlled parameters compare the different lines in growth, ion content, and water use (Paper II)
c. Identifying wheat transcription factor genes expressed during salt stress with the use of bioinformatics (Paper III and IV)

10. Main findings

*10.1 The EMS population and germination screening (**Paper I**)*

We developed a mutant wheat population starting from the moderately salt-tolerant Bangladeshi variety BARI Gom-25, with the primary goal of identify highly salt tolerant lines from the developed population. We choose EMS as a mutagen, since we wanted to introduce

Figure 6. Screening assay for salt tolerant lines
Seeds were placed on filter papers with 200 mM of NaCl. The results were recorded after 7 days of growth. To the left, 10 seeds of one of the tolerant lines. To the right, 10 seeds of the BARI- Gom 25 control variety.

a large number of point mutations, without at the same time create major deletions or DNA rearrangements in the population.

The greater the genetic variation, the higher probability to identify genes involved in salt tolerance. We developed an assay based on selection of seed germinating on agar plates with 200 mM NaCl, and screened 2000 lines from the mutagenized wheat population (Figure 6).

After the first screening, 70 promising lines were identified and planted in the field in Bangladesh. Exactly as we hoped, many of these lines germinated and grew well and produced more seeds than our reference BARI Gom-25. This strongly indicates that mutations leading to increased growth and yield had been introduced in these lines. Since all the 70 lines had a higher germination rate than the control, the initial germination screening assay we developed were relevant and reliable. Since a whole wheat genome sequencing now is available, we found it very useful to genome sequence some of our lines to further categorize genetic differences between BARI Gom-25 and OA-lines by calculating average mutation frequencies. By using the wheat genome of Chinese Spring as a reference, we first aligned the sequenced BARI Gom-25 to the reference and thereafter used BARI Gom-25 as a reference for the sequenced OA-lines. By this, we could identify the mutations that differed between OA42 and OA70 and BARI Gom-25. From this, the mutation frequency was calculated to be around 1 mutation per 20 000 bp.

*10.2 Plant phenotyping in a controlled environment (**Paper II**)*

As showed in **Paper I,** 70 of the salt tolerant OA-lines were tested in a field trial in Bangladesh. Based on the data obtained, we decided to pick 35 lines and BARI Gom-25 for a further more detailed physiological and biochemical characterizations in a very controlled environment at the Plant Phenomics Facility located in Adelaide, Australia. A benefit from using a Phenomics facility like the one in Adelaide is the ability to screen many plants without introducing human errors. The data collection is done in a non-destructive way using special cameras, which means that each plant/pot can be followed for a longer period of time and plant growth and development can be measured throughout the whole plant cycle at different salt concentrations. In this case we tested 0, 40, 80, 120, and 160 mM NaCl (Figure 7). In addition to this, at the end of the measuring period, we also obtained values for water use, Na^+, K^+, and Cl^-, and 1000 seed weight. Unlike in **Paper I**, this experiment the plants were not germinated in high salinity conditions, instead the salt was added to the soil 18 days after germination. Interestingly, several individual lines performed well both under the controlled conditions and

Figure 7. All mutagenized wheat lines in the smarthouse at The University of Adelaide Plant Phenomics Facility
To the left, the lines ordered in a row system. To the right, plants are transferred to the automatic imaging station.

during the field conditions in **Paper I**. OA23, for example, was strong in seed production per germinated seed in Bangladesh, as well as having a good yield from the Australian data.

After further analyzing the data from leaf ion concentration, growth rate, water use, and yield, six lines were selected as being highly compared to BARI Gom-25 in one or more of these parameters. When it comes to Na^+ concentration in the leaves, two lines were of particular interest since they were found on opposite side of the concentration scale. Previously in the text (Section 5.2), some examples of halophytes extracting high levels of Na^+ from the soil were given. For our lines, OA23 (Figure 8) shows a significant higher Na^+ concentration in the leaf compared to BARI Gom-25 leaving an interesting mark weather this line can remediate salinity in the soils as well. A calculation made suggests a 340 kg uptake of NaCl by OA23 at the same time as the 1000 seed weight at 160 mM is almost identical to the one for BARI Gom-25. Can this be a possible Na^+ remediating line? In contrast, OA10 have significantly lower Na^+ concentration in the leaf compared to BARI Gom-25 thorough 0-120 mM NaCl suggesting one or more Na^+ exclusion genes present. The wheat gene TaHKT1 (Referring back to section 6.3 where the genes are discussed), has been reported to be severely upregulated during salt stress in the leaf by moving Na^+ from the cytosol into the phloem, while maintain a healthy K^+ (Amirbakhtiar, Ismaili et al. 2021). Additionally, OA10 also showed a significantly lower Na:K molar ratios compared to BARI Gom-25. Yet, no gene specific studies have been made on OA10; however, it is definitely prone to future research.

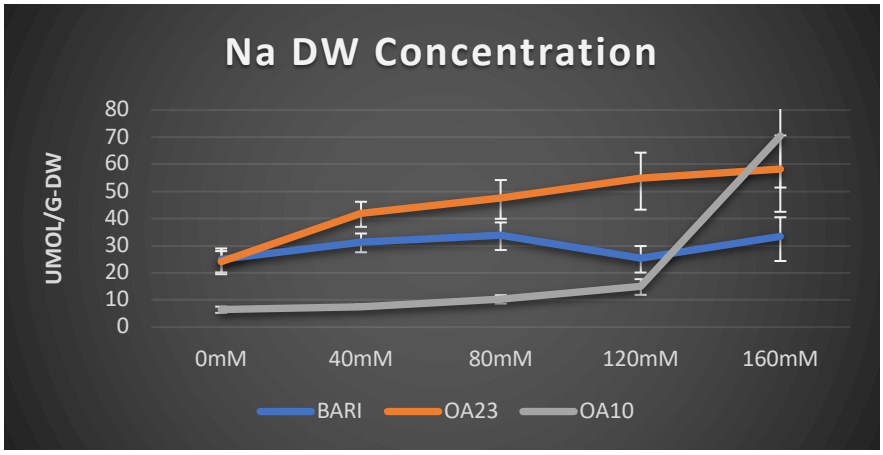


Figure 8. Na^+ Dry weight concentration in wheat leaves (flagleaf-1)
The graph shows different Na concentration ranging from 0-160 mM NaCl (x-axis). The blue line represents BARI Gom-25 and the orange and grey line represents OA23 and OA10 respectively. The y-axis is the concentration of Na in µmol/g-dw.

*10.3 WRKY transcription factor (**Paper III**)*

In this paper the aim was to achieve a more in-depth knowledge about TFs regulating salt tolerance. This to get a better idea of potential salt tolerance genes that could be up- or down regulated in the 70 lines obtained from **Paper I** and to understand the mechanisms behind. As a first example we chose to analyze the WRKY TF family since it is known to be involved in the regulation of various biotic and abiotic stress responses, including salinity. A deeper study of WRKY could provide insight into structure and diversity of the WRKY domain sequences in wheat in regards to salt tolerance and genes involved. To scan for genomic regions that contain cis elements to which the WRKY can bind, we first needed to determine the binding site sequences of the WRKY, a highly conserved DNA sequence. Through literature search we identified the W-box element ([T][T]TGAC[C/T]) as the WRKY binding site. By searching for W-

box elements 2000 bp upstream of 107,891 high-confidence protein coding locis, identified by a specific algorithm (IWGSC RefSeq v1.0). However, in a large genome as wheat, with 15 billion base pairs, several of the identified W-boxes could be false positives.

To make the search more specific, we inserted more criteria. First, we checked where in sequence the false positives occurred. Binding specificity of TFs not only depends on DNA interaction but also protein-protein interaction with other factors in the promoter region. Therefore, by looking for typical promoter elements like TATA and CAAT boxes, correct distances between the motifs etc., more false regions could be eliminated. After this, 31,296 genes remained as target genes involved in WRKY TF. However, since WRKY are involved in many different kinds of stresses, all the identified genes will not specific for salt. Through literature search we identifies 144 genes involved in salt tolerance, and when matching those against the 31,296 genes, we got a match on 47 genes. Thus, these genes could be involved in both salt tolerance and regulated by the WRKY transcription factor. These 47 genes will be studied in more detail in future research and tested on findings from **Paper I** and **Paper II**.

*10.4 MYB transcription factor and (**Paper IV**)*

From **Paper III** where 47 target genes were isolated, the same bioinformatical pipeline was used to obtain a deeper understanding of the MYB transcription factor with structure and function in relation to salt tolerance. *In silico* studies showed the MYB TFs MYB3, MYB4, MYB13 and MYB59 to be involved in salt stress, and qPCR from these four genes confirmed the involvement in salt stress by being upregulated in shoot. To isolate potential target genes involved in those four MYB TFs, the 144 salt genes retrieved from literature research **(Paper III)** was used to match ca 10 000 genes retrieved from searching 2000 bp upstream of 107,891 high-confidence protein coding loci's, identified by IWGSC RefSeq v1.0. From these, nine target were extracted and being distributed such as two at MYB3, three at MYB4, two at MYB13, and four at MYB59. Interesting is that MYB3 and MYB4 are sharing one gene (TraesCS5D02G411800), and MYB3 and MYB59 are sharing another (TraesCS3D02G350100), making these genes prioritized for further studies. The first of these two genes, TraesCS5D02G411800, is also one of the genes regulated by both MYB and WRKY transcription factor.

11. Conclusions and future perspectives

The creation of a mutagenized population has provide us with about 2000 different lines. From these, I have focused this thesis on finding salt tolerant lines and to understand the mechanism behind. The use of bioinformatics has been a great approach in order to narrow down potential salt tolerant genes to test directly on our population. Seventy lines have shown increased germination rate compared to BARI Gom-25 in field trial, and six lines have been shown enhanced abilities in either growth rate, leaf ion concentration, water use efficiency or yield in a controlled environment. One of these lines, OA6, has in comparison to BARI Gom-25, showed an increased growth rate at 80 mM NaCl (Figure 9) from 23 days after planting (DAP23-28), suggesting that OA6 carries one or many mutations that cause up- or down-regulation in critical gene(s) involved in mediating increase growth during salinity. Possible activities from such a gene(s) or pathways could be ion exclusion from sensitive cells by modifications of transport proteins leading to less uptake, more efficient transport to the vacuole, increased protein stabilization and repair, etc. Such mutations could give a higher

tolerance to salt stress in the selected mutated lines when compared to BARI Gom-25, as exemplified by OA6 (Figure 9).

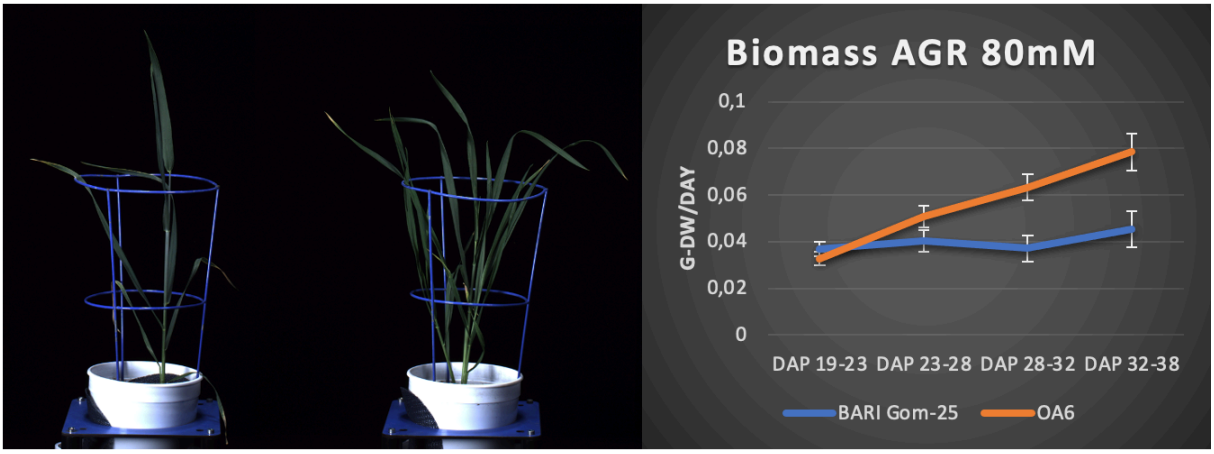


Figure 9. Biomass in g-dw/day for BARI Gom-25 and OA6
Panel to the left: BARI Gom-25 and OA6 on DAP 38 of imaging at 80 mM NaCl. Panel to the right: Average growth rate (AGR) of BARI Gom-25 and OA6 at 80 mM NaCl 19 to 38 days after planting (DAP 19-38).

With OA6 as an example above, the pathways involved in salt tolerance are rather large and involved in many interactions, one cannot exclude a crosstalk between both WRKY and MYB, and the other TFs as well. This is something we have seen as well during the target gene extraction when three genes, TraesCS5D02G411800, TraesCS7D02G063900, and TraesCS5A02G492800 showed up as target genes for both WRKY and MYB transcription factor. The goal for the closest future is therefore to test these three genes (among others) through gene expression in qPCR to confirm or deny if they are involved in our OA-lines.

With a population of 2000 lines with all carrying different mutations, future research is not limited to only look for salt tolerance, there three major components in wheat such as starch proteins, polysaccharides, and minor components such as lipids, terpenoids, phenolics, minerals, and vitamins. The protein content in wheat lies around 9-15% depending if the grain is bred for high protein or not, but normally around 12%. An initial screening of protein content in some of the OA-lines showed contents as high as 19% on some of the lines. These lines may hold higher interest for crossing in the future.

Furthermore, this population is not limited to salt or protein, but there are various other parameters also possible in the future such as heat, drought, plant diseases etc. A great advantage of doing studies of drought on this population is the high resemblance drought has with salinity stress. Many mechanisms are similar and the genes extracted for salinity could possibly be used in research for drought tolerance as well.

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