REGISTRATION FORM (BASIC DATA)

1a. Details of applicant

In case the applicant is a professor, it is assumed that he/she is also the promoter. In case the applicant is an associate or assistant professor (UHD or UD) the promoter must be mentioned under 1b.

Name, first name, title(s):	Bsc. Nakisa Echobardo Female
Institution:	Institute of Environmental Biology, Utrecht University
Department:	Science Faculty, Dept. of Biology
Position:	Student
Permanent position:	Yes

1b. Promoter

Name, first name, title(s):	
Institution:	Institute of Environmental Biology, Utrecht University
Department:	Science Faculty, Dept. of Biology

1c. Proposed PhD candidate

Name, first name, title(s):	Nakisa Echobardo, BSc Female

2. Title of research proposal

Reactive oxygen species and their influence on mediating salinity tolerance mecahnisms in *Triticum aestivum*

3. Summary of research proposal

(scientific summary in English, max 250 words)

Soil salinity has detrimental impact on wheat productivity and since wheat is amongst the most important crops for human consumption, soil salinization imposes a major threat to global food security. Currently we know three mechanisms in which plants withstand salinity stress, namely, ion exclusion, tolerance to osmotic stress and tissue tolerance. Although significant research efforts have elucidated the genetic regulations occurring in the exclusion of ions, as well as compartmentalization of ions at the cellular level and thereby enhancing tissue tolerance, it still remains largely unknown which genes mediate osmotic stress tolerance. Osmotic stress causes oxidative damage because of the accumulation of reactive oxygen species (ROS). Plants are capable of removing ROS with antioxidant systems. The mechanisms in which ROS remains in balance across the three salinity tolerance mechanisms in wheat have never been studied yet. In short, there are two gaps in our understanding of salinity tolerance in wheat, namely, the limited amounts of candidate genes discovered that influence salinity tolerance and the lack of understanding on the role of ROS metabolism in mediating the known salinity tolerance mechanisms. In this research, I aim to identify novel gene candidates associated with salinity tolerance through genome-wide association mapping. Subsequently, I aim to identify how ROS metabolism influences salinity tolerance in genes previously identified to be associated with the salinity tolerance mechanisms, as well as possible gene candidates we discover in the GWAS. The outcome of this research will improve our understanding on salinity tolerance and is pivotal for salinity tolerant crop breeding.

4. Layman's summary

(general public summary in English or dutch, max 500 words)

Soil salinity is an abiotic stress for plants that leads to a decline in plant growth and productivity. Due to rising levels of ground water that have excessive levels of salt content, poor drainage and bad irrigation processes, allot of arable lands deal with high soil salinity levels. It is estimated that by 2050, around 50% of arable land is affected by soil salinity, imposing a major thread to our global food security. Bread wheat

(*Triticum aestivum)* is the second most cultivated cereal crop on Earth, and acquaints to roughly 50% of human calories consumption. Bread wheat is also incredibly susceptible to soil salinity. To ensure food security for the world population by 2050, we need to increase our understanding on **how** salinity tolerance of wheat works and how we can use this information to breed new wheat lines that are less susceptible to salinity stress. There are three main mechanisms that helps wheat plants be tolerant to soil salinity. The first mechanism is the ability of a plant to be able to remove Na^+ or Cl^- ions from the roots, also called ion exclusion. The second mechanism is the ability of a plant to compartmentalize Na^+ or Cl^- ions in organelles and the third mechanism is osmotic stress tolerance that regulated by long distance signals. Currently, it is unknown which genes are associated with osmotic stress tolerance. However, given that salinity tolerance is controlled by many genes, it is best to use genome wide association mapping (GWAS) to new discover salinity associated genes. This research proposal aims at identifying new candidate genes using GWAS. Next to that, we also want to better our understanding on how salinity tolerance in wheat works. To do this, we will look at the impact of reactive oxygen species (ROS) in mediating salinity tolerance. ROS are very well known and important signalling molecules for many biochemical processes in plants. However, ROS is also toxic to plants and this is why plants are constantly trying to create a balance with between enough ROS to help as a signalling molecule and remove ROS whenever its concentrations accumulate at unfavourable rates. The ability of a wheat plant to remove ROS under salinity stress may be a mechanism that mediates salinity tolerance. We thereby also aim to investigate to which extend ROS metabolism in wheat plants influence salt tolerant wheat crops. This research allows us to view salinity tolerance through a more integrated lens, by taking account of biochemical and physiological pathways that take place within the cell. The outcome of this research will improve our understanding on salinity tolerance and is pivotal for salinity tolerant crop breeding.

DESCRIPTION OF THE PROPOSED RESEARCH

6. Description of the proposed research

Max. 4 pages, including figures, excluding literature references. Include details of objectives, scientific approach, impact, innovative aspects, and literature references (please include full bibliographical details).

Introduction

Soil salinity is amongst the most severe challenges facing agriculture and crop production. It is estimated that soil salinization will impact up to 50% of arable land by 2050 and the economic loss of such effects may be over tens of billions dollar annually[1]. High soil salinity causes a lowered osmotic potential around a seed, which prevents its water and nutrient up-take, leading to a slow seed germination, delay in flowering and sometimes complete crop failure[1]. In order to develop salinity tolerant crops, we must improve our understanding of soil tolerance mechanisms in relevant agricultural crops.

Tolerance of plant species towards salinity is a polygenic trait controlled by multiple genes[2]. A plant is able to adapt to salinity through three distinct mechanisms, namely; **osmotic stress tolerance**; which is regulated by long distance signals that reduce shoot growth[3], **ion exclusion**; where sodium (Na^+) and chloride (Cl^-) transporters in the roots, prevent toxic accumulation of these ions in the leaves[4] and lastly, **tissue tolerance**, whereby a tissue can withstand accumulation of Na^+ or Cl^- because the ions are compartmentalized at the cellular and intracellular levels of a plant[3–8]. Next to that, tissue tolerance is also mediated by the synthesis of multiple osmolytes (e.g. proline and ononitol) and aquaporins that have increased salinity tolerance when their production is induced[9–12]. Candidate genes associated with salinity tolerance have mainly elucidated genes associated with ion exclusion (namely *the high affinity potassium transporter (HKT)*[13] and salty overly sensitive (SOS)[14] pathway related genes) and tissue tolerance (Na^+/H^+ antiporters (NHX)[15]). However, we have yet to identify genes associated with the regulation of osmotic stress response under saline environments[16].

When a plant is under osmotic stress, there is an accumulation of reactive oxygen species (ROS), a key signaling molecule for many biochemical and physiological processes, that may also cause oxidative damage if not removed or "scavenged" properly by other plant cell components (Figure 1)[17]. The ability of a plant to scavange ROS in stressful conditions is an important indicator whether a plant is tolerant to osmotic stress. Multiple studies have highlighted both enzymatic and non-enzymatic antioxidants systems (AOS) as key components to detoxify and maintain ROS homeostasis[18]. However, there is minimal research studies that have focused on purely elucidate ROS generation under salinity stress[19–21]. Additionally, it is only highlighted that ROS-mediated salt stress is associated with osmotic stress tolerance, however no research have disclosed the possibility of ROS scavenging being a mediator in salinity tolerance associated with ion exclusion or tissue tolerance (Figure 1).

Improving our understanding in salinity tolerance needs to be species specific, since translating our understanding of salinity tolerance from the model organism *Arabidopsis thaliana* to other cereal plants

has not been successful as results that were translated from *A. thaliana* to other cereal crops showed contradictory and sometimes completely opposite outcomes in other cereal crops[22].

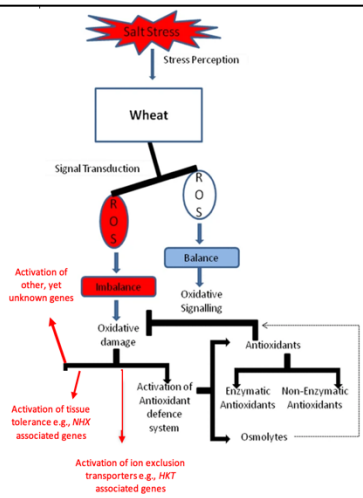


Figure 1 Visual representation of ROS metabolism upon salinity stress. Black lines indicate known biochemical strategies for ROS scavenging. This proposed research investigates whether the red lines are also associated with the ROS metabolism

Bread wheat (*Triticum aestivum*) is amongst the most cultivated crops in the world for human consumption and accounts for roughly 55% of the world populations caloric intake[23,24]. Wheat is incredibly susceptible to salinity stress, most notably during early stages of plant development are the most damaging to these plants[6,25]. Natural variation to salinity tolerance exists across wheat species and ranges anywhere from 20mM to 250mM NaCl[26,27], however we have limited understanding of their related mechanisms and associated genes. In short, it is important to identify genes associated with salinity tolerance as well figure out the mechanism this enhanced salinity tolerance is conferred to[28].

To ensure food security for the world populations, it is pivotal to design crops that tolerate hyperosmotic constraints and thereby have high production levels of wheat even under salinity stress. Historically, researching these pathways has been challenging due to the wide varieties of physiological and biochemical responses that occur during this process and a lack of suitable screening assays to take into account many of these parameters[29–31]. Wheat has a large genome of roughly 16000 Mb which inherently results in less progress on genomic and genetic engineering research, compared to corn and rice that have significantly smaller genomes[32]. The hexaploid nature of wheat also makes it a challenging species for plant breeding, since the most common method to introduce genes in wheat is through backcrossing, which may take up to seven breeding cycles for successful incorporation[33]. Next to that, is it routinely observed that introduced genes in a polyploid plant species, result in gene silencing, making the effects that desired engineered traits redundant [34,35].

Genome wide association studies (GWAS) are a powerful and widely applied tool in many crop plants to identify quantitative trait loci (QTLs) that are associated with multiple or complex traits such as response to salinity[36–38]. From there, one can take advantage of high-density single nucleotide polymorphism genotyping arrays such as illumina to rapidly analyze genetic variations[39]. In wheat, there are multiple successful studies that applied GWAS to identify QTLs associated with abiotic stress tolerance[36], frost tolerance[40] and disease resistance[41]. However, there is only one study published that applied GWAS to identify QTLs associated with salinity tolerance[42]. Although this study did identify novel genes associated with salinity tolerance, they failed to elucidate which mechanisms are associated with this tolerance.

This proposed research aims to identify genes associated with salinity tolerance by performing a GWAS. By using previously identified wheat accessions that are either incredibly susceptible to salinity versus mildly susceptible to salinity, we will attempt to identify marker- trait associations that are particularly associated with the germination potential, the most critical developmental stage of plants under 100mM and 150mM NaCl stress. If I find interesting genes associated with increased salinity tolerance, with the ultimate aim to create transgenic wheat lines that express these candidate genes.

My second aim in this proposed research is to investigate how ROS metabolism functions under salinity stress. To do this, I will look at the generation rates of ROS in wildtype bread wheat, three separate wheat lines that have increased salinity tolerance either because of their high production of the osmolyte proline[11] , overexpression of the key transporter gene related to Na^+ exclusion (TaHKT1;5-D)[13], overexpression of an Na^+/H^+ antiporter gene (*TaNHX2)*[15] and our own created transgenic wheat lines. I hypothesize that the salinity tolerance previously seen in wheat plants with high proline production and overexpression of the Na^+ exclusion transporter are mediated by ROS scavenging. Finally, by monitoring gene expression across plants that showed intesting effects in ROS generation, I aim to identify AOS genes associated with ROS detoxification.

With this proposal we will be able to, for the first time ever, assess the influence of ROS metabolism across two out of three salinity tolerance mechanisms. This is an essential part in our understanding of salinity tolerance in wheat as this can pave way to identifying more ROS dependent genes and evaluate whether we should stop perceiving ROS metabolism in a reductionistic way (i.e. only mentioned for osmotic stress tolerance) and start integrating it for screening of all plant mechanisms.

Key objectives of the proposed research are the following:

1. Identify novel candidate genes associated with salinity tolerance by using genome-wide association mapping.
2. Perform assays to determine if reactive oxygen species are accumulated at germination and flowering time between wildtype and transgenic wheat lines treated with multiple concentrations of NaCl.
3. Determine if changes in ROS metabolism across wildtype and transgenic wheat lines treated with NaCl, causes changes in gene expression of genes associated with ROS detoxification.

Scientific background

The initial mechanism that is expressed when wheat plants are exposed to saline soil is osmotic stress and later on also ion toxicity [6,43]. During this first phase, there are multiple physiological, morphological and developmental changes that are observed, including, an imbalance in nutrients, particularly N and potassium K^+, accumulation of ROS, decreased opening of the stomata and changes in antioxidant enzymes activity [44].

It is known that plant cells contain multiple well evolved antioxidant defence system (AOS) to avoid ROS induced damages, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), ascorbic acid (AsA), tocopherol, glutathione and phenolic compounds [18,45–47]. Each cellular component (nucleus, chloroplast, vacuoles, etc) contains more than one enzymatic activity that detoxifies a particular ROS. In *Nicotiana tabacum,* it was observed that an overexpression of the *APX* gene allowed for salt and drought tolerance[48]. Moreover, in a trangenic rice line, researchers were able to detect higher CAR and APX activity that was associated with drought and salinity tolerance[49]. However currently we do not know which AOS plays the most important role in ROS scavanging, therefore limiting our ability to apply our understanding of these antioxidant systems, to improve the defense mechanisms against ROS generation.

Improving our understanding in the balance between ROS generation and ROS scavenging under salinity stress in wheat, requires us to fundmentally analyse how a ROS metabolism is influenced across the multiple salt tolerance mechanisms in plants. By doing so we are able to create an initial base of understanding whether ROS plays a more critical role across all salinity mechanisms than currently elucidated. **I hypothesize that the salinity tolerance we have previously seen due to accumulation of the osmolyte proline and overexpression expression of *NHX* is mediated through enhanced levels of AOS genes.**

I propose 3 main work packages to test the following hypothesis

1. Genome wide association mapping to identify candidate genes associated with salinity tolerance
2. Investigate whether there is an associationg between ROS metabolism (both ROS generation and ROS scavenging) and salinity tolerance across previously identified salinity tolerance mechanisms.
3. Investigate whether Ta*NHX2* and Ta*P5CS* seeds are tolerant to salinity via enhanced levels of AOS genes

Scientific approach

Our primary focus is to identify gene candidates that are associated with salinity tolerance using a GWAS.

1. <u>Identify novel candidate genes associated with salinity tolerance by using genome-wide association mapping.</u>

<u>1a. Genome-wide association studies:</u> Roughly 250 wheat accessions and cultivars are selected from a worldwide collection to evaluate salinity response at the germination stage. A set of 100 seeds of each genotype is placed in a 90mm Petri dish on Whatman filter paper to germinate.The treaments will contain water (control), 100mM NaCL or 150 mM NaCl.After 72 hours we can count which the germinated seeds. All wheat accessions and cultivars are genotyped with wheat 90 K single-nucleotide polymorphism chip[50]. Physical SNPs can be obtained from the International Wheat Genome Sequencing Consortium website. After this we can map our GWAS and select our candidate genes using the GEMMA software[51]. To identify candidate genes for the loci of salt tolerance, the sequences of SNPs that were associated with salt tolerance–related traits were used to BLAST against the Chinese Spring IWGSC Refseq v1.0 genome assembly

<u>1b: Production of transgenic wheat plants:</u> DNA transformation of identified gene candidates will be performed using agrobacterium-mediated transformation. A transformation vector is created using the candidate gene and *β-glucuronidase* (*gus*) as a selection marker. Mature bread wheat zygotic embryos are inoculated with our agrobacterium suspension and we expected that after three generations we have transgenic seeds.

<u>1c. Evaluation of the salt tolerance enhancement in transgenic wheat plants</u>: Seeds from the transgenic lines and wild-type wheat plants are left for germination in Hoagland medium using 0mM, 100mM and 150mM NaCl. The salt tolerance is determined by counting the amount of days till germination and the plant dry weight after 21 days of cultivation.

2. <u>Investigate whether there is an association between ROS metabolism and salinity tolerance across transgenic wheat lines treated with multiple salinity concentrations</u>

Comparing ROS generation across plant species that show enhanced salinity tolerance phenotypes with differential mechanisms allows us to further understand how ROS mediates salinity tolerance.

<u>2a. Obtain transgenic wheat lines</u>: We will collect previously created transgenic wheat lines of three highly associated genes with a specific salinity tolerance mechanism. For ion exclusion we use a *T. aestivum* that has an overexpression in the *NHX2* gene (*TaNHX2*)[15]. For tissue tolerance we use two transgenic lines: one that has an overexpression in the *HKT1;5-D*[13] gene and one that is overexpressed in the *P5SC* gene[11]. Subsequently, we will also use wheat lines that have a knock-out in *NHX2, HKT1;5-D* and *P5SC* gene in orer to have a negative control in the experiment.

<u>2b. Direct ROS generation assays under salinity treatments</u>: To measure the ROS generation we will grow wild-type bread wheat, Ta*NHX2, TaHKT1;5-D, Ta P5SC, Tanhx2, Tahkt1;5-d, Ta5psc* and our yet to created transgenic lines in 90mm Hoagland well dishes medium containing 0mM, 100mM or 150mM NaCl. Each

plant seed required an n=12 and all experiments will be repeated three times. At 21 days after germination, the total ROS content is detected by performing Electron paramagnetic resonance (EPR) spectroscopy.
2c. Indirect ROS generation assays under salinity treatments: Since ROS generation in a stress environment causes changes in chlorophyll, anthocyanin, solutes and membrane integrity we can use biochemical methods to indirectly measure ROS content. At 21 days after germination, I will harvest our leaves and measure the total chlorophyl content of leave pigments, anthocyanin production, as reported by Jeong et al, 2010 and free proline content using isatin (1H-indole-2,3-dione) paper assays. The use of both direct and indirect ROS generation assays ensures the reliance of our data and deals with unforeseen biases.

3. Ta*NHX2* and Ta*P5CS* seeds are tolerant to salinity via enhanced levels of multiple AOS genes

To test our hypothesis of whether there are enhanced levels of AOS genes in overexpression of genes associated with ion exclusion and tissue tolerance, we will look at the content of both enzymatic and non-enxymatic antixidants as well as gene expresison of enzymatic antioxidants.
3a. Assays for measurement of total non-enzymatic antioxidant content in wild-type and transgenic wheat lines: I selected five of the most relevant non-ezymatic antioxidants (ascorbic acid, reduced glutathione, a-tocopherol, total phenolics and flavonoids)[18] to estimate their content in our transgenic and wildtype wheat lines. The methods for the quantification of ascorbic acid are spectrophotometric measurements[52]. The total phenolics, flavonoids and tocopherol can be measured using the rainbow protocol.[53]
3b. Measurement of total enzymatic antioxidant content in wild-type and transgenic wheat lines: For the enzymatic assays, we need to extract leaves and ground them in fine powder and freeze them with liquid nitrogen. All enzymatic assays will be performed in a 96-well plate setup in a Biotex synergy HTX plate reader[54]. Enzymatic activities can be normalized by fresh weight. We will measuring activity of the main enzymatic components of the defense mechanism, namely CAT, DHAR, GR, POX,SOD and APX using well known methodes used for these enzymatic assays [54–59]. Alternatively, we can also evaluate gene expression of antioxidant enzymes using RT-qPCR analysis. To do this, we need to quantify the total amount of RNA in each plant sample and from there, we use the Bio-Rad CFX Real Time Thermo Cycler using the fluorophore LightCycler® 480 SYBR Green I Master. The primers required for the qRT-PCR have previously been designed by Jiang et al 2012[60].

Innovative aspects and societal impact

Salinity tolerance mechanisms in plants have been a topic of exploration for multiple decades[6,19]. In the past, research focused on salinity tolerance have taken a reductionistic approach in perceiving salinity tolerance by only looking at genes associated with a mechanism and not how alternative biochemical and physiological components may influence this mechanism. The results of this methodology is evident by the lack of actual applicability of our current understanding of salinity tolerance in agricultural field trials. Given that soil salinization is expected to impact 50% of arable land by 2050[1], enhancing our understanding on how biochemical components in a plant impacts salinity tolerance is a key priority. This study aims to identify novel gene candidates associated with salinity tolerance as well as unravel how ROS metabolism influences salinity tolerance across previously known salinity tolerance mechanisms. This will give us a better glance in how not only candidate genes influence salinity tolerance, but also how the biochemical metabolism may corporate with this. By working with bread wheat, we will increase the chances of the translatability of our understanding on salinity tolerance to agricultural field trials.

References

1. Butcher, K., Wick, A. F., DeSutter, T., Chatterjee, A. & Harmon, J. Soil Salinity: A Threat to Global Food Security. *Agron J* **108**, 2189–2200 (2016).
2. Roy, S. J., Negrão, S. & Tester, M. Salt resistant crop plants. *Curr Opin Biotechnol* **26**, 115–124 (2014).
3. Møller, I. S. & Tester, M. Salinity tolerance of Arabidopsis: a good model for cereals? *Trends Plant Sci* **12**, 534–540 (2007).
4. Roy, S. J., Negrão, S. & Tester, M. Salt resistant crop plants. *Curr Opin Biotechnol* **26**, 115–124 (2014).
5. van Zelm, E., Zhang, Y. & Testerink, C. Salt Tolerance Mechanisms of Plants. *Annu Rev Plant Biol* **71**, 403–433 (2020).
6. Munns, R. & Tester, M. Mechanisms of salinity tolerance. *Annual Review of Plant Biology* vol. 59 Preprint at https://doi.org/10.1146/annurev.arplant.59.032607.092911 (2008).

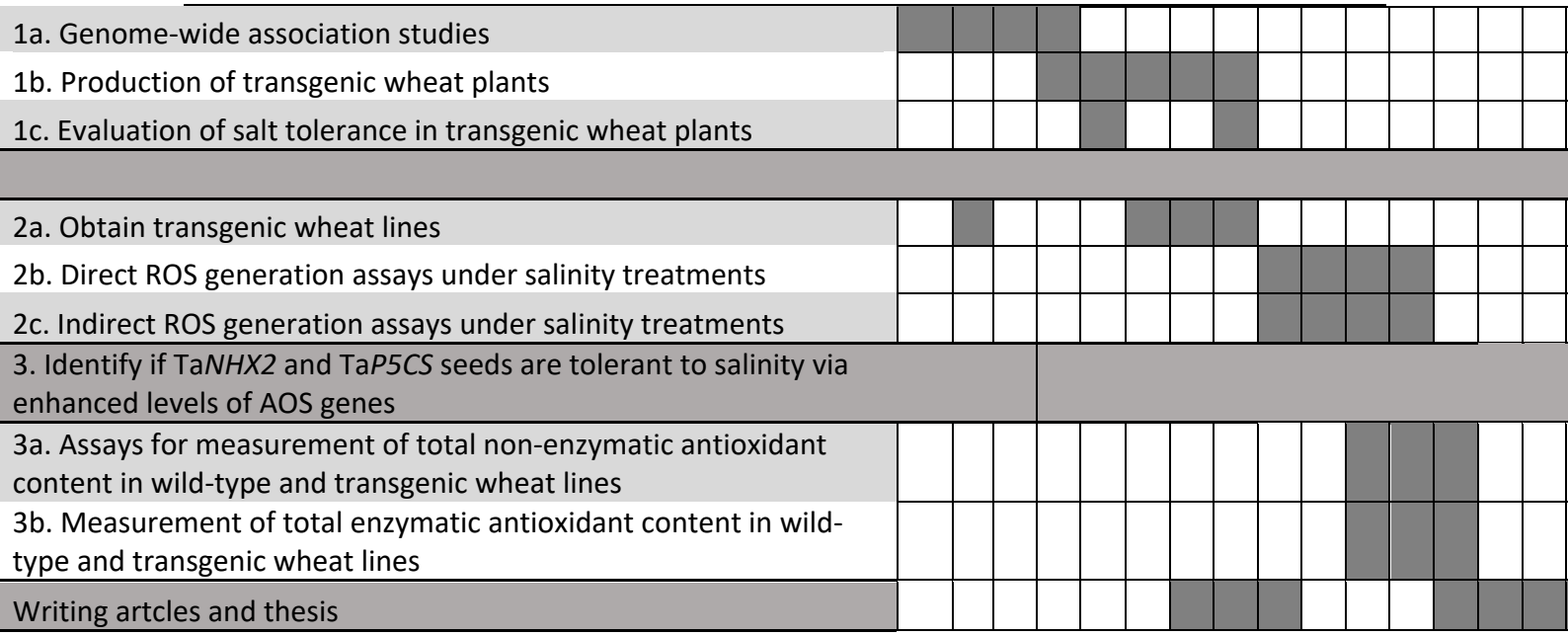
7. Shi, H., Lee, B., Wu, S.-J. & Zhu, J.-K. Overexpression of a plasma membrane Na+/H+ antiporter gene improves salt tolerance in Arabidopsis thaliana. *Nat Biotechnol* **21**, 81–85 (2003).
8. Xue, Z. Y. *et al.* Enhanced salt tolerance of transgenic wheat (Tritivum aestivum L.) expressing a vacuolar Na+/H+ antiporter gene with improved grain yields in saline soils in the field and a reduced level of leaf Na+. *Plant Science* **167**, 849–859 (2004).
9. Abebe, T., Guenzi, A. C., Martin, B. & Cushman, J. C. Tolerance of Mannitol-Accumulating Transgenic Wheat to Water Stress and Salinity. *Plant Physiol* **131**, 1748–1755 (2003).
10. Bhauso, T. D. *et al.* Overexpression of Bacterial *mtlD* Gene in Peanut Improves Drought Tolerance through Accumulation of Mannitol. *The Scientific World Journal* **2014**, 1–10 (2014).
11. Kishor, PBK., Hong, Z., Miao, G. H., Hu, CAA. & Verma, DPS. Overexpression of [delta]-Pyrroline-5-Carboxylate Synthetase Increases Proline Production and Confers Osmotolerance in Transgenic Plants. *Plant Physiol* **108**, 1387–1394 (1995).
12. Sheveleva, E., Chmara, W., Bohnert, H. J. & Jensen, R. G. Increased Salt and Drought Tolerance by D-Ononitol Production in Transgenic Nicotiana tabacum L. *Plant Physiol* **115**, 1211–1219 (1997).
13. Xu, B., Hrmova, M. & Gilliham, M. High affinity Na^+ transport by wheat HKT1;5 is blocked by K^+. *Plant Direct* **4**, (2020).
14. Zhao, C., William, D. & Sandhu, D. Isolation and characterization of Salt Overly Sensitive family genes in spinach. *Physiol Plant* **171**, 520–532 (2021).
15. Yarra, R. The wheat NHX gene family: Potential role in improving salinity stress tolerance of plants. *Plant Gene* vol. 18 Preprint at https://doi.org/10.1016/j.plgene.2019.100178 (2019).
16. Isayenkov, S. v. & Maathuis, F. J. M. Plant Salinity Stress: Many Unanswered Questions Remain. *Front Plant Sci* **10**, (2019).
17. Das, K. & Roychoudhury, A. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Front Environ Sci* **2**, (2014).
18. Ahmad, P., Jaleel, C. A., Salem, M. A., Nabi, G. & Sharma, S. Roles of enzymatic and nonenzymatic antioxidants in plants during abiotic stress. *Crit Rev Biotechnol* **30**, 161–175 (2010).
19. Hernández, J. A. Salinity Tolerance in Plants: Trends and Perspectives. *Int J Mol Sci* **20**, 2408 (2019).
20. Hasanuzzaman, M. *et al.* Reactive Oxygen Species and Antioxidant Defense in Plants under Abiotic Stress: Revisiting the Crucial Role of a Universal Defense Regulator. *Antioxidants* **9**, 681 (2020).
21. Halliwell, B. Reactive Species and Antioxidants. Redox Biology Is a Fundamental Theme of Aerobic Life. *Plant Physiol* **141**, 312–322 (2006).
22. Møller, I. S. & Tester, M. Salinity tolerance of Arabidopsis: a good model for cereals? *Trends Plant Sci* **12**, 534–540 (2007).
23. Arora, N. K. Impact of climate change on agriculture production and its sustainable solutions. *Environmental Sustainability* **2**, 95–96 (2019).
24. Giraldo, P., Benavente, E., Manzano-Agugliaro, F. & Gimenez, E. Worldwide Research Trends on Wheat and Barley: A Bibliometric Comparative Analysis. *Agronomy* **9**, 352 (2019).
25. Flowers, T. J., Munns, R. & Colmer, T. D. Sodium chloride toxicity and the cellular basis of salt tolerance in halophytes. *Ann Bot* **115**, 419–431 (2015).

26. Qureshi, R. H., Rashid, A. & Ahmad, N. A procedure for quick screening of wheat cultivars for salt tolerance. *Genetic Aspects of Plant Mineral Nutrition* 315–324 (1990) doi:10.1007/978-94-009-2053-8_48.
27. Salam, A., Hollington, P. A., Gorham, J., Wyn Jones, R. G. & Gliddon, C. Physiological genetics of salt tolerance in wheat (Triticum aestivum L.): Performance of wheat varieties, inbred lines and reciprocal F1 hybrids under saline conditions. *J Agron Crop Sci* **183**, 145–156 (1999).
28. Mickelbart, M. v., Hasegawa, P. M. & Bailey-Serres, J. Genetic mechanisms of abiotic stress tolerance that translate to crop yield stability. *Nat Rev Genet* **16**, 237–251 (2015).
29. Huang, X. *et al.* Overexpression of the wheat salt tolerance-related gene TaSC enhances salt tolerance in Arabidopsis. *J Exp Bot* **63**, 5463–5473 (2012).
30. Nishimura, A., Nasuno, R. & Takagi, H. The proline metabolism intermediate Δ 1-pyrroline-5- carboxylate directly inhibits the mitochondrial respiration in budding yeast. *FEBS Lett* **586**, 2411–2416 (2012).
31. Aycan, M., Baslam, M., Asiloglu, R., Mitsui, T. & Yildiz, M. Development of new high-salt tolerant bread wheat (Triticum aestivum L.) genotypes and insight into the tolerance mechanisms. *Plant Physiology and Biochemistry* **166**, 314–327 (2021).
32. Arumuganathan, K. & Earle, E. D. Nuclear DNA content of some important plant species. *Plant Mol Biol Report* **9**, (1991).
33. Saravanan, K. & Sabesan, T. Physical and Chemical Mutagenesis Methods for Development of Insect-Resistant Crop Varieties. in *Experimental Techniques in Host-Plant Resistance* (2019). doi:10.1007/978-981-13-2652-3_30.
34. Alvarez, M. L. *et al.* Silencing of HMW glutenins in transgenic wheat expressing extra HMW subunits. *Theoretical and Applied Genetics* **100**, (2000).
35. Janakiraman, V., Steinau, M., McCoy, S. B. & Trick, H. N. Recent advances in wheat transformation. in *In Vitro Cellular and Developmental Biology - Plant* vol. 38 (2002).
36. Challa, S. & Neelapu, N. R. R. Genome-Wide Association Studies (GWAS) for Abiotic Stress Tolerance in Plants. in *Biochemical, Physiological and Molecular Avenues for Combating Abiotic Stress Tolerance in Plants* 135–150 (Elsevier, 2018). doi:10.1016/B978-0-12-813066-7.00009-7.
37. Tam, V. *et al.* Benefits and limitations of genome-wide association studies. *Nat Rev Genet* **20**, 467–484 (2019).
38. Saini, D. K. *et al.* Comprehensive evaluation of mapping complex traits in wheat using genome-wide association studies. *Molecular Breeding* vol. 42 Preprint at https://doi.org/10.1007/s11032-021-01272-7 (2022).
39. Wang, S. *et al.* Characterization of polyploid wheat genomic diversity using a high-density 90 000 single nucleotide polymorphism array. *Plant Biotechnol J* **12**, (2014).
40. Soleimani, B. *et al.* Genome wide association study of frost tolerance in wheat. *Sci Rep* **12**, 5275 (2022).
41. Naruoka, Y., Garland-Campbell, K. A. & Carter, A. H. Genome-wide association mapping for stripe rust (Puccinia striiformis F. sp. tritici) in US Pacific Northwest winter wheat (Triticum aestivum L.). *Theoretical and Applied Genetics* **128**, 1083–1101 (2015).
42. Quamruzzaman, Md., Manik, S. M. N., Shabala, S., Cao, F. & Zhou, M. Genome-wide association study reveals a genomic region on 5AL for salinity tolerance in wheat. *Theoretical and Applied Genetics* **135**, 709–721 (2022).
43. Arzani, A. & Ashraf, M. Smart Engineering of Genetic Resources for Enhanced Salinity Tolerance in Crop Plants. *CRC Crit Rev Plant Sci* **35**, 146–189 (2016).

44. Gill, S. S. & Tuteja, N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry* **48**, 909–930 (2010).
45. Ashraf, M. Biotechnological approach of improving plant salt tolerance using antioxidants as markers. *Biotechnology Advances* vol. 27 Preprint at https://doi.org/10.1016/j.biotechadv.2008.09.003 (2009).
46. Shi, Q. & Zhu, Z. Effects of exogenous salicylic acid on manganese toxicity, element contents and antioxidative system in cucumber. *Environ Exp Bot* **63**, (2008).
47. Jaleel, C. A. *et al.* Endogenous hormonal and enzymatic responses of Catharanthus roseus with triadimefon application under water deficits. *C R Biol* **331**, (2008).
48. Badawi, G. H. *et al.* Enhanced tolerance to salt stress and water deficit by overexpressing superoxide dismutase in tobacco (Nicotiana tabacum) chloroplasts. *Plant Science* **166**, (2004).
49. Yang, Z., Wu, Y., Li, Y., Ling, H. Q. & Chu, C. OsMT1a, a type 1 metallothionein, plays the pivotal role in zinc homeostasis and drought tolerance in rice. *Plant Mol Biol* **70**, (2009).
50. Ahmadi, N. *et al.* Targeted association analysis identified japonica rice varieties achieving Na+/K+ homeostasis without the allelic make-up of the salt tolerant indica variety Nona Bokra. *Theoretical and Applied Genetics* **123**, 881–895 (2011).
51. Zhou, X. & Stephens, M. Genome-wide efficient mixed-model analysis for association studies. *Nat Genet* **44**, (2012).
52. Hewitt, E. J. & Dickes, G. J. Spectrophotometric measurements on ascorbic acid and their use for the estimation of ascorbic acid and dehydroascorbic acid in plant tissues. *Biochemical Journal* **78**, 384–391 (1961).
53. López-Hidalgo, C., Meijón, M., Lamelas, L. & Valledor, L. The rainbow protocol: A sequential method for quantifying pigments, sugars, free amino acids, phenolics, flavonoids and <scp>MDA</scp> from a small amount of sample. *Plant Cell Environ* **44**, 1977–1986 (2021).
54. Fimognari, L. *et al.* Simple semi-high throughput determination of activity signatures of key antioxidant enzymes for physiological phenotyping. *Plant Methods* **16**, 42 (2020).
55. McCord, J. M. & Fridovich, I. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *Journal of Biological Chemistry* **244**, (1969).
56. Polle, A., Otter, T. & Seifert, F. Apoplastic peroxidases and lignification in needles of Norway spruce (Picea abies L.). *Plant Physiol* **106**, (1994).
57. Edwards, E. A., Rawsthorne, S. & Mullineaux, PhilipM. Subcellular distribution of multiple forms of glutathione reductase in leaves of pea (Pisum sativum L.). *Planta* **180**, (1990).
58. Dalton, D. A., Russell, S. A., Hanus, F. J., Pascoe, G. A. & Evans, H. J. Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules. *Proceedings of the National Academy of Sciences* **83**, 3811–3815 (1986).
59. Aebi, H. [13] Catalase in vitro. in 121–126 (1984). doi:10.1016/S0076-6879(84)05016-3.
60. Jiang, L. *et al.* Real-time quantitative PCR monitoring of antioxidant enzyme gene expression in wheat radicles treated with Cu2+ and Cd2+. *Life Sci J* **9**, (2012).

7. Timetable of the project

Activity	Year 1	Year 2	Year 3	Year 4
1. Identify novel candidate genes associated with salinity tolerance				



FINANCIAL DETAILS

10a. Budget

Please use the table below for the description of the personnel and material resources required for the project. The maximum budget available from NWO per project is € 231,250. In this case the maximum project budget, including cash matching, is € 250,000.

Project budget (k€)	**Year 1**	**Year 2**	**Year 3**	**Year 4**	**Total**
Personnel costs					
Salary PhD student (including bench fee)	47,3	50,7	53,7	57,4	209,2
Research costs					
Consumables	15,0	15,0	7,8	3	40,8
Total	62,3	65,7	61,5	60,4	250

STATEMENTS BY THE APPLICANT

11. Statements by the applicant

YES/~~NO~~ I endorse and follow the Code Openness Animal Experiments (if applicable)

YES/~~NO~~ I endorse and follow the Code Biosecurity (if applicable)

YES/~~NO~~ By submitting this document I declare that I satisfy the nationally and internationally accepted standards for scientific conduct as stated in the Netherlands Code of Conduct for Scientific Practice 2012 (Association of Universities in the Netherlands (VSNU)).

YES/~~NO~~ I have completed this form truthfully

Name: Nakisa Echobardo
Place: Utrecht