Exploring interactions of GABA and alternative oxidase pathways under salinity stress

By

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ABSTRACT

Dryland salinity effects ~2.5 million hectares of Australian farmland and is expected to increase to 17 million hectares by 2050 leading to a loss in income of $270 million annually. Salinity affects growth, development and crop yield and poses a challenge to global food security. Plants growing in saline conditions show ionic imbalance, hyperosmotic stress, increased accumulation of gamma aminobutyric acid (GABA) and reactive oxygen species (ROS) leading to oxidative stress.

Studies suggest that non-phosphorylating alternative respiratory pathways mediated by NAD(P)H Dehydrogenase and Alternative Oxidase (AOX) play an important role during stress by uncoupling carbon metabolism from adenylate control and minimization of ROS. Further, increase in GABA, a non-protein amino acid helps plants cope with salinity induced osmotic stress by maintaining a balance between accumulation of sodium (Na^+) and loss of potassium (K^+).

This study investigated the interaction between GABA and AOX pathway under salinity stress. This first aim was addressed by exploring the effects of AOX overexpression on endogenous GABA concentration, expression of GABA-related (*GAD2, GAD1*) and AOX pathway related (*AOX1a, AOX1d, NDB2*) genes and levels of a marker (MDA) for reactive oxygen species, in the presence and absence of salinity stress. *Arabidopsis thaliana* wild type (Col0) and AOX1a overexpression line were grown in hydroponic system for 6 weeks and were treated with 100 mM NaCl for 1 and 9 days. Shoots and roots samples were harvested 1 day and 9 days after salt treatment. Transcript levels of *AOX1a, AOX1d, NDB2* and *GAD2* (relative gene expression for all the genes were obtained by $2^{-\Delta\Delta CT}$ method) , MDA content and endogenous GABA content in both the wild type and AOX1a overexpression line were increased under salinity stress. GABA content was higher in both shoot and root tissues of the AOX1A overexpression line relative to wild type, under control conditions.

The second aim of this study was to investigate the combined roles of AOX and GABA on salinity tolerance in *Arabidopsis thaliana* plants that were either overexpressing, or lacking, AOX1a. This

aim was addressed by exploring the effects of exogenous GABA application on plant physiology measurements, including shoot and root growth, stomatal conductance and ion accumulation in the presence and absence of salinity stress. Six-week-old wild type, AOX1a overexpression and AOX1a knock-out plants grown in soil were treated with sodium chloride (NaCl, 150 mM) with or without GABA (10 mM) for 10 days and harvested to measure fresh weight, dry weight, and sodium and potassium ion concentrations. Seedlings were also transferred to agar plates containing different concentrations of NaCl and GABA, for the measurement of total root length and relative growth rate.

Stomatal conductance was greatly reduced by salinity stress independent of the presence of exogenous GABA. However, sodium ion concentration, which can be toxic to the cell during salinity stress, was significantly lower in AOX1a knock-out plants if they were treated with GABA simultaneous to the salt treatment. Relative growth rate of roots in wild type and knock-out lines were reduced significantly when GABA was applied in the absence of salinity stress. However, under salinity stress conditions this negative growth effect was gone, and GABA improved the relative growth rate of AOX1a overexpressing roots, relative to wild type roots.

According to these results, there is some interplay between AOX and GABA, based on the increase in endogenous GABA levels in the AOX1a overexpression line, as well as decreased sodium ion concentrations and increased plant root growth in AOX1A knock-out lines treated with GABA. The AOX pathway may facilitate GABA metabolism, which in turn could act as a carbon or nitrogen source during stress, or there might be a common signalling pathway for both AOX and GABA metabolism. However, more work is required to support these findings and determine whether there is a direct interaction between the AOX and GABA shunt pathways.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Kanapaddalagamage Madhuka Hansamali

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LIST OF ABBREVIATIONS

AOX	Alternative oxidase
cDNA	Complementary DNA
Ct	Cycle Threshold
$CaSO_4$	Calcium sulphate
DNA	Deoxyribose Nucleic Acid
dNTP	Deoxy Nucleotide Tri Phosphate
DW	Dry weight
EDTA	Ethylenediaminetetraacetic Acid
ETC	Electron transport chain
FW	Fresh weight
GABA	Gamma aminobutyric acid
H_2O	Water
KCl	Potassium chloride
MDA	Malondialdehyde
MgSO4	Magnesium sulphate
MgCl2	Magnesium chloride
NAD(P)H DH	NAD(P)H dehydrogenase
NaCl	Sodium chloride
NO	Nitric oxide
Na2SO4	Sodium sulphate
Na2CO3	Sodium carbonate
O2	Oxygen
O2-	Superoxide
PC2	Physical Containment Level 2
PCR	Polymerase Chain Reaction

qRT-PCR	Quantitative real-time-polymerase chain reaction
RNA	Ribo Nucleic Acid
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
WT	Wild type
XX1	AOX1a overexpression line

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Heat, drought, salinity, soil alkalinity and acidity are all abiotic stresses that are major limiters of crop and food production in Australia. It is predicted that these adverse conditions (stressors) will escalate over the next decade causing a decline in crop and food production, with potential impacts including food shortage, loss of pasture for livestock and economic losses (ABARES 2019). Thus, there is an immediate need for research aimed at mitigating the effect of these stressors on plant growth and yields. This project seeks to explore the interactions between different stress signalling pathways in *Arabidopsis thaliana* under salinity.

1.1. Impact of abiotic stress on plants.

Plants experience abiotic stresses such as drought, salinity, flood, cold, heat and heavy metals due to rapid change in climate. Abiotic stress negatively affects plant growth and development, energy production and crop yield. Ultimately, it limits agricultural productivity, challenges food security, and costs more money for agriculture. As an example, government of Australia spends $98 million a year for agricultural production in Murray–Darling Basin due to dryland salinity (Wilson 2004). Abiotic stress such as drought and heat impact the efficiency of photosynthesis (Zhu 2016), energy production (inhibition of key enzymes in tricarboxylic acid -TCA cycle), nutrient and water uptake, mitochondrial respiration (ETC dysfunction – electron transport chain) and generation of reactive oxygen species (ROS) (Bouché et al. 2003; Millar et al. 2011; Selinski et al. 2018; Shelp, Mullen & Waller 2012; Vanlerberghe 2013). Agricultural productivity and environmental sustainability can be enhanced by improving stress tolerance as plants susceptible to stresses utilise large amount of resources such as water and fertilizers that add extra burden to the environment (Zhu 2016).

1.2. Salinity stress in plants

Numerous salts such as NaCl, Na_2SO_4, $MgSO_4$, $MgCl_2$, $CaSO_4$, KCl, and Na_2CO_3 are present in the soil and the excessive presence of NaCl, $MgCl_2$, KCl and Na_2CO_3 cause salinity (Tavakkoli et al. 2010). Salinization is the term used to describe the process of increasing salt content in the soil (Narsing Rao et al. 2019). In general, soil is considered to be saline when the electrical conductivity (EC) of saturation extract of soil in the root zone is higher than 4 dS m^{-1} (approximately 40 mM NaCl) at 25 °C and composed of 15% of exchangeable sodium (Jamil et al. 2011; Munns 2005). Sodium chloride is the most available salt in saline soils and negatively impacts plants causing reduction in plant growth, photosynthesis, and seed germination (Narsing Rao et al. 2019; Tavakkoli et al. 2010). Around 20% of the cultivated land and 30% of the irrigated lands are severely affected by salinity. The saline soil areas are increasing approximately at a rate of 10% annually due to weak precipitation, elevated surface evaporation, weathering of native rocks, use of saline water for irrigation, and inadequate cultural practices (Jamil et al. 2011). According to estimations, more than 50% of the arable lands will be salinized by 2050 (Jamil et al. 2011; Shrivastava & Kumar 2015). Different physiological and metabolic processes such as photosynthesis, transpiration, respiration, vegetative growth, germination, and flowering are adversely affected by salinity stress (Gupta & Huang 2014; Kaleem et al. 2018).

Crop yield around the world is severely affected due to salinity and therefore it is essential to understand how salinity stress affects plants (Figure 1.1) (Narsing Rao et al. 2019). According to the model by Munns (1988), plants experience osmotic and ionic stress when subjected to salinity. Roots of the plants instantly experience osmotic stress due to expansion of cells and inhibition of the water uptake (Munns et al. 1995). Accumulation of toxic ions such as sodium (Na^+) cause ionic stress and as a result necrosis and chlorosis occurs and metabolic activities such as photosynthesis, biosynthesis of protein and absorption of potassium ions ($K^{+)}$ are negatively affected in plants (Deinlein et al. 2014; Panuccio et al. 2014). Osmotic and ionic stresses give rise to secondary stresses such as oxidative

stress which can damage lipids, proteins, and nucleic acids in the membrane (Pérez-López et al. 2009).

Plants uptake water due to the high-water potential in the soil solution compared to the roots (Bliss et al. 2019). Water potential of the soil decreases due to the salinity and as a result water uptake through the roots is inhibited (Bliss et al. 2019). Salinity affects the root system of the plants by reducing length of roots, diameter of roots, number of roots, number of root hairs, diameter of xylem vessels and the width of root cortical cells (Acosta et al. 2017; Arif et al. 2019).

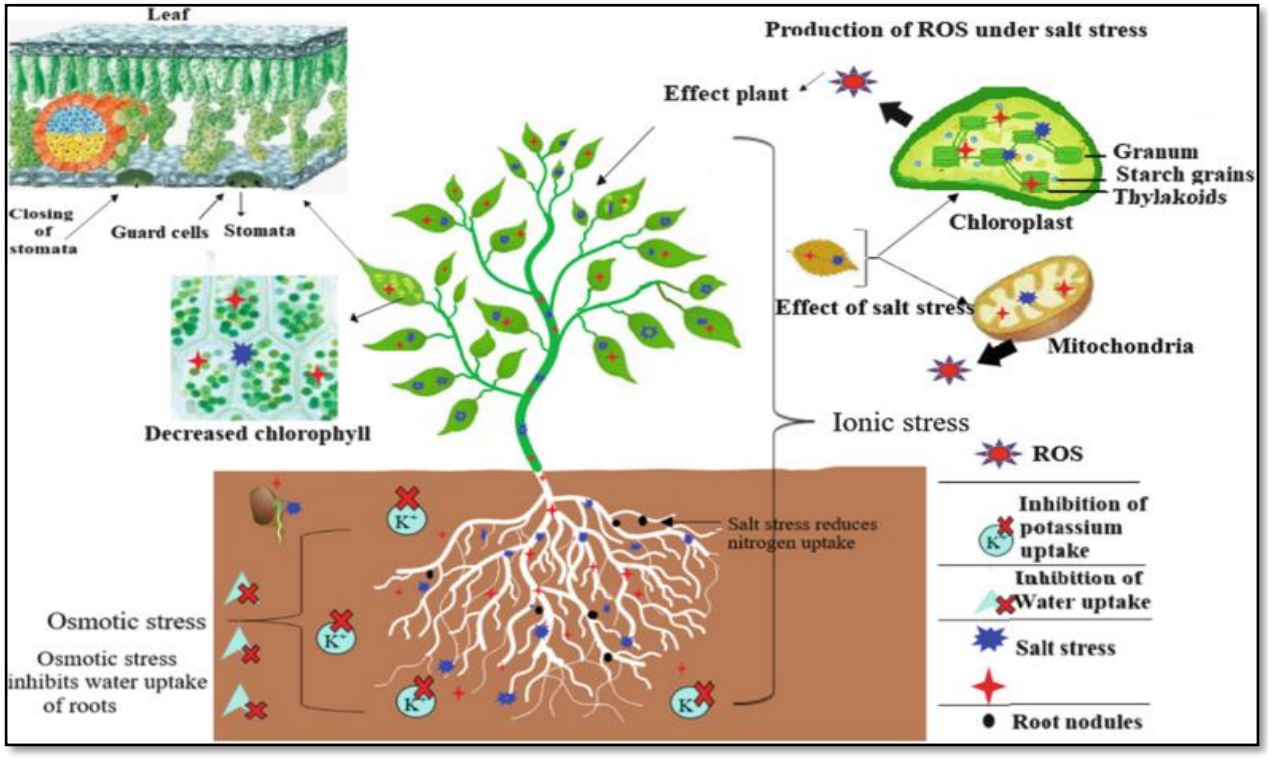

Figure 1.1: Physiological and biochemical changes occurring during salinity stress (Adapted from the permission of Springer eBook, Narsing Rao et al. 2019).

Water carries salts into the xylem of the vascular system through roots via apoplastic or symplastic pathways (Das et al. 2015). Immense vacuolization and lack of general organization of the apical tissue in roots can be seen under salt stress. Parenchyma cells and epidermal cells in the cortex and pith show shrinkage under salt stress (Carillo et al. 2011; Munns & Rawson 1999) and root growth is

hindered due to reduction in cyclin level and suppression of cell elongation and division (Duan et al. 2015).

Adaptation to the stress is affected by mineral nutrition of the plant (Paul & Lade 2014). Imbalance in the plant nutrition reduces growth, development, and yield of the plants (Paul & Lade 2014). K^+ is an important cation present in plant cells that helps to maintain membrane potential and activity of several enzymes. High Na^+ abundance around the exterior region of the root disrupts the uptake of K^+ due to the similarity in chemical properties of Na^+ and K^+. The cells are damaged by the inhibition of the activity of many proteins and enzymes because of the Na^+ entry into the cytosol (Das et al. 2015; Xiong & Zhu 2002).

Seed germination is affected adversely by salinity (NaCl concentrations between 200 mM to 400 mM) in different plants such as *Posidonia, Oryza sativa, Triticum aestivum, Zea mays* and sweet sorghum cultivars (Fernández & Sánchez 2013), (Xu et al. 2011), (Eskandari & Kazemi 2011), (Shahzad et al. 2019) and (Zhang et al. 2014) respectively. Multiple studies have shown that germination (rate of germination and germination initiation) is negatively affected by salinity stress because salinity stress reduces absorption of water and accumulates sodium or chloride ions that cause toxicity in seeds (Abdel et al. 2020; Shu et al. 2017; Singh et al. 2012).

Photosynthesis is negatively affected by salinity stress. When the salinity stress continues, chlorosis occur in old leaves, and they senesce. The overall photosynthetic capacity of the plant will be reduced if the death rates of the leaves are higher than the production rate, leading to the scarcity of carbohydrates in the young leaves, ultimately resulting in growth rate reduction (Carillo et al. 2011). Photosynthesis is affected by long term salinity stress as salt accumulates in young leaves leading to the reduction in chlorophyll and carotenoid content (Saravanavel et al. 2011). Development in shoot and reproduction also negatively influenced by salinity stress (Ilangumaran & Smith 2017).

Lipid content in plants is crucial for cell permeability and the total lipid content is reduced under salt stress (Keshtehgar et al. 2013). Compatible solutes namely, glycine, betaine, proline, polyols, and

sugars are noticeably increased under salt stress (Khan et al. 2000; Singh et al. 2000). The production of ROS in mitochondria, chloroplast and peroxisomes also increases under salinity stress. Significant increases in activities of antioxidative enzymes namely glutathione reductase, ascorbate peroxidase, dehydro-ascorbate reductase and monodehydroascorbate reductase also observed under salinity stress (Parida & Das 2005).

Nitric oxide is responsible for the regulation of processes such as flowering, respiration, closure of stomata, root growth, germination of seeds and cell death (Numan et al. 2018). Activity of nitrate reductase is reduced during salinity stress because of the presence of excessive Cl^- ions in the external medium. Nitrogen fixation is also inhibited due to decreasing activity of nitrogenase under salinity stress (Parida & Das 2005).

In summary, plants respond to salinity according to a biphasic model where the first phase is osmotic stress followed by the second phase ion toxicity. During the first phase the plant growth is reduced significantly, and closure of stomata occurs due to the decline in water potential. Oxidative stress increases and photosynthetic pigments and enzymes are also affected due to the accumulation of Na^+ ions during the second phase. Reactive Oxygen Species (ROS) have a dual role in response to salinity stress; namely as a toxic by product of stress, but also as a signalling molecule that activates pathways to help combat the stress (Ma et al. 2020).

1.3. Reactive oxygen species (ROS)

ROS are derivatives of highly reactive oxygen, composed of oxygen radicals and certain nonradicals. Oxygen radicals such as singlet oxygen, superoxide radicals ($^{\bullet}O_2^-$) and the hydroxyl radical ($HO^{\bullet}$) are composed of unpaired electrons. ROS are involved in the regulation of ion channel activity and gene expression (Demidchik 2018; Miller et al. 2008). However, levels of ROS increase considerably during salinity stress and excess ROS causes oxidative damage (Munns & Tester 2008).

Generation of ROS mainly occurs in chloroplasts, mitochondria, and peroxisomes during both biotic and abiotic stress. Biomolecules are subjected to oxidation when ROS accumulates excessively,

triggering processes leading to damage of proteins, nucleic acids, and lipids and peroxidation of thylakoid membranes (Emma et al. 2000; Partelli et al. 2011). The structure and volume of the chloroplasts changed and caused damage to the structure and integrity of cell membranes and mitochondria under waterlogging stress in maize plants (Ren et al. 2016).

Studies show that salt stress affects mitochondria leading to a decrease in electron transport activities and increase in ROS levels leading to induction of ROS-scavenging systems in the mitochondria (Rodríguez & Taleisnik 2012). The alternative oxidase (AOX) pathway in the mitochondria also has an important role in plants, including the prevention of ROS over-accumulation.

1.4. Alternative oxidase

The alternative oxidase (AOX) is an enzyme of the mitochondrial ETC in various organisms including plants. The electron transport chain of the plant mitochondria is branched to pass the electrons present in the ubiquinone pool to oxygen, either through the classical pathway of Complex III, cyt *c* and Complex IV, or through AOX (Selinski et al. 2018; Vanlerberghe 2013). The flow of electrons through the classical cytochrome pathway, is coupled with proton translocation and thus ATP generation. However, the flow of electrons via AOX is not coupled with proton translocation and therefore ATP is not generated as efficiently. The presence of these two pathways with different yields of energy help maintain the balance of carbon, redox potentials (e.g., ratio of NAD(P)H: NAD(P)) and energy (e.g., NAD(P)H: ATP and ATP:ADP) during stress (Chai et al. 2010; Cvetkovska & Vanlerberghe 2012; Dahal & Vanlerberghe 2018; Giraud et al. 2008; Martí et al. ; Millar et al. 2011; Smith et al. 2009).

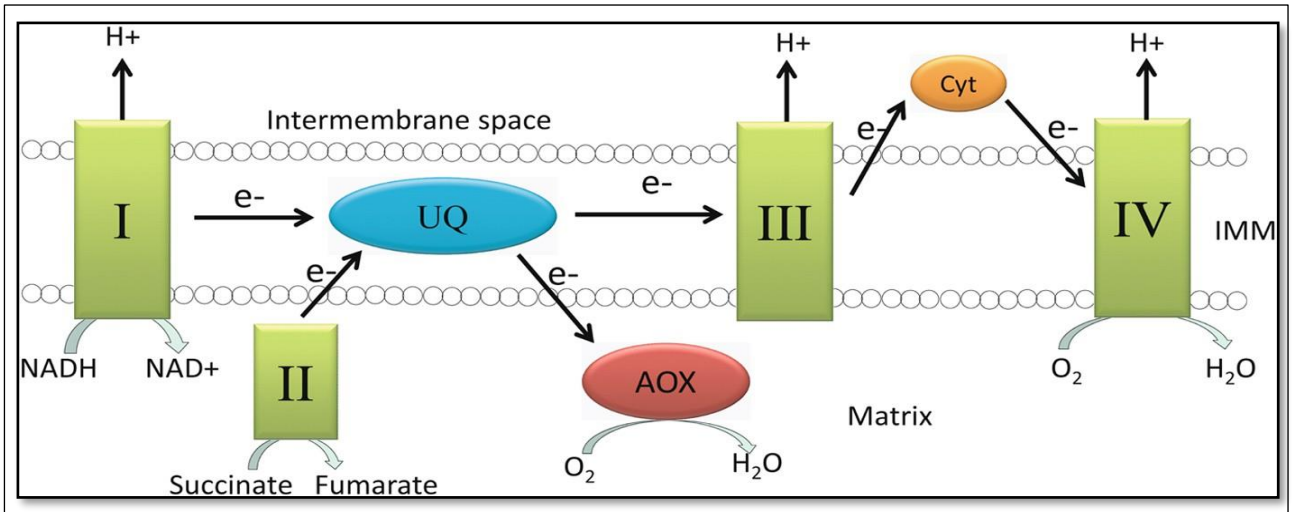

Figure 1.2: The mitochondrial respiratory ETC including the alternative oxidase (AOX). AOX accepts electrons from ubiquinol (UQ). Synthesis of ATP is driven by the production of a proton (H^+) gradient across the inner mitochondrial membrane (IMM). Complexes I, III and IV are responsible for moving protons from the matrix to the intermembrane space (Cheng et al. 2018; Liu & Guo 2017).

AOX activity prevents the accumulation of high energy electrons in the mitochondrial electron transport chain, which can react with oxygen and influence ROS production and subsequently reactive nitrogen species (RNS) production. Thus, active AOX can prevent or decrease ROS formation (Jayawardhane et al. 2020).

AOX has also been proposed to optimize photosynthesis when abiotic stress reduces the photosynthetic rate (Feng et al. 2013; Smith et al. 2009). It was proposed that respiration, photosynthesis, and chlorophyll synthesis are controlled by AOX during drought stress for the maintenance of overall homeostasis and to increase the lifespan of the plants (Vanlerberghe et al. 2016). Salt tolerance was increased in *Medicago truncatula* due to increased AOX expression, which protected the photosystems by regulating ROS levels, since ROS are produced in the reaction centres in Photosystems I and II during abiotic stress conditions (Tripathy & Oelmüller 2012). *Arabidopsis thaliana* plants overexpressing AOX1a and another enzyme of the alternative respiration pathway, NDB2, were more tolerant to a combined drought and high light stress (Sweetman et al. 2019). Several other studies also reported that AOX gene expression and AOX protein increased during

salinity stress in *A. thaliana* (Kreps et al. 2002; Seki et al. 2002), poplar (Ottow et al. 2005), tobacco (Andronis & Roubelakis 2010) and pea (Martí et al. 2011) which helps to reduce oxidative stress induced damages by reducing ROS production.

There are five gene isoforms of AOX in *A. thaliana*, named *AOX1a*, *AOX1b*, *AOX1c*, *AOX1d* and *AOX2* (Polidoros et al. 2009). Functions performed by AOX1a cannot be replaced by other isoforms of AOX, for example one study reported that *AOX1d* expression increased in *AOX1a* knock out plants, but these plants were unable to tolerate antimycin A treatment (an inhibitor of Complex III in the cytochrome *c* pathway) (Alexandre & Lehninger 1984; Kühn et al. 2015; Strodtkötter et al. 2009).

As well as AOX, the alternative pathway of respiration includes type II NAD(P)H dehydrogenases (NDs). NDs are localised to the inner mitochondrial membrane (IMM) either at the inner (matrix-facing) side (NDA and NDC) or the outer (intermembrane space facing) side (NDB) (Millar et al. 2011). The study by Sweetman et al. (2019) showed that in Arabidopsis, AtNDB2 is the main external NADH dehydrogenase which is co-expressed with AtAOX1a and can help to handle stress caused by photoinhibition (Sweetman et al. 2019).

1.5. Gamma aminobutyric acid (GABA)

Under both abiotic and biotic stress, GABA increases in plants and is thought to play a role in stress tolerance. GABA was discovered in 1949 in potato tubers (Steward 1949) before it was found in extracts of animal brain in 1950s (Awapara et al. 1950). Research progressed rapidly as GABA was observed to be a neurotransmitter in the central nervous system of mammals (Elliott & Jasper 1959). GABA is a non-protein amino acid consisting of four carbons and contributes to different physio-biochemical functions to regulate plant growth and stress tolerance including signalling, regulating redox status, maintaining cytosolic pH, osmotic pressure, C and N metabolism, and C-N fluxes (Carroll et al. 1994; Shelp et al. 1999). GABA is a significant stress responsive metabolite because of its accumulation in response to different stimuli (Renault et al. 2010). Several studies showed that GABA levels rapidly increase in response to abiotic stresses such as waterlogging, heat shock, salt

stress, mechanical stimulation, low temperature, and plant hormones (Sharma & Zheng 2019; Shelp et al. 1999). Under stress certain enzymes of the tricarboxylic acid cycle (TCA) are inhibited and to maintain energy production GABA shunt is activated in the cytosol (Bouché et al. 2003; Shelp et al. 2012). The GABA pathway of plants is composed of three enzymatic reactions that bypass two steps of TCA cycle; therefore, it is known as the GABA shunt. The enzymes involved are 2-oxoglutarate dehydrogenase and glutamate decarboxylase (GAD), which synthesise glutamate and GABA respectively, and GABA transaminase (GABA-T), which converts GABA to succinate semialdehyde for reintroduction to the TCA cycle. Activation of the GABA shunt leads to increased GABA production, which provides anaplerotic succinate to maintain TCA cycle activity in the mitochondria (Bouché et al. 2003; Shelp et al. 2012). GABA has a signalling role and modulates nitrate uptake (Beuve et al. 2004), regulates *14-3-3* genes (Lancien & Roberts 2006) and growth and the guidance of pollen tube in plants (Palanivelu et al. 2003).

A proper, functioning GABA shunt is necessary to limit the increase in ROS production because this process supplies NADH and/or succinate to the electron transport chain in mitochondria when there is high level of ROS and respiration is impaired (Lee et al. 2021). The mitochondrial electron transport chain (mETC) and the TCA cycle are provided with substrates obtained by GABA catabolism to supply energy and carbon skeletons during salinity stress. Under stress the activity of the GABA biosynthetic enzyme, GAD, increases due to changes in calcium concentrations leading to an increase in GABA synthesis. Lack of GAD has been shown to cause necrosis and accumulation of ROS in Arabidopsis (Bouché et al. 2004).

In addition, oversensitivity to salinity can be seen in an *A. thaliana* mutant lacking GABA-T (*pop2-1)*, which showed severe phenotypes when exposed to 150 mm NaCl in agar plates (Renault et al. 2010). Accumulation of GABA occurs under salinity stress in various plants such as alfalfa, Arabidopsis, barley, tobacco, and soybean (Renault et al. 2010; Widodo et al. 2009; Zhang et al. 2011).

Leaves of soybean (*Glycine max*) accumulate GABA 25-fold when subjected to salinity and cold stress (Ramputh & Bown 1996). *Arabidopsis thaliana* mutant plants that produce lower levels of GABA were more vulnerable to drought stress due to defective in stomatal closure and this defect could be overcome by enhancing the internal level of GABA using reversed phenotype of the gad1/2 mutant by functional complementation (Mekonnen et al. 2016). The study by Xu et al. (2021) showed that stomal opening and loss of water via transpiration were reduced by the production of GABA in guard cells in *Arabidopsis thaliana* and this helps plants to tolerate drought stress (Xu et al. 2021). Overall, studies shows that GABA metabolism is crucial in the mitigation of abiotic stressors, including salinity (Jacoby et al. 2011).

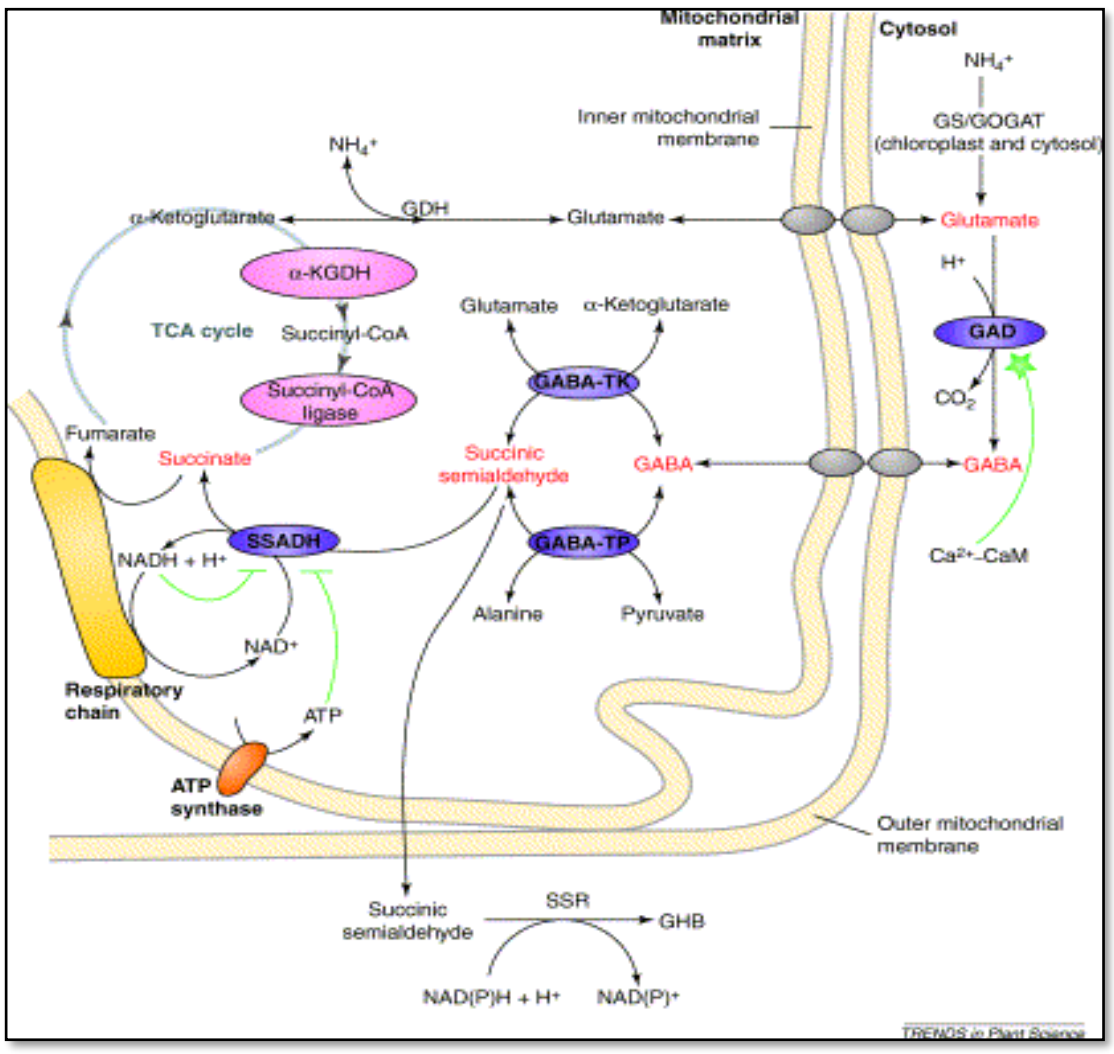

Figure 1.3: Regulation of GABA shunt metabolic pathway in plants (Adapted from the permission of Trends in Plant Science, Bouché & Fromm 2004).

1.6. Exogenous γ-Aminobutyric Acid (GABA) application on plants under salinity.

Several studies have shown that exogenous GABA application is useful to alleviate abiotic stresses. When GABA was exogenously applied to two varieties of maize seedlings subjected to waterlogging stress (Salah et al. 2019; Sheteiwy et al. 2019), it enhanced seedling growth, and resulted in downregulation of ROS producing enzymes, activation of antioxidant defence systems, and improvement of photosynthetic traits and chloroplast ultrastructure (Salah et al. 2019; Sheteiwy et al. 2019). In a study carried out in sunflower plants, 2 mg/L of GABA was exogenously applied for 3 weeks at intervals of 5 days 35 days after planting (Abdel et al. 2020). According to this study, GABA application under drought stress enhanced the expression of aquaporin (AQP) genes and increased the resistance to drought and heat stress due to increased levels of antioxidant enzymes, chlorophyll content and total soluble sugars. Several studies have shown that exogenous application of GABA to *Oryza sativa* seedlings, *Piper nigrum* and creeping bent grass under heat and drought stress enhanced their performance (Li et al. 2016; Nayyar et al. 2014; Vijayakumari & Puthur 2016). Studies related to exogenous application of GABA under salinity stress are shown in Table 1.1

Table1.1: Effects of exogenous application of GABA under salinity stress

Plant	Salt stress	GABA Conc	Effect on plants under salinity stress with exogenous application of GABA	Reference
white clover	100 mM NaCl	(0.25, 0.5, and 1 μM)	50% percent of white clover seeds would not germinate if treated with salinity stress however soaking in low concentrations of GABA reduced the effect of salt stress on germination.	(Cheng et al. 2018)

Caragana intermedia	300 mM NaCl	(1 – 20 mM)	Reduced the production of H_2O_2 and oxidative damage by the regulation of expression of genes responsible to produce H2O2 and genes encoding peroxidases in Caragana intermedia roots (endogenous GABA levels also increased).	(Shi et al. 2010)
Tomato (Solanum lycopersicum L.)	175 mM NaCl	5 mM	Tomato seedlings were positively affected by exogenous GABA to resist salinity stress due to the reduction of Na+ flux to the leaves through roots, enhancing the content of amino acids and increasing antioxidant metabolism. Endogenous GABA was enhanced under salinity stress and exogenous GABA application.	(Wu, et al. 2020)
Maize	150 mM NaCl (moderate stress) and 300 mM (severe salt stress)	0.5 mM	Endogenous GABA content increased. Exogenous GABA reduced damages to membrane and loss of water also reduced. Activity of antioxidant enzymes increased.	(Wang et al. 2017)

1.7. Potential interplay between γ-Aminobutyric Acid (GABA) metabolism and other stress responses.

Plants counter environmental stresses by altering their metabolism, hormones (e.g., abscisic acid, ethylene) and expression of genes (Fernando & Schroeder 2016; Gamalero & Glick 2012; Kinnersley & Turano 2000; Sah et al. 2016; Sharma et al. 2019). To sustain energy production under stress, the GABA shunt is activated in the cytosol, to maintain TCA cycle activity in the mitochondria (Bouché et al. 2003; Shelp et al. 2012). Interestingly, stresses (Al, salinity, cold, drought) that trigger increases in GABA concentrations also result in the activation of the alternative oxidase (AOX) pathway in the mitochondria (Erdal et al. 2015; Kumari et al. 2008; Nunes et al. 2014; Saha et al. 2016; Smith et al. 2009b, 2009a; Wang et al. 2011; Wanniarachchi et al. 2018). AOX activity can alter the levels of other potential signalling molecules such as superoxide, nitric oxide and important redox couples, and thus influence signalling pathways that regulate gene expression, prevent excessive ROS production and maintain electron flow to oxygen (Erdal et al. 2015; Kumari et al. 2008; Nunes et al. 2014; Saha et al. 2016; Smith et al. 2009b; Vanlerberghe 2013; Wang et al. 2011; Wanniarachchi et al. 2018). However, we do not understand if there is an interplay between increased GABA levels and activation of AOX pathways in ameliorating salt stress. If there is cross talk between GABA and AOX, this would have implications for understanding and promoting salinity tolerance in plants.

1.8. Hypothesis

There is an interaction between GABA and AOX pathways under salinity stress. AOX overexpression helps the plant to tolerate salinity stress and knockout results in plants that are more sensitive to salinity stress, while exogenous GABA application further helps the AOX overexpression plants to alleviate salt stress.

1.9. Aims

1.To investigate the interaction between GABA and AOX pathway under salinity stress. This aim will be addressed by exploring the effects of AOX overexpression on endogenous GABA concentrations and expression of GABA-related genes.

2.To investigate the effect of exogenous GABA application in mitigating salt stress in *Arabidopsis thaliana* plants that are either overexpressing, or lacking, AOX1a. This aim will be addressed by exploring the effects of exogenous GABA application on shoot and root growth, stomatal conductance and ion accumulation in the presence and absence of salinity stress.

1.10. Significance of the project for biotechnology

This project will enhance the fundamental knowledge of potential interactions between GABA and AOX under salinity stress which will enable us to engineer crops with improved stress tolerance and performance in saline soils.

CHAPTER 2

MATERIALS AND METHODS

2.MATERIALS AND METHODS

2.1. MATERIALS

All the reagents were obtained from Sigma-Aldrich and autoclaved milliQ water was used unless mentioned specifically (Refer to Appendix 5.1 for reagents, software and equipment used).

2.2. METHODS

2.2.1. Transcriptomic data analysis

Genevestigator (https://genevestigator.com/gv/) (Hruz et al. 2008) a transcriptomics database was used to investigate the expression of genes of interest that changed under environmental stresses such as salinity in *Arabidopsis thaliana.* The genes used were Glutamate dehydrogenase 1 (GAD1), glutamate dehydrogenase 2 (GAD2), glutamate dehydrogenase ¾ (GAD3/4), glutamate dehydrogenase 5 (GAD5), succinic semialdehyde dehydrogenase (SSADH) and gamma aminobutyrate transaminase (GABA-T) are related to GABA shunt and Alternative Oxidase 1a (AOX1a), Alternative Oxidase 1b (AOX1b), Alternative Oxidase 2 (AOX2), Alternative Oxidase 1d (AOX1d) and external alternative NAD(P)H-ubiquinone oxidoreductase B2 (Ndb2) are related to alternative oxidase pathway. Some genes were not represented in the data, which is mostly from microarray experiments and likely there were no appropriate probes for these genes on the microarray chips. The Log 2 ratio of the available gene transcripts for Treatment/Control, were entered in an excel file (Microsoft Excel 2010) relevant to each salt study and data with statistical significance ($P<0.05$) is denoted by asterisks (*). This is an adjusted p value generated by Genevestigator. A heatmap was prepared by representing larger up-regulation and larger down-regulation by darker reds and darker greens, respectively. These salt studies were conducted in other laboratories with *A. thaliana* wildtype (WT). Salt treatments ranged from 100-250 mM NaCl, from 0.5 hours to 6 days, and either leaf or root tissues were used for the transcriptomic analyses. Refer to Appendix table 5.15 for further experimental details.

2.2.2. Salinity treatments using an agar plate growth system.

2.2.2.1. Seed sterilization.

Seeds of *A. thaliana* Col0 wildtype (WT), an AOX1a overexpression line (XX1) developed in a previous study (Umbach et al. 2005) and an AOX1a knockout line (KO; SALK_084897) developed as part of the SALK Institute Genomic Analysis Laboratory (Alonso et al. 2003) and sourced from the Arabidopsis Biological Resource Center), were used for the plate assays. Seeds were sterilized in 1.5 ml Eppendorf tubes using a mixture of 2% sodium hypochlorite (active ingredient) and 0.01% (v/v) tween-20 for 10 mins, with shaking. The solution was removed by pipetting and the seeds were washed a minimum five times using sterile milli Q water. These washing steps were done in a laminar flow cabinet to avoid contamination. Then seeds were placed onto sterilized filter papers, allowed to dry, and stored in Petri dishes covered by aluminium foil at 4 ^{0}C in the dark until further use.

2.2.2.2. Plate assays for root measurements.

The media were prepared according to the recipes listed in the Appendix table 5.11, autoclaved and poured into the plates (100 x100x 20 mm sterile square plates, Adelab Scientific) inside the laminar flow cabinet and stored at 4 ^{0}C until further use. Sterilised seeds were germinated on the plates with ½ MS media and sucrose under 10 h light and 14 h dark photoperiod, 55% atmospheric humidity, 22 ^{0}C temperature and irradiance of 150 µmol photons $m^{-2}s^{-1}$ for 4 days and then seedlings were transferred to new plates containing various combinations of salt NaCl, calcium chloride ($CaCl_2$), sucrose and/or GABA as detailed below in Appendix table 5.11. In Experiment 1, the germinated seedlings were transferred to plates containing different concentrations of salt (0, 50, 100 and 150) with and without sucrose (2% w/v) and $CaCl_2$ (0, 0.6. 1.2 and 1.8 mM respectively, to match the increasing concentrations of NaCl) (Appendix table 5.11). In Experiment 2, the seedlings were transferred to plates with or without GABA (10 mM) and NaCl (150 mM) (Appendix table 5.12). Sterilized toothpicks were used for transferring seedlings from germination plates to assay plates. Assay plates were kept vertically in a growth cabinet under 10 h light and 14 h dark photoperiod, 55% atmospheric humidity, 22 ^{0}C temperature and irradiance of 150 µmol photons $m^{-2}s^{-1}$ for 7 days.

Plants were allowed to grow until the roots reached the bottom of the plates and the growth of the roots were marked on days 0, 3, 5 and 7. Root length was measured using the Image J software (Schneider et al. 2012) and refer Appendix figure 5.5 to see root growth of plants grown in plates under different treatments. The relative growth rates were calculated according to the formula listed in Appendix 5.2.1.

2.2.3. Growing Arabidopsis thaliana plants in Hydroponic systems

Seeds of *A. thaliana* Col0 wildtype (WT) and AOX la overexpression line (XX1) lines were used in these experiments. The growth setup was based on a previously published method (Conn et al. 2013). Germination medium (GM) was prepared (Recipe Appendix table 5.1) with 0.7 g agar (final conc. 0.7% w/v). A 1.2–1.8 mm diameter hole was made in the center of a black microcentrifuge tube lid using a hypodermic needle. About 250 microcentrifuge tube lids were prepared and attached top-face-down onto an adhesive tape. Then these microcentrifuge tube lids were filled with approximately 250-300 µl germination medium and allowed to solidify for 15 minutes. Lids were inverted onto floating racks with the agar plug in contact with liquid Germination media (GM) (Appendix table 5.1 for recipe) to create the functional seed plant holder. A maximum of three seeds were placed into the small hole of each lid using a moistened toothpick, ensuring the seeds were in contact with the agar surface to maximise chances of seed germination. Then, the entire rack was covered with plastic clingfilm to maintain humidity. Seeds were stratified in the dark at 4°C for 48 h. Fifteen germination racks were transferred into a growth cabinet under a 10:14 h, light: dark cycle, with 55% atmospheric humidity, at 22°C and an irradiance of 150 µmol photons $m^{-2}s^{-1}$. Plants were then thinned to single plant per lid and the GM solution was incrementally replaced with a standard growth solution called Basal Nutrition Solution (BNS) (Appendix table 5.2 for recipe). On day 1 of the solution change, 1/3 of the GM was replaced with BNS. On day 2, 50% of the existing solution was exchanged with BNS and on day three the entire solution was replaced with BNS. After day 14, the clingfilm was punctured to gradually decrease humidity and then clingfilm was removed completely after day 17.

Approximately 21 days post-germination, each lid containing a single, healthy plant was transferred to a modified 50 mL centrifuge tubes, with the roots passing through a 11 mm diameter hole drilled in its lid to support the lip of the seedling holder. Then this unit was inserted into a tank containing 10 L of growth solution. Eight tanks were set up and aerated using Aquarium air pumps. Plants were allowed to grow for 10 days in the tanks and then salt treatment was started in fresh BNS medium. The salt treatment consisted of 100 mM NaCl and 1.8 mM CaCl2, which was added incrementally over three days. Shoot and root tissues were harvested before the first addition (T0), then on 1 day, 3 days, and 9 days after the final salt addition (i.e., once a final concentration of 100 mM NaCl had been reached). Tissues were frozen in liquid nitrogen and stored at -80 ^{0}C.

2.2.3.1. Isolation of Total RNA using TRIzol

Total RNA was extracted as described in (Chomczynski & Sacchi 1987) and (Shavrukov et al. 2013) with some modifications. TRIzol-like reagent contained: 38% (v/v) phenol; 1 M guanidine thiocyanate; 1 M ammonium thiocyanate; 0.1 M sodium acetate; and 5% (v/v) glycerol.

Approximately 50-100 mg of frozen *A. thaliana* (WT and XX1) root and shoot tissues from the hydroponic system (control (T0), 1day and 9 days after 100 mM NaCl treated plants) were ground and weighed into 1.5mL tubes and 1 mL of TRIzol-like reagent was added into the tube and vortexed. The tubes were centrifuged at maximum speed (16,000 g) for 10 min at 4 °C and the supernatant from each tube was transferred to a new 1.5 mL tube. Then 200 µl of chloroform was added, vortexed vigorously for 10 s, centrifuged again at 16,000 g for 10 mins, and the aqueous phase transferred to new tubes. The chloroform extraction was repeated, and the final aqueous phase was mixed with 500 µl of iso-propanol and left to precipitate overnight at -20 °C. Samples were then centrifuged at 16,000 g for 10 min at 4 °C. Supernatant was discarded by tipping off, leaving gel-like pellets of RNA that were then washed twice with 1 mL of 75% ethanol and centrifuged at 7,500 g for 5 min at 4 °C. After removing the ethanol by pipette, tubes were kept upside down in the laminar flow for drying for 20-30 min. RNA was re-suspended in 25 µl of Rnase-free water by pipette. RNA concentration was

measured, and RNA purity was estimated using 260/230 ratio using NanoDrop 1000 spectrophotometer (refer Appendix 5.3.1.0 section for more details) and the samples were stored at -80 ^{0}C until further use.

2.2.3.2. Agarose gel electrophoresis

RNA quality was checked by running a 1% (w/v) agarose gel (Sambrook 2001) (Appendix figure 5.2 for gel image). Briefly, 1X TAE buffer was used to dissolve agarose in a microwave and GelRed™ (Biotium, U.S.A.) stain was added after cooling. Promega 6X loading dye was used to load 5 µl aliquots of each sample and a 1 kb DNA ladder (Promega) was used as a size marker. The gel was run in PowerPac ^{TM} Basic (Bio-Rad) at 100 V for 50 mins. The gel was visualized using BioRad EZ Imager.

2.2.3.3. Synthesis of cDNA

Complementary DNA (cDNA) was synthesized using 2 µg of RNA. First, a master mix was prepared by mixing 50 µM Oligo d(T)20, 10 mM dNTP and sterile autoclaved water (Appendix table 5.4 for recipe) and then 2 µg of RNA was added separately to each tube. The solution was mixed and heated at 65 ^{0}C for 5 min then transferred to ice for 1 min. Then each sample was treated with 1µl Dnase (RQ1 Rnase-Free Dnase, Promega) and incubated for 15 min at room temperature. An aliquot (1 µl) of each sample was taken at this point to make sure all genomic DNA had been degraded, by running as a negative control in Quantitative Reverse Transcriptase PCR (qRT-PCR) assays. PhotoScript (M0368S, NEB) mixture (7 µl) containing 1x PhotoScriptII Reaction Buffer, 0.1 M DTT, Murine RnaseOut, PhotoScriptII RT (Appendix table 5.5 for quantities) was added to each sample and placed in BioRad MyCylcer^{TM}. The PCR protocol for samples included incubation for 45 min at 42 ^{0}C for Reverse Transcriptase reaction and incubation for 5 min at 80 ^{0}C to inactivate the enzyme. The cDNA samples were diluted 1:20 prior to use.

2.2.3.4. Quantitative Reverse Transcriptase PCR (qRT-PCR)

The gene expression of two reference genes (*Ubiquitin* (*UBQ*) and *Tubulin*), *Alternative oxidase 1a* (*AOX1a*), *Alternative oxidase 1d* (*AOX1d*), *Glutamate decarboxylase 1* (*GAD1*), *Glutamate decarboxylase 2* (*GAD2*) and *external NADH-dehydrogenase 2* (*NDB2*) genes in root and shoot tissues of WT and XX1 under control and treated conditions were detected by qRT-PCR. Forward and reverse primers of above-mentioned genes were designed during previous studies (appendix table 16 for primer sequences used in this study). These primers (except *AOX1a* and *AOX1d*) were designed at splice junctions to prevent amplification of genomic DNA. First, Specific annealing temperatures for *GAD1*, *GAD2* and *Tubulin* were detected using a temperature gradient (Appendix table 5.16). Annealing temperatures of *AOX1a*, *AOX1d* and *NDB2* were obtained from previous studies by other members of the lab. Each reaction included: 5 µl KAPA SYBR (2x); 1 µl forward/reverse primer (1 µM); and 4 µl cDNA and placed in BioRad C1000^{TM} Thermal Cycler. Thermal cycling steps consisted of 3 minutes of 95 ^{0}C, followed by 40 cycles of: 95 ^{0}C for 5 seconds; and 60 ^{0}C (depend on the annealing temperature of the specific primer) for 15 seconds. These steps were followed by a melt curve including 5 second increments of 0.5 ^{0}C from 65 ^{0}C to 95 ^{0}C.

2.2.3.5. Gene expression data Analysis

The Cycle threshold (Ct) values and melt curve data were derived from CFX Manager^{TM} software version 2.0 in BioRad C1000^{TM} Thermal Cycler and analysed after exporting to an excel file. Relative gene expression for all the genes were obtained by $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001). *Ubiquitin* and *Tubulin* housekeeping genes were used to normalize the gene expression levels. The upregulation or downregulation of gene expression was calculated as a fold change relative to the control sample.

2.2.3.6. TBARS assay

A thiobarbituric acid reactive substances (TBARS) assay was used to measure MDA equivalents (Singh et al. 2012). Homogenized tissue (50-100 mg) was weighed using electronic balance (Mettler

Toledo), ROWE Scientific Pty Ltd) in Eppendorf (2 mL) tubes. Then 1 ml of 80% (v/v) ethanol was added to the each sample and vortexed. Samples were centrifuged at 13,000 rpm (Eppendorf centrifuge 5425) for 15 minutes. The supernatant was divided into aliquots (2 x 300 µL) in new centrifuge tubes (a & b). Each sample (a) was treated with 300 µL 20% (w/v) TCA containing 0.5% (w/v) thiobarbituric acid (TBA), 0.01% butylated hydroxytoluene (BHT) and each sample (b) was treated with 300 µL 20% (w/v) TCA containing 0.01% butylated hydroxytoluene (BHT) but no thiobarbituric acid (TBA). Samples were heated to 96 °C for 30 minutes after piercing the lids with a syringe to prevent pressure build-up. Samples were cooled on ice and then centrifuged at 9,500g for 10 minutes to remove any precipitate in the sample that could interfere with the readings. Supernatants (100 µL) were loaded into a 96 well plate in duplicate. The change in absorbance was measured by a spectrophotometric plate reader (FLUOstar Omega) using the MARS Data Analysis Software at 532 nm (TBA-MDA complex); 600 nm (nonspecific turbidity); and 440 nm (interfering soluble sugars) (Hodges et al. 1999). The modified equation proposed by Du and Bramlage (Du & Bramlage 1992) was used to calculate MDA equivalents: MDA equivalents = ((A532-A600) – (A440-A600) x (0.0571)) / 157 000) x 10^6.

2.2.3.7. Gabase enzyme assay

Frozen tissues (From Section 2.2.3) were ground to a fine powder in liquid nitrogen and 100 mg tissue was weighed into microfuge tubes. The exact weight of each sample was recorded. Methanol (400 ul) was added to each tube and incubated at 25 degrees for 10 min. The tubes were placed in a vacuum centrifuge (Savant SC100 Speed Vac Concentrator with Savant RH40-12 Rotor) to dry for approximately 3 h. The pellets were mixed with 1 ml of 70 mM lanthanum chloride and placed on a platform shaker for 15 min. Tubes were centrifuged at 12,000 rpm (Eppendorf centrifuge 5425) for 5 min. Supernatant (800 µL) was transferred to fresh microfuge tubes with 160 µl of 1N KOH and then placed on a platform shaker for 5 min. Tubes were centrifuged at 12,000 rpm for 5 min and supernatants were frozen at -20 degrees in fresh tubes, until further use.

The Gabase enzyme assay was carried out in two steps. In step 1, Potassium pyrophosphate (75 mM), 2 β-Mercaptoethanol (3.3 mM), alpha ketoglutarate (5 mM) and NADP (1.25 mM) (recipe in table 5.6) were added into 96 well plate (COSTAR) and 45.2 µl of the samples were added to each well. Readings were taken at 340 nm in the plate reader (FLUOstar Omega) for 3 cycles. Then enzyme was added and read for 35 cycles to ensure the reaction reached completion. Change in absorbance was used in calculations.

GABA dilutions (0, 5 µM, 10 µM, 20 µM, 50 µM, 100 µM, 200 µM) were prepared from an initial 10 mM stock solution (Appendix table 5.13 for calculations for preparation of dilutions). These dilutions were added instead of samples to form the standard curve according to the same protocol described above for the samples. Data were obtained using MARS Data Analysis software program (version 3.20 R2) and the standard curve used to calculate concentrations of GABA in unknown samples using GraphPad Prism 9.2.0.

2.2.4. Growing Arabidopsis thaliana plants in soil.

Small pots were filled with approximately the same amount of soil and watered until the weight was 260 g. Then 3-4 seeds were added to each pot and covered with cling film to enhance humidity. The pots were kept in a glass house with growth conditions maintained as close as possible to 10:14 h, light: dark cycle, with 55% atmospheric humidity, at 22 °C and an irradiance of 150 µmol photons $m^{-2}s^{-1}$. The plants were watered every three days and at 4-5 weeks old, were separated into four groups to start treatment. The treatments included Control (C: treated with water), Control with 10 mM GABA (G+G), 150 mM NaCl (S), and 150 mM NaCl with 10 mM GABA (S+G). The salt concentration was increased gradually to avoid shock to the plants, with one addition of 50 mM followed by another 100 mM the following day. Pots were also topped up with water to the same approximate volume every few days, then harvested for fresh and dry weight measurements of shoot tissues 10 days after the treatment. Fresh weight was measured immediately after harvest, then the

shoots were stored in paper bags at 37°C for two days before measuring dry weight, as modified from (Sakuraba et al. 2017).

2.2.4.1. Determination of Na^+ and K^+ concentration

Na^+ and K^+ concentrations were determined in dried tissues of WT, XX1 and KO plants treated with GABA (10 mM), NaCl (150 mM) and combination of GABA (10 mM) and NaCl (150 mM) in soil system. Dried shoot samples (rosette) were put into yellow capped tubes and the dry weights were measured and recorded. The shoot samples were fully emerged in 10 mL of 1% v/v Nitric acid (HNO_3 in milli Q water Appendix 5.2.4.0**)** and shaken well. Then samples were incubated at 80 ^{0}C in an oven (SCIENTRONIC HB 900) for 4 hours, with mixing every 30 minutes. Then after 4 hours, the samples were allowed to cool to room temperature overnight.

Measurements were made using a flame photometer (Model 420, Sherwood). In brief, 2 µl of sample solution was dissolved in 1998 µl of milli Q water prior to analysis. Two technical replicates were done for each sample and Na^+ and K^+ concentration was measured based on the mean of these two technical replicates. Diluted samples and Na^+ and K^+ standards with different concentrations were used to maintain the readings within the flame photometer limits as plants were subjected to different treatments. That is, for salt-treated samples, a higher concentration standard for Na^+ was used to calibrate the machine. The concentration of Na^+ or K^+ was measured per gram of dry tissue weight (µmoles. G^{-1} DW) using the equation listed below.

$$[\mathrm{Na+}] \text{ or } [\mathrm{K+}] \text{ mol. g} - 1 \text{ Dry Weight } = \frac{\text{Flame photometer reading x coefficient}}{\text{Sample Dry weight}}$$

Where:

$$\text{Dilution Factor} = \frac{\text{Total dilution volume (µL)}}{\text{Total sample volume (µL)}}$$

$$\text{Standard coefficient} = \frac{\text{Standard (µM)}}{\text{Maximum units}}\ x \text{ Dilution factor x Volume of digestion (L)}$$

CHAPTER 3

RESULTS

3. RESULTS

3.1. Transcriptomic data analysis

Publicly available transcriptomic data was investigated using Genevestigator (Hruz et al. 2008). *A. thaliana* Affymetrix-based experiments under salinity stress were used to investigate changes in transcript levels of GABA shunt and alternative respiration pathway genes. *Glutamate dehydrogenase 1* (*GAD1*), *glutamate dehydrogenase 2* (*GAD2*), *glutamate dehydrogenase ¾* (*GAD3/4*), *glutamate dehydrogenase 5* (*GAD5*), *succinic semialdehyde dehydrogenase* (*SSADH*) and *gamma aminobutyrate transaminase* (*GABA-T*) are related to GABA shunt and *alternative oxidase 1a* (*AOX1a*), *alternative oxidase 1b* (*AOX1b*), *alternative oxidase 2* (*AOX2*), *alternative oxidase 1d* (*AOX1d*) and external type II NADH dehydrogenase B2 (*NDB2*) are related to the alternative pathway of respiration. GABA shunt genes *GAD3/4*, *SSADH* and *GABA-T* and all alternative pathway genes were upregulated in at least one salinity stress treatment (Table 3.1).

Based on the data, *AOX1a*, *AOX1d* and *NDB2* genes were selected for analysis in subsequent experiments. *GAD1* and *GAD2* genes were selected for quantitative real-time RT-PCR instead of *GAD3/4* and *GAD5* because GAD1 and GAD2 are the most abundant GADs in *A. thaliana*. Also, previous studies indicated transcriptional up-regulation of *GAD1* and *GAD2* under salinity stress (Akçay et al. 2012; Su et al. 2019; Wu et al. 2020).

Table 3.1: Transcript levels of GABA shunt and alternative oxidase pathway genes respond to salinity stress. Numbers in the table show log 2-fold change in expression. Statistical significance (P<0.05) is denoted by asterisks (*). Highly up-regulated or down-regulated genes are represented by dark red and dark green respectively in the heatmap. All studies were carried out using *Arabidopsis thaliana* wildtype Col-0. Refer Appendix table 5.15 for further experimental details.

Experiment	NaCl stress	Tissue	Treatment period	GAD1	GAD2	GAD 3/4	GAD 5	SSADH	GABA-T	AOX1a	AOX1b	AOX2	AOX1d	Ndb2
Salt study 2	150 mM	root	0.5, 1 and 3 h	-0.1	-0.3	1.8	0.1	0.0	0.0	1.4	0.1	*0.6	2.0	*1.3
Salt study 2	150 mM	root	6, 12 and 24 h	*-0.2	*-0.9	*4.6	*0.3	*0.5	*0.3	*3.6	*1.8	*1.5	*7.4	*2.4
Salt study 3	250 mM	rosette	24 h	0.2	0.1	1.1	0.0	0.9	*1.4	2.3	0.6	0.3	1.2	1.8
Salt study 4	100 mM	leaf	6 days	-0.2	-0.4	*0.7	-0.1	*2.0	0.6	-0.3	-0.1	-0.3	0.4	0.4
Salt study 5	140mM	root tip	1 h	*-0.1	-0.1	0.2	0.1	0.0	0.3	*1.1	0.0	-0.2	-0.2	*0.7

<-2.5	<-2	<-1.5	<-1	<-0.5	0	>0.5	>1	>1.5	>2	>2.5
Down regulated									Up regulated	

3.2. Obstacles faced during sample collection

Whole batches of plants had to be discarded three times due to several reasons, as summarised below. Only subsequent batches of plants that were healthy, were used for experiments.

1. Flowering

An initial attempt at growing plants in the hydroponic system led to plants that flowered in the age of 4 weeks, i.e., before starting the salinity treatment. The reason for early flowering was due to a problem in lighting (day length was too long). The photoperiod was fixed to 10 h light and 14 h dark. Plants that had flowered were discarded because gene expression changes dramatically during flowering and it can confound results.

2. Lock down due to Covid-19

The first attempt at growing plants in soil was impacted because plants were ready for treatment in the lead up to a 7-day state lock-down, and upon return to the campus, these plants were too old for the experiment and had to be discarded.

3. Plants were sick

In a second attempt with growing plants in soil, all the plants on one bench became sick after the first day of the treatment: even the control plants. Unfortunately, we couldn't determine the reason for this, although the chlorotic lesions on the leaves (Figure 3.1) seemed to indicate a harmful spray or an infection. All these plants were discarded.

Figure 3.1: Discarded sick plants grown in the soil system.

3.3. Effects of salinity on *Arabidopsis thaliana* plants grown in a hydroponic system.

3.3.1. Changes in gene expression of GABA shunt and alternative oxidation pathway under salinity stress.

Relative gene expression of *AOX1a* in the shoots of both WT and XX1 increased 1 day after treatment but returned to control levels after 9 days (Figure 3.2 A and B), however none of these changes were significant ($p < 0.05$). In the root tissues, relative gene expression of *AOX1a* increased in both WT and XX1 at 9 days after the salt treatment, however none of these changes were significant (Figure 3.2 C and D).

Relative gene expression of *GAD2* (Figure 3.3 A) and *AOX1d* (Figure 3.4 A) in WT shoots only showed a significant increase 1 day after treatment and expression returned to control levels 9 days after the treatment. All the other changes in WT roots (Figure 3.3 C and Figure 3.4 C) and XX1 both shoots (Figure 3.3 B and 3.4 B) and roots (Figure 3.3 D and 3.4 D) were not significant. Relative gene

expression of *NDB2* (Figure 3.5) in WT shoots showed a significant increase 1 day after treatment and that expression returned to control levels 9 days after the treatment (Figure 3.5 A). In XX1 shoots, relative gene expression of *NDB2* increased significantly at 1 day (S1) and remained high at S9 (Figure 3.5 B). There was no significant difference in the gene expression of *NDB2* in both WT and XX1 root tissues under salinity stress (Figure 3.5 C and D).

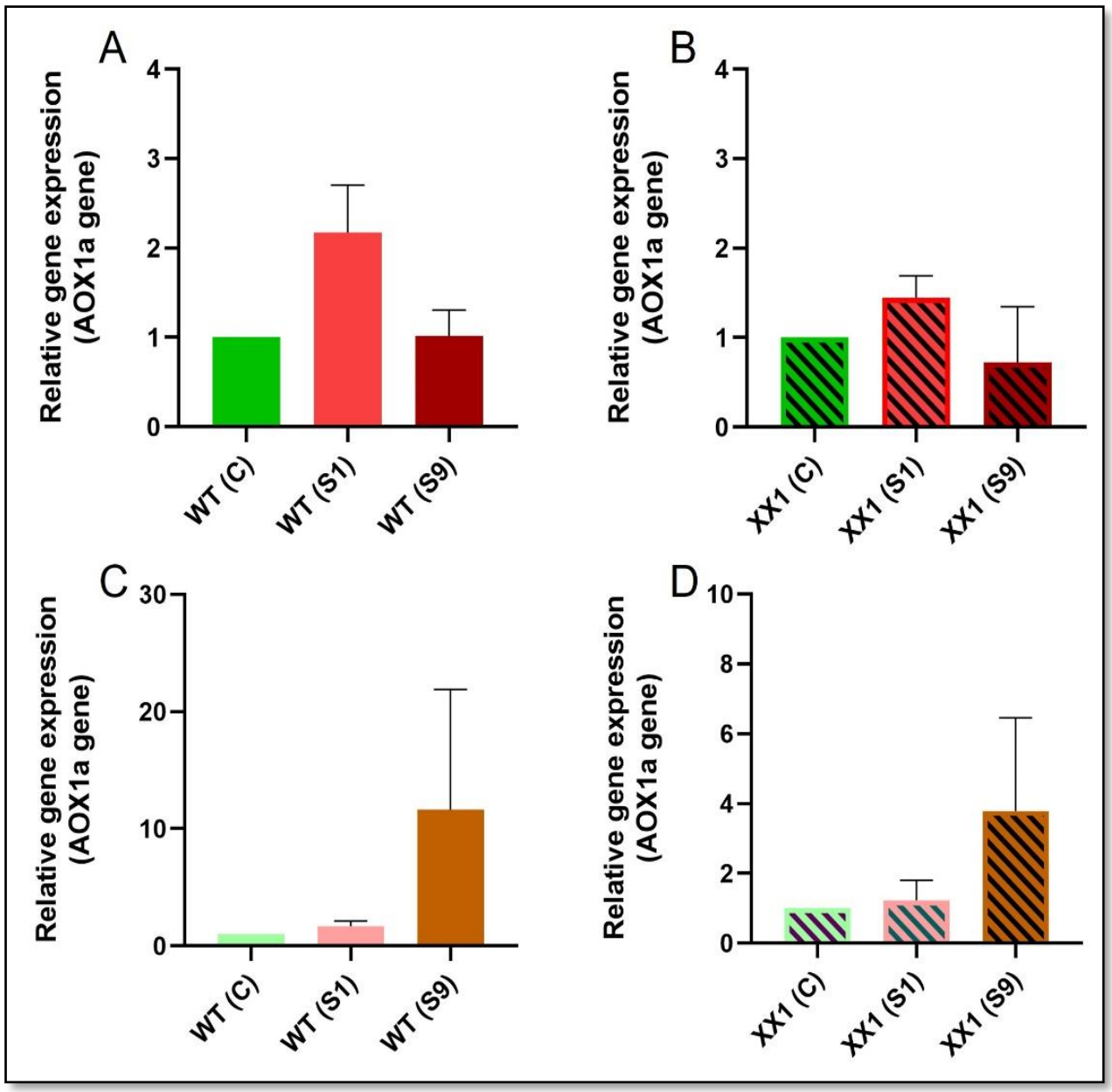

Figure 3.2: Relative gene expression of *AOX1a* in wild type (WT) (A) shoots, (C) roots and AOX1a over expressor (XX1) (B) shoots and (D) roots under control and 100 mM salt treatment. Control (C),

1 day after salt treatment (S1), 9 days after salt treatment (S9). N=4; data analysed using 2-way ANOVA with Tukey post hoc testing. Error bars represent standard error of mean (SEM).

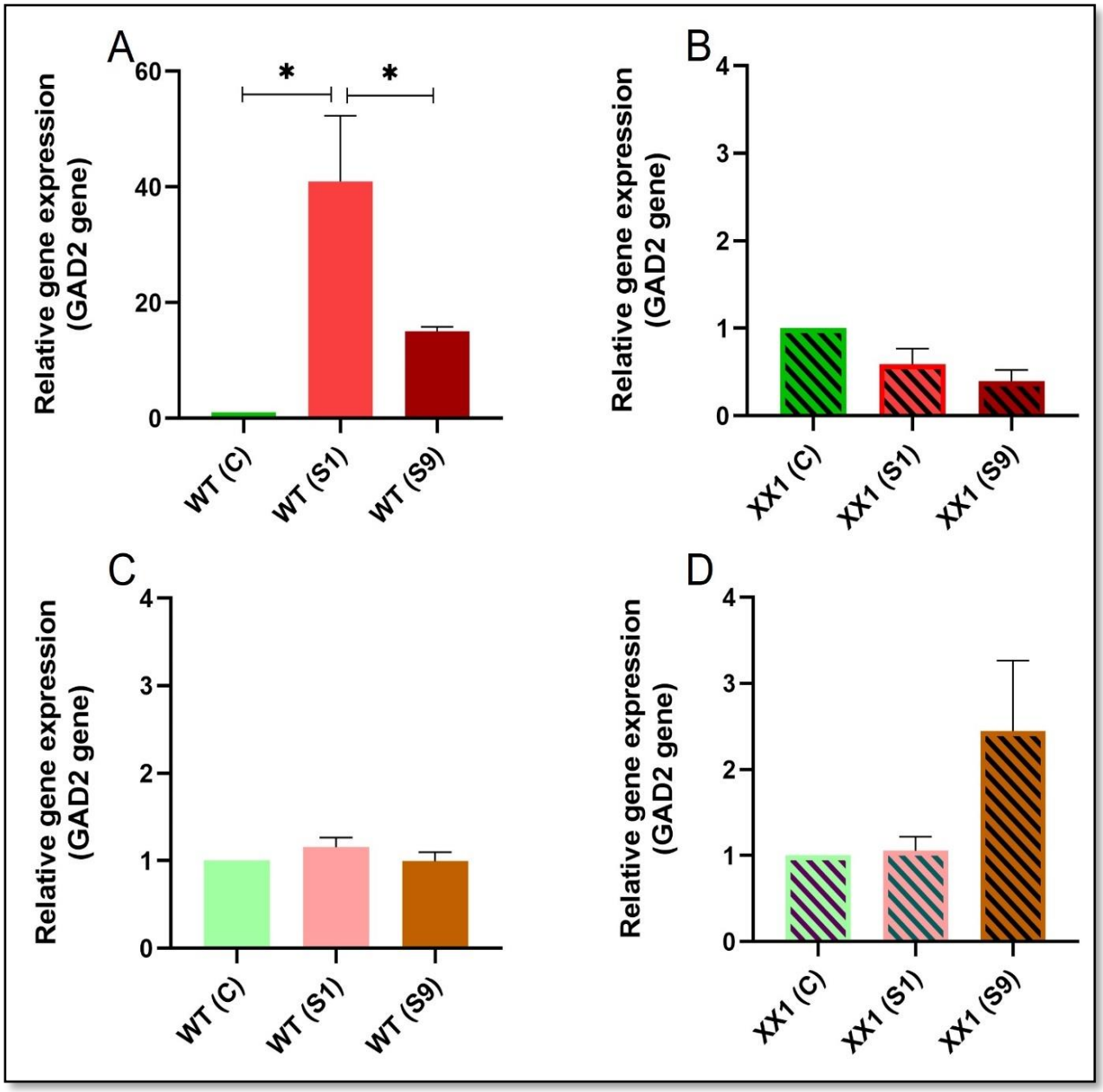

Figure 3.3: Relative gene expression of *GAD2* in wild type (WT) (A) shoots, (C) roots and AOX1a over expressor (XX1) (B) shoots and (D) roots under control and 100 mM salt treatment. Control (C), 1 day after salt treatment (S1), 9 days after salt treatment (S9). N=4; data analysed using 2-way ANOVA with Tukey post hoc testing. Statistical significance ($P<0.05$) is denoted by asterisks (*). Error bars represent SEM.

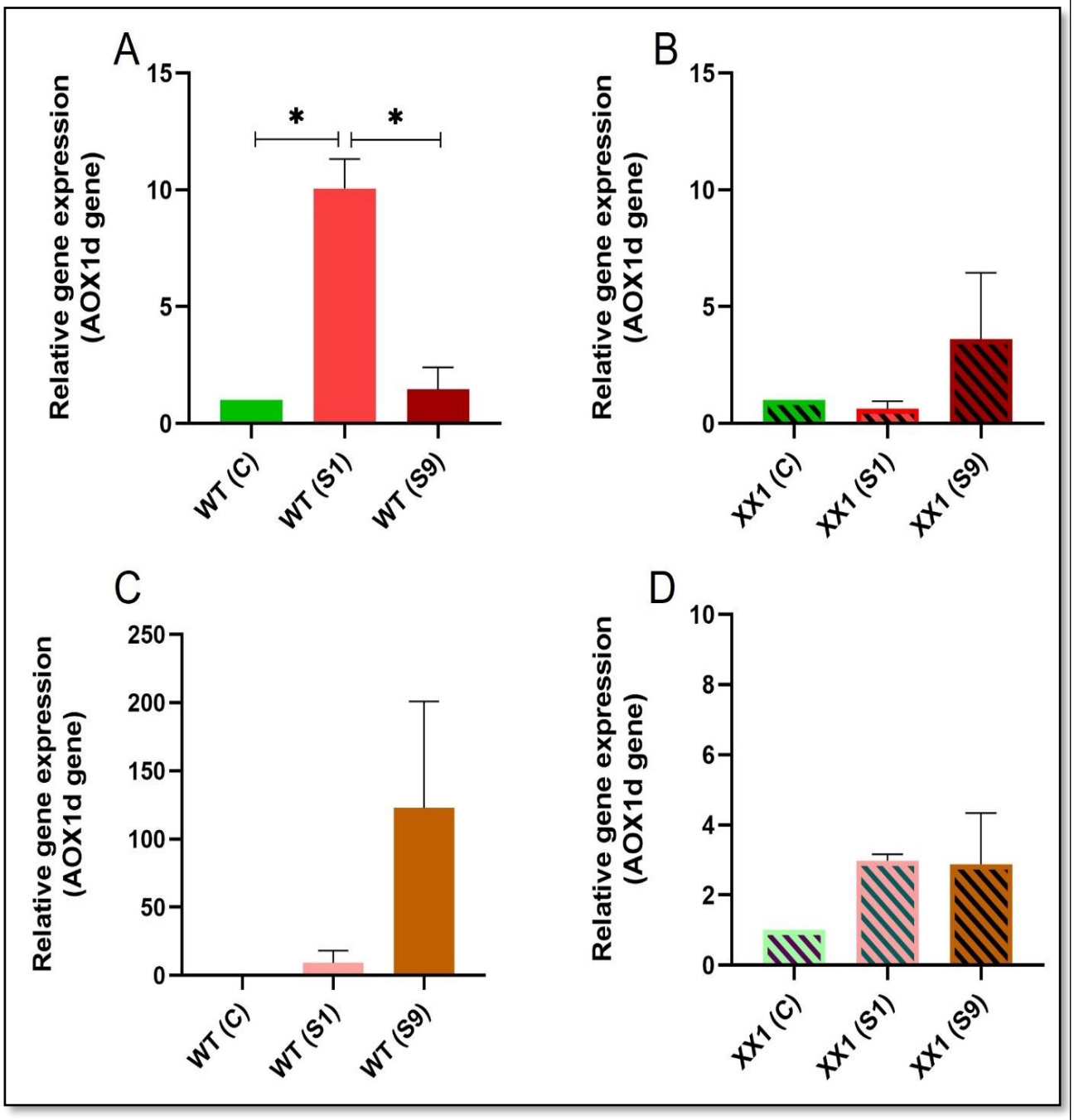

Figure 3.4: Relative gene expression of *AOX1d* in wild type (WT) (A) shoots, (C) roots and AOX1a over expressor (XX1) (B) shoots and (D) roots under control and 100 mM salt treatment. Control (C), 1 day after salt treatment (S1), 9 days after salt treatment (S9). N=4; data analysed using 2-way ANOVA with Tukey post hoc testing. Statistical significance ($P<0.05$) is denoted by asterisks (*). Error bars represent SEM.

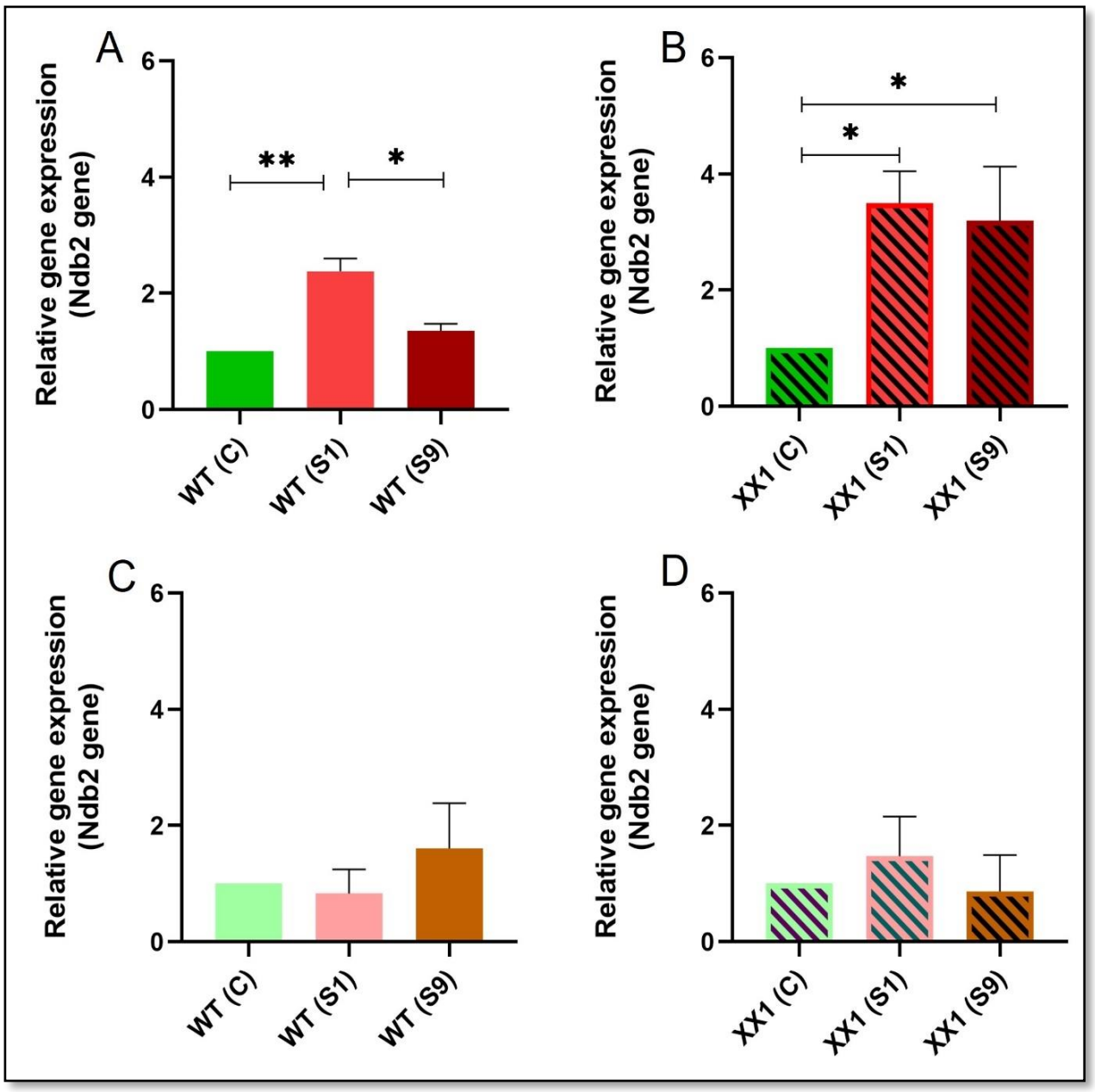

Figure 3.5: Relative gene expression of *NDB2* in wild type (WT) (A) shoots, (C) roots and *Arabidopsis thaliana* AOX1a over expressor (XX1) (B) shoots and (D) roots under control and 100 mM salt treatment. Control (C), 1 day after salt treatment (S1), 9 days after salt treatment (S9). N=4; data analysed using 2-way ANOVA with Tukey post hoc testing. Statistical significance ($P<0.05$) and ($P<0.01$) are denoted by asterisks (*) and (**) respectively. Error bars represent SEM.

3.3.2. Endogenous GABA content increased with salinity stress in wildtype and AOX1a overexpression line.

GABA content of WT plants increased significantly in both shoots and roots after 1 day of salinity stress, and remained high after 9 days, compared to the control (Figure 3.6). The XX1 shoots also showed an increase in GABA content but the increase was significant only after 9 days of salt treatment, when compared to its control (Figure 3.6 A). This could be because GABA levels were already higher in XX1, compared to WT, therefore the salt treatment had less of an effect. GABA content of XX1 roots showed a significant increase 1 day after salt treatment compared to XX1 control, although decreased towards control levels by day 9 (Figure 3.6 B).

3.3.3. Malondialdehyde (MDA) content changes during salinity stress in wildtype and AOX1a overexpression line.

Malondialdehyde (MDA) content is used to detect the membrane lipid peroxidation in plants and MDA is a marker to recognize oxidative lipid injury due to environmental stress (Kong et al. 2016). A significant increase in MDA equivalents could be seen in shoots of WT and XX1 after one day of treatment. Unfortunately, there was not enough root tissue to measure MDA equivalents at day 1, as these roots were small, and the tissue samples were prioritised for gene transcript and GABA measurements. After 9 days of treatment, MDA equivalents returned to control levels in WT but not XX1 (Figure 3.7 A). Lower MDA equivalents were observed in both WT and XX1 root tissues relative to shoot tissues and the changes in MDA equivalents under salinity stress were not significant (Figure 3.7 B).

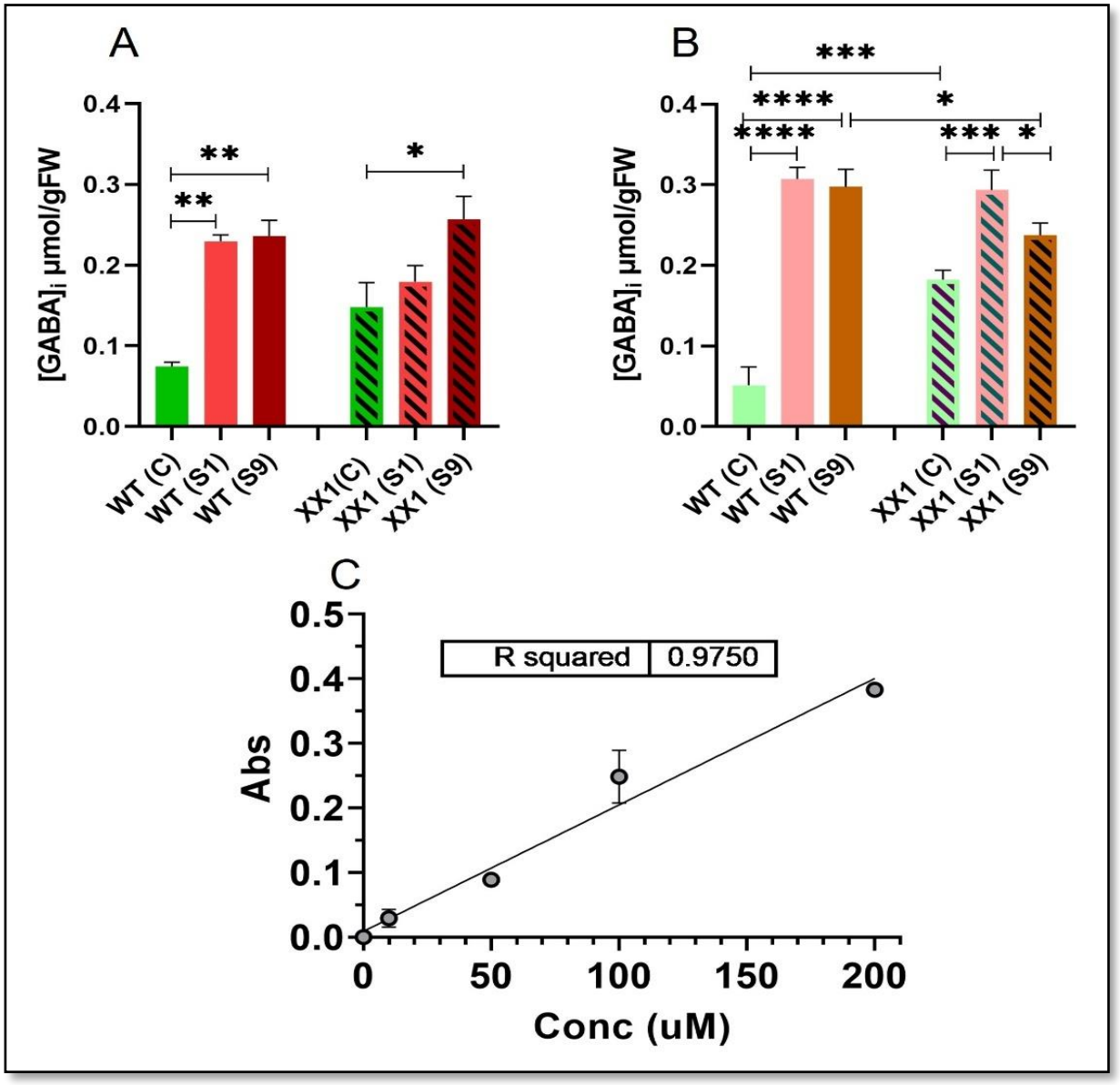

Figure 3.6: Endogenous GABA content of (A) shoots and (B) roots in wild type (WT) and AOX1a over expressor (XX1) plants under control and 100 mM salt treatment (C) Standard curve for calculating GABA content. Control (C), 1 day after salt treatment (S1), 9 days after salt treatment (S9). N=4; data analysed using 2-way ANOVA with Tukey post hoc testing. Statistical significance ($P<0.05$), ($P<0.01$), ($P< 0.001$) and ($P<0.0001$) are denoted by asterisks (*), (**), (***) and (****) respectively. Error bars represent SEM.

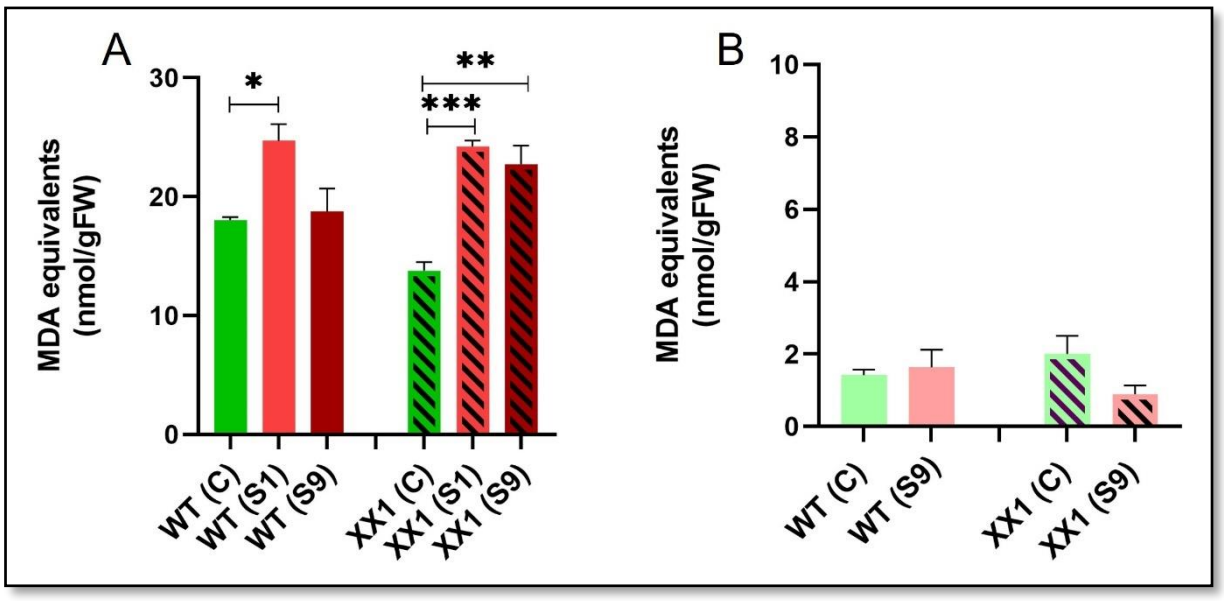

Figure 3.7: Malondialdehyde (MDA) equivalents of (A) shoots and (B) roots in type (WT) and AOX1a over expressor (XX1) plants under control and 100 mM salt treatment. Control (C), 1 day after salt treatment (S1), 9 days after salt treatment (S9). N=4; data analysed using 2-way ANOVA with Tukey post hoc testing. Statistical significance ($P<0.05$), ($P<0.01$) and ($P< 0.001$) are denoted by asterisks (*), (**) and (***) respectively. Error bars represent SEM.

3.4. Effect of exogenous GABA on root growth of wild type, AOX1a overexpression line and AOX1a knockout lines under salinity stress.

For the purposes of analysing root growth phenotypes in response to salinity and GABA application, plants were grown in agar plates. Such plate assays require less space, time, and cost, and it is feasible to observe and measure the growth of roots in response to stress treatments. Root growth shows plasticity and responds to the changes in the environment, enabling the plant to survive.

If 2% sucrose was present in the growth medium, there were no differences in total root length between wild type (WT) and AOX1a over expressor (XX1) plants grown on plates with NaCl (0 mM, 50 mM, 100 mM and 150 mM) (Figure 3.8 A). But in the absence of sucrose, there were significant differences between WT and XX1 plants grown on plates with 150 mM NaCl (Figure 3.8 B).

Relative growth rate of roots reduced significantly in WT and KO plants treated with GABA when compared to the plants in control condition (Figure 3.9 A). In contrast, XX1 showed a small increase, which was not significant. The relative growth rate of roots in the AOX1a over expression line XX1 showed a significant increase compared to WT, whether GABA was present or not (Figure 3.9 A). Meanwhile, KO plants showed a non-significant trend towards decreased relative growth rate compared to WT and a significant decrease compared to XX1 (Figure 3.9 A).

A similar trend was seen for plants grown in salt (Figure 3.9 B). Most notably, under salinity stress the relative growth rate of roots in XX1 treated with GABA (S+G (XX1)) were higher than any other sample, with a significant increase compared to WT and KO with GABA (S+G (WT) and S+G (KO)) (Figure 3.9 B).

All three genotypes showed significant decreases in relative growth rate of roots when exposed to salinity stress, whether GABA was present or not (Figure 3.9 and Table 3.2) .

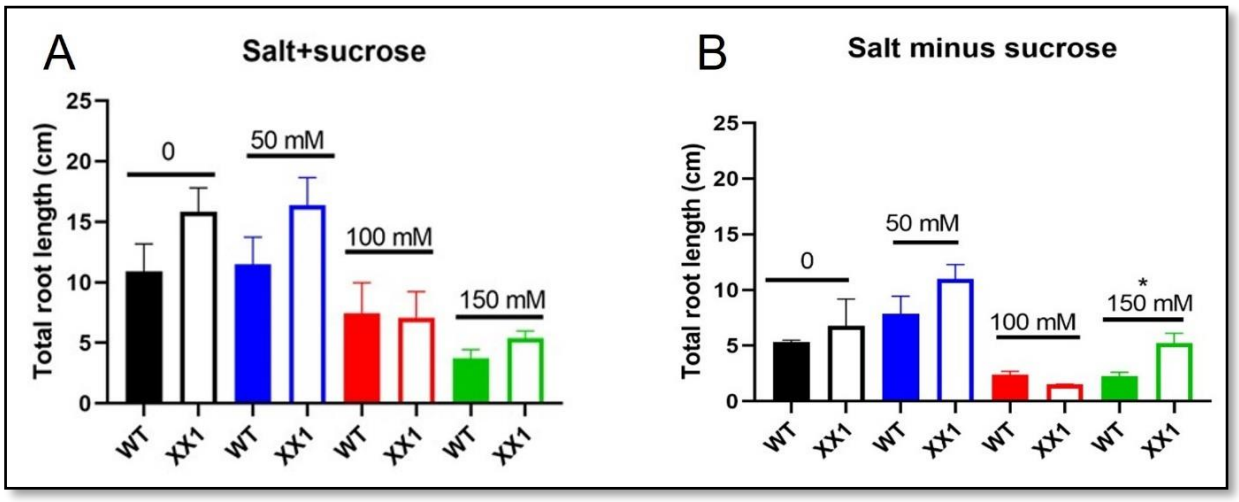

Figure 3.8: Total root length of wild type (WT) and AOX1a over expressor (XX1) exposed to 0 Mm, 50 mM, 100 mM, and 150 mM NaCl (A) with 2 % sucrose and (B) without sucrose. N=4; data analysed using 2-way ANOVA with Tukey post hoc testing. Statistical significance ($P<0.05$) is denoted by asterisks (*). Standard deviation is denoted by error bars.

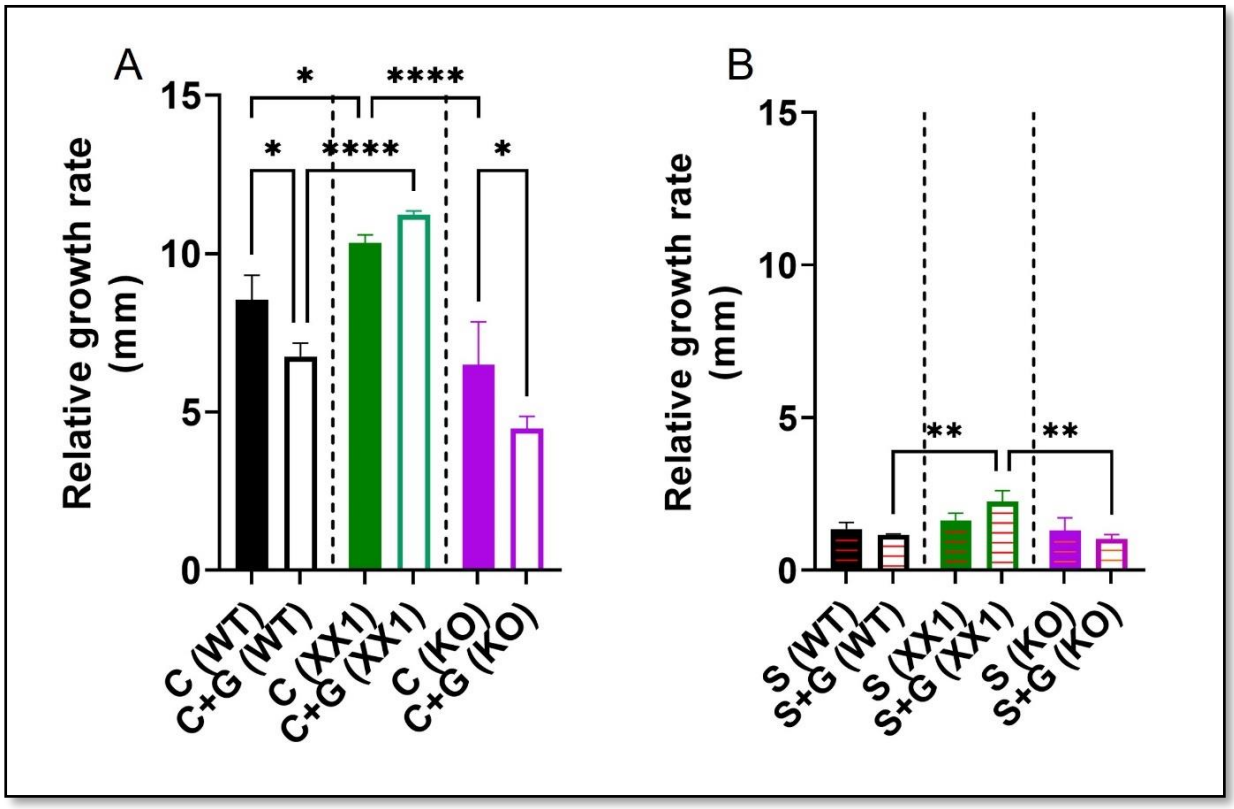

Figure 3.9: Relative growth rate of roots in *Arabidopsis thaliana* wild type (WT), AOX1a over expressor (XX1) and AOX1a knock out (KO) plants (A) exposed to GABA (B) exposed to NaCl and combination of NaCl and GABA. Control (C), Control + GABA (C+G), Salt treated (S), Salt + GABA (S+G). n=4; data analyzed using 2-way ANOVA with Tukey post hoc testing. Statistical significance ($P<0.05$), ($P<0.01$), ($P< 0.001$) and ($P<0.0001$) are denoted by asterisks (*), (**), (***) and (****) respectively.Error bars represent the standard deviation.

Table 3.2: Significant differences between relative growth rate of roots (Figure 3.9 A and B) for wild type (WT), AOX1a over expressor (XX1) and AOX1a knock out (KO) plants exposed to GABA, exposed to NaCl and combination of NaCl and GABA. Control ©, Control + GABA (C+G), Salt treated (S), Salt + GABA (S+G). n=4; data analyzed using 2-way ANOVA with Tukey post hoc testing. ($P<0.0001$) are denoted by asterisks (****).

Tukey's multiple comparisons test	Adjusted P Value	Summary
C (WT) vs. S (WT)	<0.0001	****
C+G (WT) vs. S+G (WT)	<0.0001	****
C+G (XX1) vs. S+G (XX1)	<0.0001	****
C (KO) vs. S (WT)	<0.0001	****
C+G (KO) vs. S+G (KO)	<0.0001	****

3.5. Effect of exogenous GABA on plant phenotype and ion accumulation in wild type, AOX1a overexpression and AOX1a knockout lines under salinity stress.

A soil-based experiment was designed for the measurement of plant growth and ion accumulation in response to salinity in the presence and absence of exogenous GABA in soil, as a more realistic representation of plants grown in the field compared to the plate-based root assays. WT, XX1 and KO plants were grown in soil for 6 weeks, then treated with either GABA, NaCl or a combination of NaCl and GABA for 10 days.

3.5.1. Stomatal conductance decreased significantly due to salinity stress

On day 5 after salt treatment, the stomatal conductance of WT plants was significantly higher than XX1, however by day 10 stomatal conductance in both genotypes were 100 mmol m−2 s−1 (Figure 3.10 A). The effect of salt was a rapid decline in stomatal conductance within three days, in both WT and XX1 plants. This was seen both in the presence and absence of GABA and it remained consistantly low at least until at least day 10 after treatment. There was no difference in stomatal conductance between the two genotypes when treated with salt with or without GABA (Figure 3.10). Interestingly, there was a downward trend in stomatal conductance between day 5 to 10, in all treatments except for GABA-treated XX1 plants, which, at day 10 showed significantly higher stomatal conductance compared to WT plants with GABA (Figure 3.10 B).

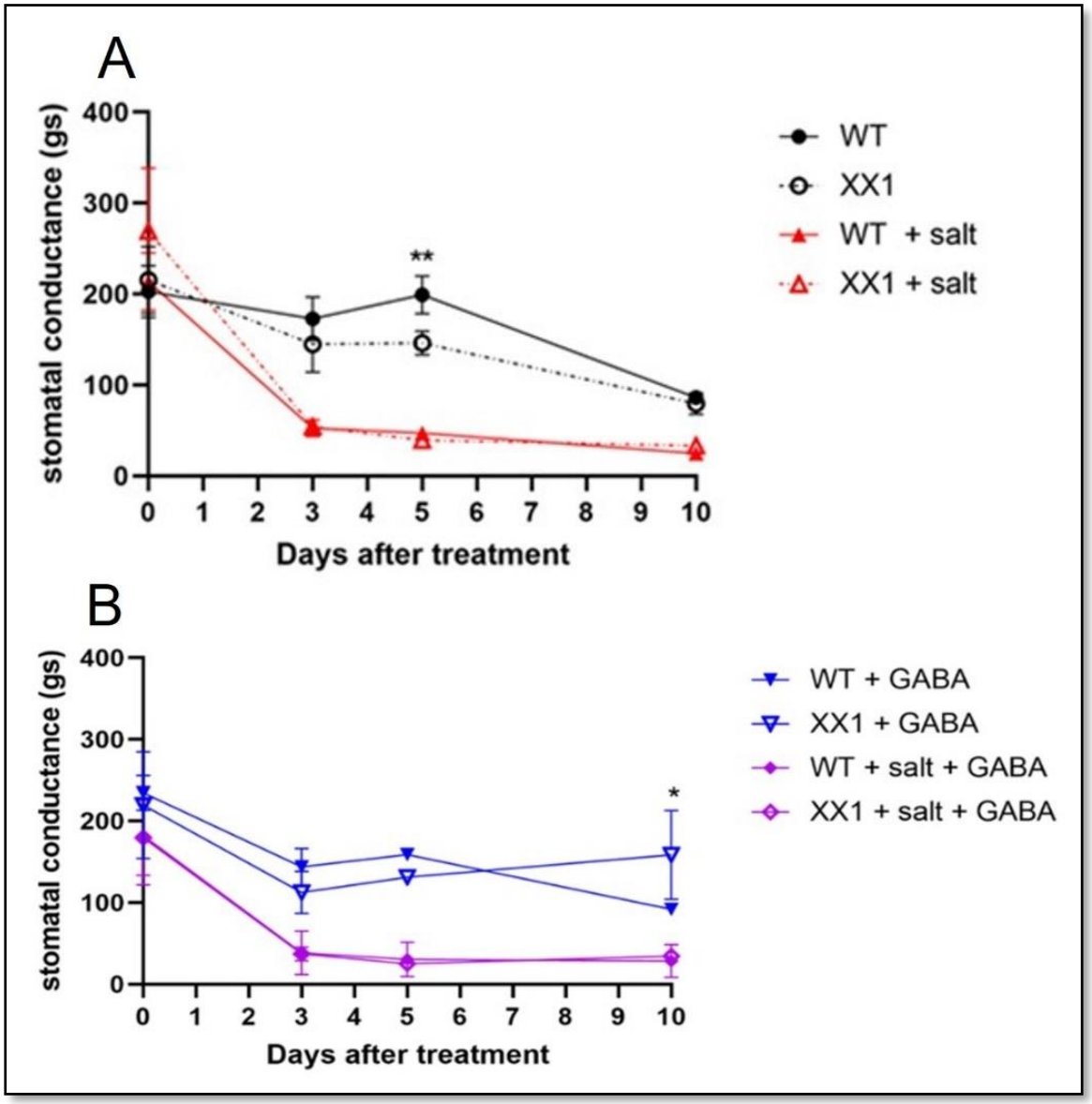

Figure 3.10: Stomatal conductance of *Arabidopsis thaliana* wild type (WT) and AOX1a over expressor (XX1) plants (A) control and exposed to 150 mM salt (B) exposed to GABA and combination of NaCl and GABA. N=4; data analysed using Unpaired t test. Statistical significance ($P<0.05$) and ($P<0.01$) are denoted by asterisks (*) and (**) respectively. Error bars represent standard deviation.

3.5.2. Changes in fresh weight and dry weights of shoots when GABA was applied exogenously under salinity stress.

Six week old WT, XX1 and KO plants in soil were subjected to different treatments with or without GABA under salinity and control conditions and harvested 10 days after treatment to measure fresh weight and dry weight. All lines showed a significant decrease in fresh weight and dry weight under salinity (Figures 3.11 and 3.12), indicating that the salt treatment was causing a stress.

The fresh weight of XX1 also decreased with the addition of GABA (C+G), when compared to control conditions (Figure 3.11 B). Shoots of KO plants exhibited a significant increase in fresh weight and dry weight when GABA was applied alongside the salinity stress (S+G), compared to salinity stress without GABA (S) (Figure 3.11 C and 3.12 C) and a similar pattern was seen for XX1 fresh weight, but this was not statistically significant and was not observed for dry weights (Figure 3.11 B and 3.12 B).

This suggests, at least for the KO line, an interaction between AOX1a loss and GABA, when it comes to regulating growth under saline conditions. The presence of GABA may overcome the decreased fitness of plants lacking AOX1a during exposure to salinity stress. However, overall there was no significant difference in fresh or dry weights of XX1 and KO relative to WT under different treatments (C, C+G, S, S+G) (tables 3.3 and 3.4).

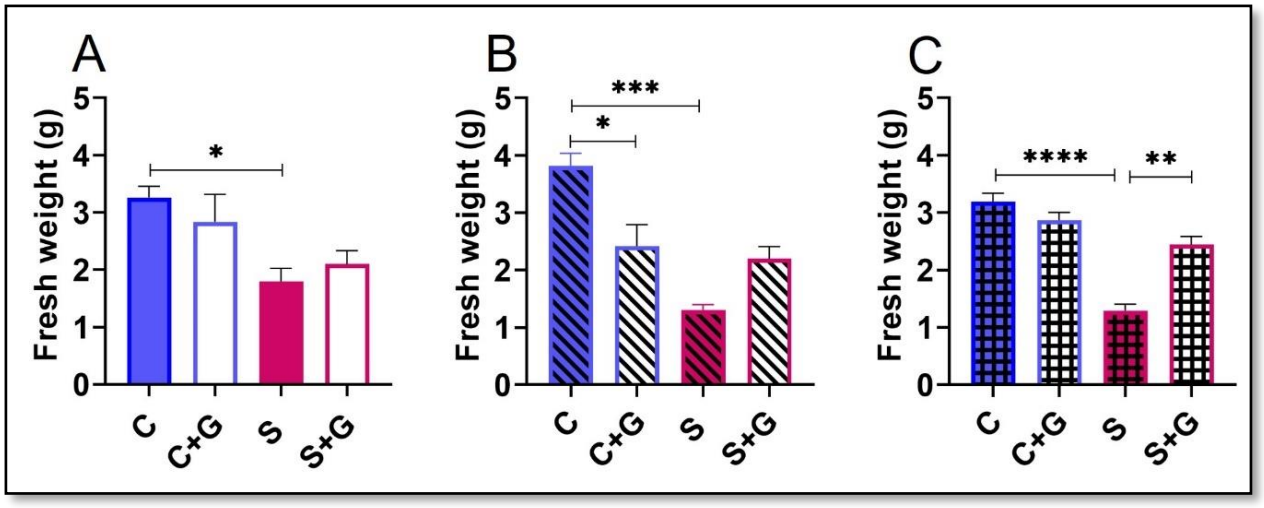

Figure 3.11: Fresh weight (FW) of (A) wild type (WT), (B) AOX1a over expressor (XX1) and (C) AOX1a knock out (KO) plants exposed to GABA (10 mM), NaCl (150 mM) and combination of NaCl and GABA. Control (C), Control + GABA (C+G), Salt treated (S), Salt + GABA (S+G). n=4; data analysed using 2-way ANOVA with Tukey post hoc testing. Statistical significance ($P<0.05$), ($P<0.01$), ($P< 0.001$) and ($P<0.0001$) are denoted by asterisks (*), (**), (***) and (****) respectively. Error bars represent standard error of mean (SEM).

Table 3.3: Statistical significance (p values) of fresh weight in XX1 and KO, relative to WT for each treatment. Wild type (WT), AOX1a over expressor (XX1) and AOX1a knock out (KO) plants exposed to GABA, exposed to NaCl and combination of NaCl and GABA. Control (C), Control + GABA (C+G), Salt treated (S), Salt + GABA (S+G). n=4; data analysed using 2-way ANOVA with Tukey post hoc testing.

		XX1				**KO**			
		C	**C+G**	**S**	**S+G**	**C**	**C+G**	**S**	**S+G**
WT	**C**	0.8902				>0.9999			
	C+G		0.9818				>0.9999		
	S			0.9496				0.9424	
	S+G				>0.9999				0.996

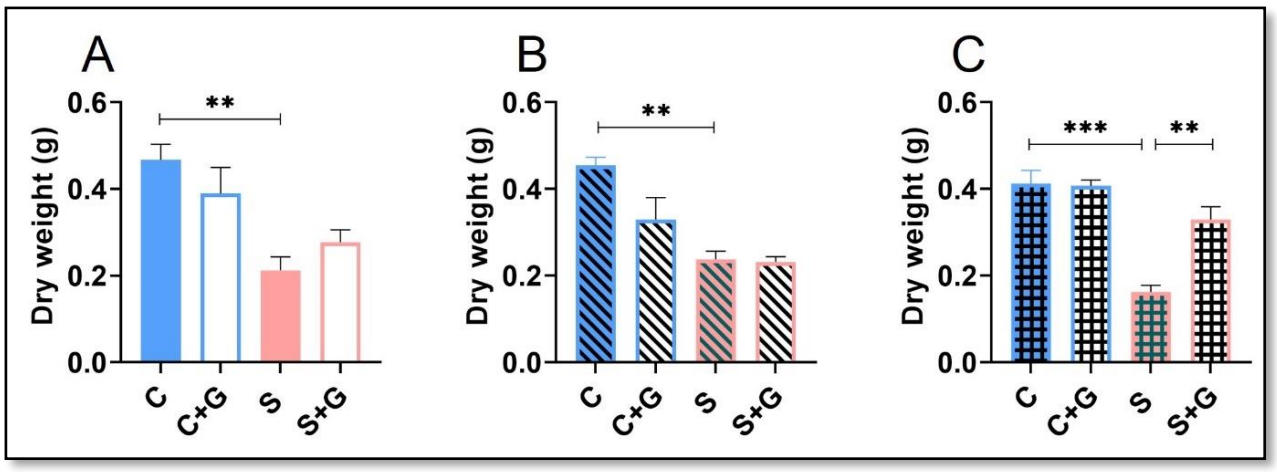

Figure 3.12: Dry weight (DW) of (A) wild type (WT), (B) AOX1a over expressor (XX1) and (C) AOX1a knock out (KO) plants exposed to GABA (10 mM) , NaCl (150 mM) and combination of NaCl and GABA. Control (C), Control + GABA (C+G), Salt treated (S), Salt + GABA (S+G). n=4; data analyzed using 2-way ANOVA with Tukey post hoc testing. Statistical significance ($P<0.01$) and ($P< 0.001$)] are denoted by asterisks (**) and (***) respectively.Error bars represent standard error of the mean (SEM).

Table 3.4: Statistical significance (p values) of dry weight XX1 and KO relative to WT. Wild type (WT), AOX1a over expressor (XX1) and AOX1a knock out (KO) plants exposed to GABA, exposed to NaCl and combination of NaCl and GABA. Control (C), Control + GABA (C+G), Salt treated (S), Salt + GABA (S+G). n=4; data analyzed using 2-way ANOVA with Tukey post hoc testing.

		XX1				**KO**			
		C	**C+G**	**S**	**S+G**	**C**	**C+G**	**S**	**S+G**
WT	**C**	>0.9999				0.9819			
	C+G		0.9659				>0.9999		
	S			>0.9999				0.9914	
	S+G				0.9939				0.9873

3.5.3. Sodium ion concentration decreased in AOX1a knock out (KO) plants when GABA was applied alongside salinity stress.

Na^+ concentration and the ratio of Na^+ and K^+ concentration ($[Na^+]/[K^+]$) of WT, XX1 and KO shoots increased significantly under salinity stress (Figure 3.13). KO plants exhibited the greatest increase, reaching almost 4,000 mol/g dry weight, compared to approximately 2,000 mol/g in the salt-treated WT and XX1.

However, this line also exhibited a significant decrease in Na^+ concentration when GABA was applied simultaneous to the salinity stress (S+G), when compared to salinity stress alone (S) (Figure 3.13 C). This suggests an interaction between AOX1a loss and GABA, with respect to ion accumulation during salinity stress. There was no significant difference in K^+ concentration between WT, XX1 and KO shoots (Figure 3.13 D, E and F).

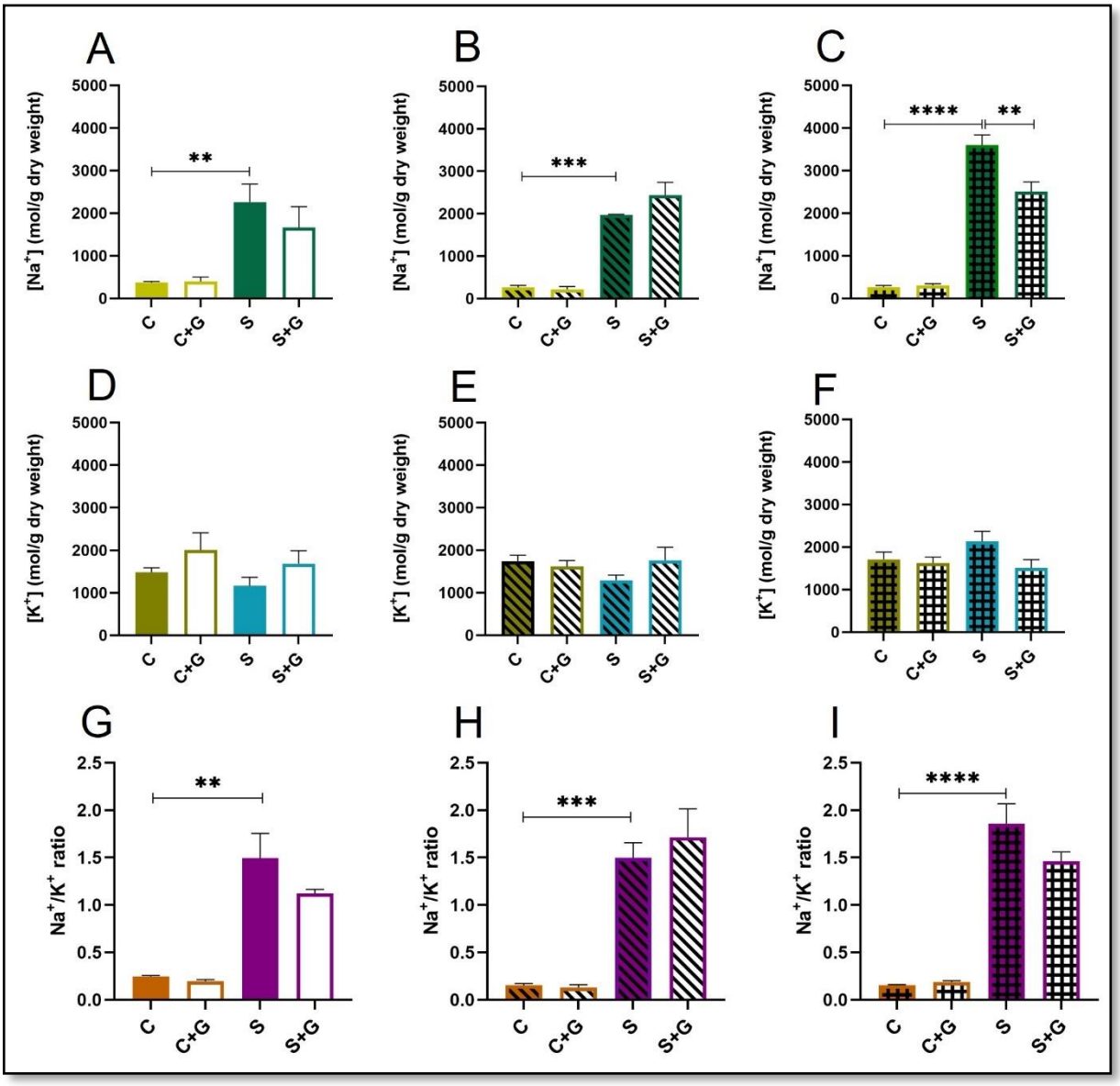

Figure 3.13: Effect of exogenous GABA on ion accumulation in AOX1a overexpressor and knockout plants exposed to salinity stress. Na^+ concentration of (A) WT, (B) XX1 and (C) KO plants exposed to GABA (10 mM) , NaCl (150 mM) and combination of NaCl and GABA. K^+ concentration of (D) WT, (E) XX1 and (F) KO plants exposed to GABA (10 mM) , NaCl (150 mM) and combination of NaCl and GABA. Na^+ and K^+ ratio ($[Na^+]/ [K^+]$) of (G) WT, (H) XX1 and (I) KO plants exposed to GABA (10 mM) , NaCl (150 mM) and combination of NaCl and GABA. Control (C), Control + GABA (C+G), Salt treated (S), Salt + GABA (S+G). n=4; data analyzed using 2-way ANOVA with Tukey post hoc testing. Statistical significance ($P<0.01$), ($P< 0.001$) and ($P<0.0001$) are denoted by asterisks (**), (***) and (****) respectively. Error bars representstandard error of the mean (SEM).

CHAPTER 4

DISCUSSION

4. Discussion

4.1. Summary of findings

The results obtained in this study suggest that *Arabidopsis thaliana* WT and XX1 plants subjected to 100 mM NaCl in the hydroponic system, and WT, XX1 and KO plants subjected to 150 mM NaCl in the soil were impacted by salinity stress. Plants showed decrease in fresh weight, dry weight, and growth rate as well as impaired stomatal conductance and accumulation of the lipid peroxidation product, MDA, an indicator of oxidative stress. Alternative oxidase pathway genes *AOX1a*, *AOX1d* and *NDB2* showed higher relative transcript levels under salinity stress in shoots, in line with analysed data from Genevestigator (Table 3.1), although the *AOX1a* response was not significant. In addition, a gene encoding glutamate dehydrogenase, *GAD2* (of the GABA shunt) also showed higher relative expression in shoots under salt stress. Together, these findings indicate that the application of salt to plants in hydroponics, agar plates and soil-based systems were sufficient to generate a stress. More specific effects of the salinity treatments in combination with AOX1a overexpression or knock-out, and exogenous GABA treatments are given below.

4.2. Effect of altered AOX1a expression on oxidative stress markers and GABA content during salinity stress

Salinity and osmotic stress increase the generation and accumulation of reactive oxygen species (ROS) and excessive ROS production and accumulation causes damages to the cell such as lipid peroxidation, protein oxidation, inhibition of enzymes, damages to nucleic acids, stimulation of programmed cell death (PCD) pathway and finally cause cell death (Abbasi et al. 2007; Borsani et al. 2005; Giraud et al. 2008; Miao et al. 2006; Serrato et al. 2004; Zhu et al. 2007). Relative gene expression, endogenous GABA and MDA content were measured in this study under 100 mM salt stress in WT and XX1 grown in the hydroponic system. The aim was to investigate the effect of AOX

expression on endogenous GABA under salinity stress and to understand the relationship between AOX and GABA.

MDA content is a marker of oxidative damage and increases due to increasing ROS (Sweetlove et al. 2002). In this study, MDA content in both WT and XX1 increased significantly after salt treatment (Figure 3.7) which is similar to a study with perennial ryegrass (*Lolium perenne L.*), whereby salt stress (250 mM NaCl) led to an increase in MDA content relative to the control (Wu et al. 2017). Lower MDA content was expected in XX1 compared to WT under salt treated conditions, because numerous studies have mentioned that AOX plays a role in preventing ROS over-accumulation (Maxwell et al. 1999; Purvis 1997; Sweetlove et al. 2002) and reduces the cellular damage to the plants during abiotic stresses such as excess light and drought stress (Wang & Vanlerberghe 2013). In particular, AOX1a knockout plants have previously shown high sensitivity to high light and drought stress, including an over-accumulation of ROS (Giraud et al. 2008; Nadarajah 2020). There seemed to be a slight decrease in MDA levels in XX1 compared to WT when grown under control conditions, but this was not significant and was not observed in salt-treated samples (Figure 3.7). Reasons for the lack of effect of AOX1a overexpression on MDA levels may include duration and the concentration of NaCl treatments. In a previous study the MDA content of wild type *Arabidopsis* was 80 nmol g^{-1}DW under control conditions, and 130 nmol g^{-1}DW and 140 nmol g^{-1}DW after 24 h and 72h of 100 mM NaCl treatment respectively, overall, a 75% increase (Ellouzi et al. 2011). Another study showed that MDA content was also significantly higher in wild type Arabidopsis under 100 mM NaCl for 24 h (38 µg g^{-1}FW) relative to control (22 µg g^{-1}FW), a 72% increase (Ding et al. 2016). In the present study the MDA levels were 18 nmol g^{-1}FW and 15 nmol g^{-1}FW in WT and XX1 shoots under control conditions while 25 nmol g^{-1}FW under salinity stress for 1 day in both WT and XX1(Figure 3.7 A), that is, an increase of 38% and 66% respectively. In the present study, 100 mM NaCl was used. Perhaps this was not enough to allow a difference to be observed between lines with high or low AOX1a expression.

Gene expression of alternate oxidase pathway genes (*AOX1a*, *AOX1d* and *NDB2*) were higher after salinity stress in both WT and XX1 shoots (few results showed significant differences) in this study (Figure 3.2,3.4,3.5). It is possible that increased AOX expression may alleviate salinity stress by managing ROS production. Similar increase in transcripts levels of *AOX1a* and *NDB2* were observed under 150 mM salinity stress after 72 h in *A. thaliana* (Smith et al. 2009) and hydrogen peroxide levels (direct marker of oxidative stress) were lower than WT under salinity stress confirming the role of AOX in the minimization of ROS production as described in the (Umbach et al. 2005) study.

Abiotic stresses including salinity stress also enhance the accumulation of GABA in variety of plants (Kinnersley & Turano 2000). Salt treatments induced accumulation of GABA in tobacco (Zhang et al. 2011) and *A. thaliana* (Renault et al. 2010; Su et al. 2019). In the present study, the application of salt stress for one day, induced GABA accumulation in both WT and XX1. However, the fold increase was larger in WT than in XX1, probably because GABA levels were already higher in XX1 under control conditions (Figure 3.6). It was interesting that under control conditions, XX1 roots had higher endogenous GABA content than WT roots (Figure 3.6 B). There was also a similar trend in shoots, but this was not significant. Endogenous GABA content of XX1 roots decreased back towards control levels after 9 days of salt treatment while levels in WT roots remained high. Interestingly, *GAD2* transcript levels were higher (although not significantly) in XX1 roots at day 9 compared to day 1 and control samples (Figure 3.3 D), therefore the decrease in GABA levels might not be due to a decrease in biosynthesis. This suggests that the plants overexpressing AOX1a had lower requirements for GABA accumulation during the later stages of stress, or that the elevated AOX1a protein provided a mechanism for enhanced GABA catabolism during later stages of stress.

Glutamate (Glu) is subjected to decarboxylation for the synthesis of GABA and this irreversible reaction is catalysed by glutamate decarboxylase (GAD) (Wu et al. 2020). Regulation of enzyme activity and transcriptional expression of GAD regulate GABA levels (Wu et al. 2020). GAD genes generally change in expression in response to abiotic stress (Wu et al. 2020). In the present study,

WT shoots showed an increase in *GAD2* expression 1 day after treatment, but this effect was reduced by 9 days after treatment (Figure 3.3 A). There was no significant difference in XX1 shoot (Figure 3.3 B and D), although there was a trend towards decreasing transcript levels throughout the treatment. There were also no significant changes in *GAD2* transcript levels in roots of both WT and XX1 (Figure 3.3 C and D). The increase in *GAD2* transcript levels after salt stress might account for the increased GABA content in WT. However, in WT roots and XX1 shoot and root tissues, an increase in GABA content occurred despite no significant increase in *GAD2* expression. There are additional GAD genes in *A. thaliana* such as *GAD1*, *GAD3*, *GAD4* and *GAD5* (Miyashita & Good 2008). There is a possibility of having high expression of other GAD gene or genes that were not examined in this study. Published *GAD1*, *GAD3/4* and *GAD5* primers did not work when tested and due to limitations of time, new primers were unable to be tested. Miyashita & Good (2008) reported that expression of *GAD1* was predominant in roots while *GAD2* expression was distributed in every part of the plant and other GAD genes (*GAD3*, *GAD4* and *GAD5*) showed a weak expression under hypoxia (Miyashita & Good 2008). In that study, root tissues had higher GABA content relative to the shoot tissues, and it might be due to the higher expression of *GAD1* gene in root tissues.

4.3. Exogenous application of GABA reduces the relative growth rate of roots in WT and AOX1a knockout plants.

Excessive GABA content retards the elongation of dark-grown hypocotyls and primary roots (Li et al. 2021). Exogenous GABA application retards the growth of primary roots and adventitious roots by alteration of balance between carbon and nitrogen (Li et al. 2021). As an example, the adventitious root growth of poplar lines was reduced due to the elevated GABA levels by exogenous application of GABA (Xie et al. 2020; Yue et al. 2018) in the absence of abiotic stress. Studies in wheat lines under aluminium toxicity have shown that GABA negatively regulates root growth (Ramesh et al. 2015). In this study, exogenous GABA application under control conditions (C+G) also reduced the relative growth rate of roots in WT and XX1 lines, relative to controls (C) (figure 3.9 A). When

GABA was applied exogenously under salinity stress (S+G), XX1 showed higher root relative growth rate compared to WT (S+G) and KO (S+G) (figure 3.9 B). These results suggest that overexpression of AOX1a may help alleviate negative regulation of root growth by GABA under salinity stress.

4.4. Stomatal conductance decreased significantly due to salinity stress and was not greatly affected by GABA application.

Osmotic stress is induced by the salt stress and leads to stomatal closure and gas exchange inhibition which may conserve water but can also lead to lipid peroxidation and damage to cell membrane due to accumulation of ROS (Wu et al. 2017). Studies have proved that during non-stressed conditions GABA plays a role in the modulation of stomatal opening in *A. thaliana* (Xu et al. 2021). Transcription of *GAD2* and accumulation of GABA show diurnal regulation; GABA normally reaches its highest at the latter part of dark cycle before the opening of stomata and reaches lowest at mid-day when the stomatal conductance reaches maximum (Espinoza et al. 2010). Abundance of *GAD2* transcript and accumulation of GABA increase during stress conditions and it has been recently proposed that stomatal opening is minimized by GABA under drought stress helping the plants minimise transpiration (Espinoza et al. 2010; Xu et al. 2021).

In the present study, stomatal conductance was significantly reduced under salinity stress with or without GABA, and GABA application had no impact on stomatal conductance under salinity stress. There was also no significant difference in stomatal conductance between WT and XX1 under salinity stress, with or without GABA (Figure 3.10).

Stomatal conductance of WT and XX1 leaves decreased from day 0 to day 10. However, in XX1 plants, when GABA was applied at Day 0, stomatal conductance decreased only until day 3, then increased, reaching close to day 0 stomatal conductance on day 10 (Figure 3.10 B). Stomatal conductance may be reduced due to aging of the leaves (close to senescence) (Reich & Borchert 1988) or due to limited uptake of water under salt stress (Ma et al. 2020; Munns & Tester 2008). Plants in this study were 7-8 weeks old by the end of the treatment and WT and XX1 plants might be showing

age related stomatal conductance decline as there was no difference under control conditions (except at Day 5) in both WT and XX1 (Figure 3.10 A). Only GABA treated (C+G) XX1 plants could regain the day 0 conductance (Figure 3.10 B). Thus, it is possible that a combination of AOX1a overexpression and exogenous GABA application could slow down age-related decline in stomatal conductance. Xu et al. (2021) showed that exogenous GABA application increased the endogenous GABA levels, causing a decrease in stomatal conductance (Xu et al. 2021). In the present study, there was no consistent impact of exogenous GABA application on stomatal conductance in WT and XX1 under salt and both lines were salt sensitive (Figure 3.10 B).

4.5. Exogenous GABA application increased fresh weight and dry weight and decreased sodium content in AOX1a knock out plants.

According to (Skirycz et al. 2010), *A. thaliana* AOX1a over expressor plants were smaller relative to wildtype under normal conditions but there was no significant difference in the relative growth rate under control conditions. During mild and severe drought stress, WT plants showed a decrease in relative growth rate compared to the AOX1a over expressor (Skirycz et al. 2010). In the present study, WT and XX1 plant sizes were similar under control conditions (Appendix figure 5.6).

Salinity stress decreases fresh weight and dry weight of plants because plant growth and development are limited, and metabolic activities are perturbed due to the induction of oxidative and osmotic stresses (Xiang et al. 2016). Many studies showed that chlorophyll metabolism is affected by salinity stress which affects photosynthesis negatively (Jin et al. 2019; Li, J et al. 2015). Exogenous application of GABA alleviates salinity stress by regulating antioxidant systems and biosynthesis of chlorophyll (Jin et al. 2019). Salinity stress inhibited main metabolic enzymes necessary for cyclic functioning of the TCA cycle in wheat leaves, but the TCA cycle was supplied with alternative carbon source to operate in mitochondria due to elevated GABA shunt activity (Che-Othman et al. 2020). Plants such as muskmelon (Jin et al. 2019), maize (Wang et al. 2017b), tomato (Wu et al. 2020a), white clover (Cheng et al. 2018) and barley (Ma et al. 2019) tolerate salt stress by reducing the damage

to the plants by increasing endogenous GABA content and activation of enzymatic antioxidant activity after exogenous application of GABA under salinity stress (Li et al. 2021).

Ion homeostasis is maintained by the uptake of ions and compartmentalization and this process is important not only for normal growth of plants but also for the growth of plants under salt stress (Gupta & Huang 2014). Metabolic processes might be perturbed by the presence of excess Na^+ ions in leaf tissues. Influx of K^+, Ca^{2+}, Mg^{2+} decreased and influx of Na^+ increased due to the high concentration of Na^+(Skirycz et al. 2010). As a result, ion toxicity and osmotic stress occur affecting the normal plant growth (Skirycz et al. 2010). Studies showed that exogenous GABA reduced Na^+ content in plant tissues but there is no evidence to prove GABA reduces Na^+ content directly (Wu et al. 2020). Root sodium flux was reduced in mutant Arabidopsis plants with high endogenous GABA compared to mutant plants with lower endogenous GABA (Su et al. 2019). Data from one study revealed that exogenous application of GABA reduced the Na^+ content in leaves of tomato because absorption of Na^+ through the roots was reduced. Regulation of Na^+ influx by exogenous GABA under salinity stress might be the reason for lower Na^+ content in shoot and root tissues of tomato (Wu et al. 2020).

In the present study, KO plants showed higher fresh weight (FW), dry weight and lower Na^+ accumulation after exogenous application of GABA under salinity stress (S+G) relative to KO plants treated only with salt (S) (Figures 3.11 C, 3.12 C and 3.13 C). Plants lacking AOX1a might be more susceptible to salinity stress, but excess GABA could overcome this susceptibility. But GABA doesn't have much of an effect on alleviating salinity stress if AOX1a is already present. That is, both pathways are working towards similar goals and GABA could partially complement the stress response of AOX1a if the latter is missing. GABA might act as a signal molecule in the shoots of *A. thaliana* to alleviate salt stress in the absence of AOX1a. But it cannot be predicted that there is a negative interaction between GABA and alternate oxidase pathway because there are other AOX genes in the plants. A recent study showed that AOX1d also plays a role in alleviating salinity stress

by reducing oxidative stress (Oh et al. 2021) and *AOX1d* increases in the absence of AOX1a (Strodtkötter et al. 2009).

4.6. General discussion

Alternative oxidase pathway genes and endogenous GABA increase with abiotic stress and studies suggest that both AOX and GABA help to manage excess ROS production and accumulation (Andronis & Roubelakis 2010; Kreps et al. 2002; Martí et al. 2011; Ottow et al. 2005; Salah et al. 2019; Seki et al. 2002; Sheteiwy et al. 2019; Shi et al. 2010; Wu et al. 2020b). Succinic-semialdehyde dehydrogenase (SSADH) enzyme catalyses the conversion of succinic-semialdehyde (SSA) into succinate (Bouché et al. 2003). Knock out mutants of SSADH *Arabidopsis thaliana* under heat stress showed tissues damage such as necrosis and cell death due to excessive accumulation of ROS (Bouché et al. 2003). Respiration catalysed by AOX is significant under abiotic stress because energy surplus is dissipated as heat, and this can decrease ROS generation by preventing over reduction of the ubiquinone pool (Vishwakarma et al. 2015). Among all genes that encode mitochondrial proteins, *AOX1a* and *AOX1d* are highly stress responsive genes in *A. thaliana* (Vishwakarma et al. 2015). Studies show that exogenous GABA application enhances the expression of stress responsive genes such as heat shock proteins (*HSP70*), monodehydroascorbate reductase (*MDHAR*), osmotin (*OSM*), glutathione reductase (*GR*), dehydrins (*DHN*s), late embryogenesis abundant proteins (*LEA* proteins) and aquaporins (*AQP*) in sunflower under heat and drought stress (Abdel Razik et al. 2021). Since *AOX1a* and *AOX1d* are also stress responsive genes, exogenous GABA application might play a role in enhancing AOX expression under salinity stress. Antioxidative systems help plants to adapt to abiotic stress conditions (Vishwakarma et al. 2015). Ascorbate (Asc) detoxifies hydrogen peroxide under abiotic stress (Bartoli et al. 2006). An *A. thaliana* AOX1a overexpression line showed enhanced production of ascorbate relative to WT in leaves under high light (Bartoli et al. 2006). Activity of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX) are upregulated by GABA as well in the presence of exogenous GABA

(Wang et al. 2017a). All these facts suggest that there might be a relationship between AOX transcript levels and endogenous GABA.

In the present study, relative growth rates of wild type and AOX1a knock-out roots were decreased when GABA was applied exogenously under control and salinity stress conditions. This wasn't observed in AOX1a overexpression lines, suggesting that high AOX1a expression may prevent root growth inhibition by GABA. Meanwhile, knock-out plants subjected to GABA treatment under salinity stress in soil-based experiments had higher shoot fresh weight and dry weight and lower sodium ion concentration relative to knock-out plants subjected to salinity treatment only, suggesting that GABA treatment reduced the impact of salinity stress on the shoot tissues of plants lacking AOX1a. Together, these two findings suggest interaction between alternative respiration and GABA shunt pathways during salinity stress in *A. thaliana*, but with tissue specificity.

As mentioned, when GABA was applied exogenously, AOX1a presence helped the root growth of plants under normal and salinity stress conditions. This might be because AOX1a had an impact on GABA metabolism, which can act as a carbon or nitrogen source and might aid in root growth. In shoots, GABA treatment of plants lacking AOX1a helped to alleviate salt stress. This might be because the absence of AOX1a prevented breakdown of GABA, facilitating the role of GABA as a signal molecule, activating antioxidant defence systems and chlorophyll biosynthesis under salinity stress. AOX1a over expression led to an increase in endogenous GABA level under control conditions, which does not support these hypotheses, however it is unknown where in the plant tissue this GABA was localised, or which cell types. The exogenous GABA was applied to the roots (through the agar or soil) and therefore likely to be present in the vasculature. It is nonetheless interesting that AOX1a overexpression led to an increase in GABA content. Future work in this area is required to determine whether there is a real interaction between the AOX and GABA shunt pathways

The second hypothesis of this study is that AOX overexpression helps the plant to tolerate salinity stress and knockout results in plants that are more sensitive to salinity stress, but from the data

presented, this hypothesis cannot be either accepted or rejected as roots favored AOX1a presence while shoots behaved opposite under salinity stress. Thus, there is a tissue-specific response. In addition, the exogenous application of GABA was predicted to enhance the protective effect of AOX1a overexpression, however this was not the case. If anything, GABA treatment seemed to assist plants that were lacking AOX1a during salinity stress.

4.7. Limitations and future directions

Findings from the current study are novel and intersting because it suggests that there might be an interaction between GABA and AOX and it is tissue specific. However, these results are preliminary, and experiments should be repeated to confirm the results. Replicate numbers can be increased to see more statistical significance beacause as an example, *AOX1a* transcript levels did not significantly increase in response to salt stress whereas it did in Genevestigator analysis and in other studies (Andronis & Roubelakis 2010; Kreps et al. 2002; Martí et al. 2011; Ottow et al. 2005;Seki et al. 2002) , therefore more replication might help to avoid high standard deviation (STD) and standard error of the mean (SEM) and improve statistical significance.

Published *GAD1*, *GAD3/4* and *GAD5* primers did not work when tested and therefore future work should include designing suitable primers and measuring the transcript levels of these genes. Other measurements could be used to complement the ones completed so far. For example, the Amplex Red Hydrogen Peroxide/Peroxidase assay or 3,3'-diaminobenzidine (DAB) staining could be carried out to measure ROS in plants treated with GABA or salt, or a combination. Chlorophyll content and photosynthesis could also be measured using gas exchange equipment such as Infra Red Gas Analysers (IRGAs, e.g. LiCor 6400) and SPAD meters. Other antioxidant enzymes (e.g. catalase, SOD, APX) could also be measured to further investigate the relationship between ROS, AOX pathway and GABA shunt.

One of the limitations of the soil experiment, was that root samples could not be collected. Roots are directly exposed to the salt stress, as it is applied to the soil or growth meduim, therefore it is important to look at the responses of this tissue. This is why plate and hydroponics systems were also used in the present study. But soil experiments are more realistic to situations in the environment. In future this could be improved by replacing soil with sand or a more suitable soil to make it easier to obtain soil-free roots for assays. In this study some of the the assays (fresh weight , dry weight and sodium ion (Na^+) and potassium ion (K^+) ratio) were done only in shoots harvested from soil.

Some results were different from the literature, potentially because of the concentration of NaCl used and duration of the salt treatments. For example, the AOX1a overexpression line did not show improved growth rates under salinity, whereas it has in another study (Smith et al. 2009). Therefore future experiments might involve different NaCl concentrations and shorter or longer treatment times. The plate-based assay indicated significant differences between lines at 150 mM NaCl but the hydroponics experiment utilised 100 mM NaCl (based on previous experiments by other lab members). Also the addition of NaCl to soil (150 mM final concentration of watering solution) was only made once, followed by top-ups of water, therefore some salt may have been lost during watering. Optimising the application of NaCl to soil can be difficult because different soil particles bind NaCl differently and therefore it is difficult to quantify the concentration of salt that the plant is exposed to. Future experiments should use more precise measurement of soil weights and adjustment of watering volumes to control the treatment between pots and to increase the intensity of the salinity stress.

In the present study, the AOX1a knockout line showed a bigger response to exogenous GABA application under salinity stress, compared to the other lines. Perhaps future work could expand on this line, and include additional stresses such as drought/high light stress to clarify whether these effects are unique and only observed under salinity stress. Another approach to explore the interaction between AOX and GABA may be to use mutant plants in which multiple AOX genes have been

knocked out, such as AOX1a and AOX1d, under same salinity stress, because *AOX1d* transcript has been shown to increase in the AOX1a knock out line when exposed to stress (Oh et al. 2021). Other than AOX1a and AOX1d it is intersting to see plants with altered alternative NAD(P)H dehydrogenases. NDB2 might be interesting because expression of this gene is also salt-responsive according to the genevestigator data (Table 3.1) and qRT-PCR data (Figure 3.5). But internal-facing NAD(P)H DHs might also be interesting (NDAs or NDC) because the NADH generated by the GABA shunt would occur in the mitochondrial matrix and the NDAs could use that NADH directly.

Furthermore, other Arabidopsis mutants or transgenics plants where GABA metabolism enzymes have been altered could be used to investigate the impact on AOX expression and activities, in the presence and absence of salinity stress to get a clearer understanding about the interaction of GABA and AOX pathways. Investigation of transcriptomic effects of GABA application, and in plant lines with altered GABA shunt genes and alternative pathway genes might help to determine if there are signalling responses. For example, if common transcription factors are affected when altering GABA-related genes and AOX-related genes. It will help to understand the role of GABA, whether it act as a carbon/nitrogen source or signalling molecule (or both) in the presence of AOX during salinity stress.

This study can be taken further by selecting a plant such as tomato that accumulate GABA in the fruit during early development then break it down during ripening (Takayama & Ezura 2015). It would be interesting to measure alternative respiration activities and gene expressionin the different phases in tomato fruit. There is a potential for improving crop stress tolerance by understanding the relevance of GABA and alternative respiration pathways in stress and in relation to each other. Therefore it would be good to follow up on some of the ideas mentioned here.

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APPENDIX

5.1 Materials and recipes, equipment and software used in this study.

Table 5.1: Recipe for Germination Medium (GM)

Germination Medium

Macronutrients	FW	g to make 1 L stock	Stock Conc (M)	Vol of stock (mL) for 1L	Final conc (mM)
NH_4NO_3	80	80	1	**0**	0
KNO_3	101.1	101.1	1	**0**	0
$CaCl_2$	1M Solution		1	**0.75**	0.75
KCl	74.55	74.55	1	**1**	1
$Ca(NO_3)_2 \cdot 4H_20$	236.1	94.4	0.4	**0.625**	0.25
$MgSO_4 \cdot 7H_20$	246.5	98.6	0.4	**2.5**	1
KH_2PO_4	136.1	13.61	0.1	**2**	0.2

Micronutrients	FW	g to make 1 L stock	Stock Conc (mM)	Vol of stock (mL) for 1L	Final conc (uM)
NaFe(III)EDTA	367.1	18.4	50	**1**	50
H_3BO_3	61.8	3.09	50	**1**	50
$MnCl_2 \cdot 4H_20$	197.9	0.99	5	**1**	5
$ZnSO_4 \cdot 7H_20$	287.5	2.875	10	**1**	10
$CuSO_4 \cdot 5H_20$	249.7	0.125	0.5	**1**	0.5
Na_2MoO_3	242	0.0245	0.1	**1**	0.1

pH with NaOH to 5.6

0.7% Agar	**7g per 1000ml**

MACRONUTRIENTS

Final Conc of i	mM	Activity
K	1.2	4.79
Ca	1	1.05
Mg	1	1.03
NH_4	0	1.72
Cl	2.51	3.19
NO_3	0.5	7.75
SO_4	1.0105	0.893
PO_4	0.2	1.8 pM
Na	0.1012	1.38

MICRONUTRIENTS

Final Conc of i	mM	Activity
Fe	0.01	25 pM
Mn	0.005	23nM
Zn	0.01	50 uM
Cu	0.0005	23 nM
Mo	0.0001	31 nM

Table 5.2: recipe for Basal Nutrient Solution (BNS)

Basal Nutrient Solution

Macronutrients	FW	g to make 1 L stock	Stock Conc (M)	Vol of stock (mL) for 1L	Final conc (mM)
NH_4NO_3	80	80	1	**2**	2
KNO_3	101.1	101.1	1	**3**	3
$CaCl_2$	1M solution		1	**0.1**	0.1
KCl	74.55	74.55	1	**2**	2
$Ca(NO_3)_2 \cdot 4H_20$	236.1	94.4	0.4	**5**	2
$MgSO_4 \cdot 7H_20$	246.5	98.6	0.4	**5**	2
KH_2PO_4	136.1	13.61	0.1	**6**	0.6
NaCl	58.44	58.44	1	**1.5**	1.5

Micronutrients	FW	g to make 1 L stock	Stock Conc (mM)	Vol of stock (mL) for 1L	Final conc (uM)
NaFe(III)EDTA	367.1	18.4	50	**1**	50
H_3BO_3	61.8	3.09	50	**1**	50
$MnCl_2 \cdot 4H_20$	197.9	0.99	5	**1**	5
$ZnSO_4 \cdot 7H_20$	287.5	2.875	10	**1**	10
$CuSO_4 \cdot 5H_20$	249.7	0.125	0.5	**1**	0.5
Na_2MoO_3	242	0.0245	0.1	**1**	0.1

pH with NaOH to 5.6

0.7% Agar	**7g per 1000ml**

MACRONUTRIENTS

Final Conc of i	mM	Activity
K	5.6	4.79
Ca	2.1	1.05
Mg	2	1.03
NH_4	2	1.72
Cl	3.71	3.19
NO_3	9	7.75
SO_4	2.0105	0.893
PO_4	0.6	1.8 pM
Na	1.5502	1.38

MICRONUTRIENTS

Final Conc of i	mM	Activity
Fe	0.01	25 pM
Mn	0.005	23nM
Zn	0.01	50 uM
Cu	0.0005	23 nM
Mo	0.0001	31 nM

Table 5.3: Recipe for RNA extraction

TRIzol – like reagent	1 mL per tube
Chloroform	200 ul per tube
Iso-propanol	500 ul per tube
75% Ethanol	1 mL per tube
Autoclaved MQW, tips and tube	

Table 5.4: Recipes for CDNA synthesis first master mix

Reagent	Quantity
2 ug of RNA	Adjustable
50 uM Oligo d(T)20	2.0 ul
10 mM dNTP	1.0 ul
sterile autoclaved water	Make up to 13 uL

Table 5.5: PhotoScript mixture

Reagent	Quantity
5 x PhotoScriptII Reaction Buffer	4.0 ul
0.1 M DTT	2.0 ul
Marine RNaseOut	0.5 ul
PhotoScriptII RT	0.5 ul

Table 5.6: Recipe for Gabase enzyme assay

Step	Reagent	Volume for one reaction (µl)
Step 1	Potassium pyrophosphate (75 mM)	15
	2-Mercaptoethanol (3.3 mM)	0.33
	Alpha- ketoglutarate	25
	NADP	12.5
	Sample	45.2
Step 2	Gabase enzyme (0.01 units)	2

Table 5.7: The apparatus utilized for the hydroponic system

Equipment to set up hydroponic system
1.5 mL microcentrifuge tube, black
50 mL polypropylene conical centrifuge tube with flat top screw cap
Hypodermic needles
10 L multi stacking container
24 well floater microtube rack, blue with hinged lid
Plastic support for tubes in hydroponics container, plastic
Aquarium air pump
Aquarium tubing
Freshwater aquarium air stones

Table 5.8: The equipment used in this study

Equipment	Manufacturer
NanoDrop™ 2000 spectrophotometer	Thermo Fisher Scientific™
Class II bio-safety Cabinet	GELAIRE®
Master cycle Eppendorf	
SCIENTRONIC HB 900 (80 ^{0}C oven)	
80 ^{0}C oven	Labmaster
Electronic balance (METTLER TOLEDO)	ROWE Scientific Pty Ltd
Dry block heater	Adel Lab Scientific
C1000^{TM} Thermal Cycler	BIO RAD
SUPER- Mixer vortex machine	LAB-LINE INSTRUMENTS, Inc
Magnetic stirrer	INDUSTRIAL INPUT & CONTROL Pty Ltd

pH meter (STARTER2100)	OHAUS®
Fume hood	SOUTHERN CROSS SCIENTIFIC
Flame photometer (Model 420)	Sherwood
Pipettors (P2, P20, P200, P1000)	
Centrifuge (Eppendorf 5425)	
Plate reader	Omega
Savant SC100 Speed Vac Concentrator with Savant RH40-12 Rotor	

Table 5.9: Software utilized during the study

Software name	Application
Microsoft Excel 2010	For all calculations in the study.
NanoDrop 2000 software 1.6	To determine nucleic acid concentration in extracted RNA samples.
GraphPad Prism 9.1.2.	For all statistical analysis.
MARS Data Analysis Software	Plate reading in the TBARS assay.
CFX Manager™ Software (version 2.0)	To analyse Rt qPCR results.
Genevestigator	To obtain publicly available data.
Image J software	To measure the root length.

Table 5.10: Primers used in the study

Gene	Primer	Sequence (5'-3')
Aox1a	AtAox1a_F	CTGGACCACGTTTGTTC
	AtAox1a_R	ACACCCCAATAGCTCG
Aox1d	AtAox1d_F	TACCGCACTCTTCGAC
	AtAox1d_R	GGCTGGTTATTCCCACT
Ndb2	Atndb2_F	CCGAAACTGATGATGTATCTAAG
	Atndb2_R	TTCTCACACTCTTCCATACGG
Ubiquitin*	AtUbiq_F	GACAGAGCAGAGAACATAAGG
	AtUbiq_R	TGGGGATTGGGTAAAGAGG
a-tubulin*	a-tubulin_F	GGTCACCACCTGGAACAACT
	a-tubulin_R	TGGCACCATCAAGACAAGACAAAGA
GAD 1	GAD1_F	CGCAGGTATGGATGGATA
	GAD1_R	AGCTCACGCATCACTTTC
GAD 2	GAD2_F	CGAGGGGTACAAAAATGTGA
	GAD2_R	CCAGCCAAAACGACGTAG

*Denotes Housekeeping genes

Table 5.11: Media preparation for plate assay with and without sucrose.

Media	Final volume (ml)	MS (g)	NaCl (g)	Sucrose (g)	$CaCl_2$ (g)	Agar (g)	pH
½ MS	250	0.554	No	No	No	2	5.62
½ MS + 50 mM NaCl	250	0.554	0.731	No	No	2	5.66
½ MS + 100 mM NaCl	250	0.554	0.461	No	No	2	5.61
½ MS + 150 mM NaCl	250	0.554	2.192	No	No	2	5.65
½ MS + 50 mM NaCl + 0.6 mM $CaCl_2$	400	0.886	1.169	No	0.035	3.2	5.75
½ MS + 100 mM NaCl + 1.2 mM $CaCl_2$	400	0.886	2.338	No	0.071	3.2	5.73
½ MS + 150 mM NaCl + 1.8 mM $CaCl_2$	400	0.886	3.506	No	0.106	3.2	5.73
½ MS + 2% Sucrose	800	0.886	No	16	No	6.4	5.74
½ MS + 2% Sucrose +50 mM NaCl + 0.6 mM $CaCl_2$	400	0.886	1.169	8	0.035	3.2	5.74
½ MS + 2% Sucrose + 100 mM NaCl + 1.2 mM $CaCl_2$	400	0.886	2.338	8	0.071	3.2	5.75
½ MS + 2% Sucrose +150 mM NaCl + 1.8 mM $CaCl_2$	400	0.886	3.506	8	0.106	3.2	5.75

Repeated plate assay including four treatments (Control, Control + GABA (10 mM), Salt treated (150 mM) and salt (150 mM) + GABA (10 mM) treated). Genotypes used include Wild type, AOX1a over expression line and aox1a knock out.

Table 5.12: Media preparation with or without GABA under control and salinity conditions.

Media	Final volume (ml)	MS (g)	NaCl (g)	Sucrose (g)	$CaCl_2$ (g)	Agar (g)	GABA (2 M) (ml)	pH
½ MS (Control)	600	1.329	No	No	No	4.8	No	5.64
½ MS (Control) + GABA (10 mM)	600	1.329	No	No	No	4.8	3	5.64
½ MS + 150 mM NaCl + 1.8 mM $CaCl_2$	600	1.329	5.259	No	0.159	4.8	No	5.6
½ MS + 150 mM NaCl + 1.8 mM $CaCl_2$ + GABA (10 mM)	600	1.329	5.259	No	0.159	4.8	3	5.6
½ MS + 2% Sucrose (for seed germination)	400	0.89	No	8	No	3.2	No	5.6

5.2 Calculations

5.2.1 Calculations for plate assay.

Preparation 2 M GABA stock (50 ml)

=2 mol/ 1000 ml x 50 ml x 103.12 $gmol^{-1}$

=10.312 g

A mass of 10.312 g was dissolved in sterilized milli Q water to make 50 ml GABA solution, and this was filter sterilized before adding to the MS media.

Sample calculation for salt and GABA containing media (½ MS + 150 mM NaCl + 1.8 mM $CaCl_2$ + 10 mM GABA)

½ MS = (4.43 g x ½)/1000 ml x 600 ml

= 1.329 g

NaCl = (150 x 10^{-3} mol/1000 ml) x 600 ml x 58.44 g mol^{-1}

= 5.259 g

$CaCl_2$= (1.8 x 10^{-3} mol/1000 ml) x 600 ml x 147.02 g mol^{-1}

=0.159 g

Volume of GABA added to the 600 ml of MS media = (10 x 10^{-3} M x 600 ml) / 2M

= 3 ml

Agar = (0.8 g / 100 ml) x 600 ml

=4.8 g

Calculation of relative growth rate of roots

$$\text{Root relative growth rate} = \frac{(\text{Total root length} - \text{Root length of Day 0})}{(\text{Root length of Day 0})}$$

= (136.82 – 13.03) mm / 13.03 mm

= 9.50

5.2.2 Calculations for Gabase enzyme assay

Preparation of GABA stock solutions

GABA needed to prepare 10 ml of 10 mM GABA stock solution

$= (10 \times 10^{-3} \text{ mol}/1000 \text{ ml}) \times 10 \text{ ml} \times 103.12 \text{ gmol}^{-1}$

$=0.01$ g

Preparation of 1 ml of 100 µM GABA stock solution using 10 mM GABA stock solution

$V_1 = (C_2 \times V_2)/C_2$

$= (100 \times 10^{-6}) \times (1 \times 10^{-3})/ (10 \times 10^{-3})$

$= 10$ µl

Therefore 10 µl of 10 mM GABA was added to 990 µl of autoclaved milli-Q water.

Table 5.13: Preparation of 1 ml of GABA dilutions for the standard curve.

Standard concentrations	Concentration of the stock used	Volume of the stock used (µl)	Volume of water used
200 µM	10 mM	20 µl	980 µl
100 µM	10 mM	10 µl	990 µl
50 µM	10 mM	5 µl	995 µl
20 µM	100 µM	200 µl	800 µl
10 µM	100 µM	100 µl	900 µl
5 µM	100 µM	50 µl	950 µl
2 µM	100 µM	20 µl	980 µl

5.2.3.0 Calculation of primer efficiency

Sample for calculation of Primer efficiency: GAD 2 primer efficiency

Dilution	Ct (rep 1)	Ct (rep 2)	Average Ct	Sample quantity	Log (Sample quantity)
stock (0)	17.97	17.06	17.52	1	0
1-Oct	19.89	20.11	20.00	0.1	-1
1/100	23.34	23.45	23.40	0.01	-2
1/1000	27.17	28.05	27.61	0.001	-3
1/10,000	30.02	30.51	30.27	0.0001	-4

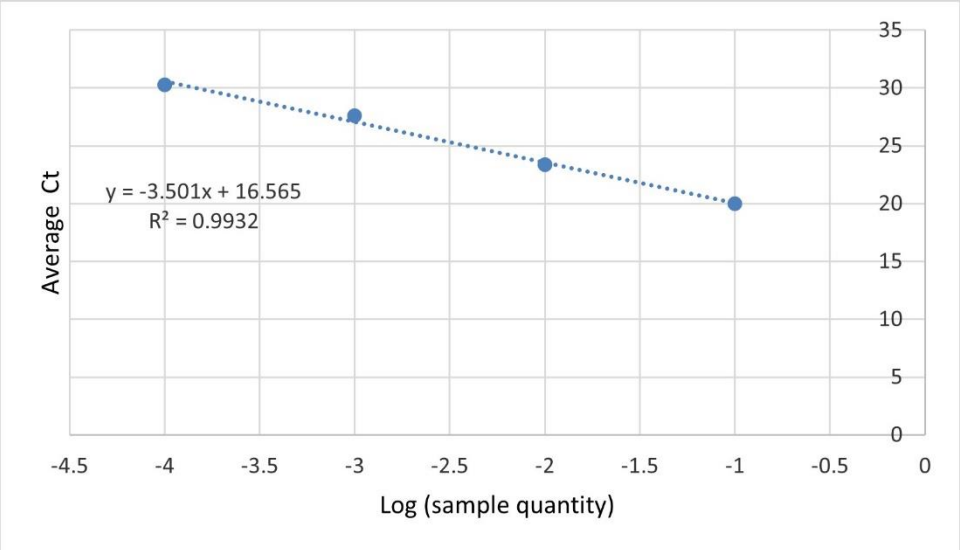


Figure 5.1: Graph to calculate GAD2 primer efficiency

Primer efficiency (%) = $(10^{(-1/\text{slope})} - 1) \times 100$

$= (10^{(-1/-3.501)} - 1) \times 100$

$= 93.03\ \%$

T**able 5.14:** Primer efficiencies of all primers used in this study

Primer	Primer efficiency (%)
GAD 1	92.8
GAD 2	93.03
Ubiquitin	90.7
Tubulin	99.1
AOX1a	90.5
AOX1d	97.3
Ndb2	92.4

5.2.4.0 Calculation for 1% HNO_3 preparation

Concentration of HNO_3 Stock used = 70 %

Number of samples = 48

Volume of 1% HNO_3 needed for 1 sample = 10 ml

Volume of 1% HNO_3 needed for 48 samples = 480 ml

Volume of 70% HNO_3 needed to prepare 500 ml of 1% HNO_3 = (1% x 500 ml) / 70%

= 7.2 ml

Add 7.2 ml 70% $HN0_3$ to 492.8 ml of non-autoclaved milli water.

5.3.0.0 Methods Elaborated

Table 5.15: Details of the salt experiments in genevestigator data

Experiment	Treatment	control
salt study 2 early (Col-0)	At day 16, stress treatment started at 3 hours of light treatment. Salt stress (150 mM NaCl) was applied. NaCl was added to a concentration of 150 mM in the Media. To add NaCl the raft was lifted out. A magnetic stir bar and a stirrer were used to mix the media and the added NaCl. After the rafts were put back in the boxes, they were transferred back to the climate chamber. Roots were harvested at 0.5, 1 and 3 h after onset of treatment.	No treatment; control of root samples for time-points 0.5, 1 and 3 h.
salt study 2 (late) (Col-0)	At day 16, stress treatment started at 3 hours of light treatment. Salt stress (150 mM NaCl) was applied. NaCl was added to a concentration of 150 mM in the Media. To add NaCl the raft was lifted out. A magnetic stir bar and a stirrer were used to mix the media and the added NaCl. After the rafts were put back in the boxes, they were transferred back to the climate chamber. Roots were harvested at 6, 12 and 24 h after onset of treatment.	No treatment; control of root samples for time-points 6, 12 and 24 h.

salt study 3 (Col-0)	Rosette leaf samples from 5-weeks-old Col-0 plants, treated with 250 mM NaCl for 24h.	Rosette leaf samples from 5-weeks-old Col-0 plants, treated with water for 24h.
salt study 4 (Col-0)	Leaf samples of Col-0 grown on soil for 14 days (10h light (350µmol photons m-2 s-1) at 23°C / 14h dark at 18°C; 70% relative humidity) and then watered for 6 days with NaCl, which was dissolved in ½ strength Hoagland solution. Plants were watered from below and sprayed with the same concentration of NaCl solution from above every 2 days, starting with 50mM NaCl and applying 100 mM NaCl during the next waterings.	Leaf samples of Col-0 grown for 14 days (10h light (350 µmol m-2 s-1) at 23°C / 14h dark at 18°C; 70% relative humidity) and then watered with ½ strength Hoagland solution (from below and sprayed from above) every two days for 6 days.
salt study 5 (Col-0)	Primary root tip (5mm long) samples of Col-0 grown for 5 days on solid standard medium (1x Murashige and Skoog salts, 0.5g/L MES, 1% sucrose, 1% agar; pH 5.7), then transferred onto solid standard medium supplemented with 140mM NaCl for 1h.	Primary root tip (5mm long) samples of Col-0 grown for 5 days on solid standard medium (1x Murashige and Skoog salts, 0.5g/L MES, 1% sucrose, 1% agar; pH 5.7), then transferred onto solid standard medium for 1h.

5.3.1.0 RNA Yield and the Purity Estimation using the Nanodrop 2000 Spectrophotometer

Concentration and the purity of RNA was determined using the NanoDrop™ 2000 spectrophotometer (Thermo Scientific™) prior to synthesis of cDNA. Nuclease-free water (1µl) was used to blank the instrument for system initiation. RNA settings were selected before measuring the samples in NanoDrop 2000 software 1.6.

5.3.2.0 Running an agarose gel

RNA was extracted from shoot and root tissues of *Arabidopsis thaliana* WT and XX1 under control and salt treated conditions. The gel was run to test quality of extracted RNA. The bands named 18S and 28S represent ribosomal RNA present in the samples (18S – 1.8 KB and 28S - 4.8 KB) and faded bands in the bottom might be contaminations or degraded RNA.

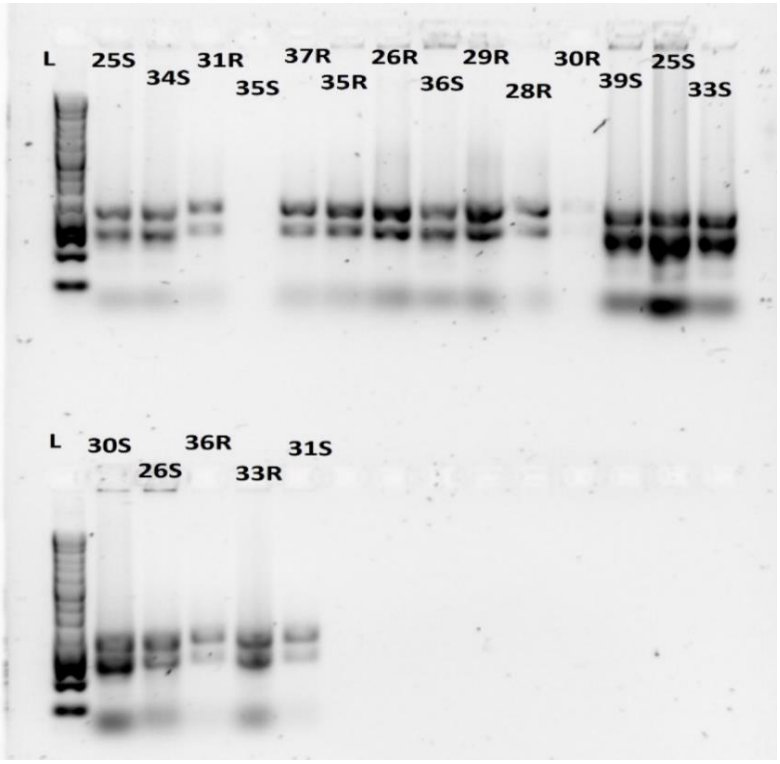


Figure 5.2: Gel image of the extracted RNA samples.

5.3.3.0 Determining of annealing temperatures of the primers

Annealing temperatures were known in AOX1a, AOX1d, Ubiquitin and Ndb2 primers from previous studies done in the lab using theses primers. The annealing temperatures of GAD1, GAD2 and tubulin were determined by a running a qRT PCR under a temperature gradient (50 ^{0}C to 60 ^{0}C). Best annealing temperature was decided by looking at quantification and melt peak graphs for each primer.

As an example, according to figure 5.3 A product amplification is higher in tubulin at 58 ^{0}C and at that temperature melt peak (FIGURE 5.3 B) also showed only one product formation since it has a single peak.

(A) (B)

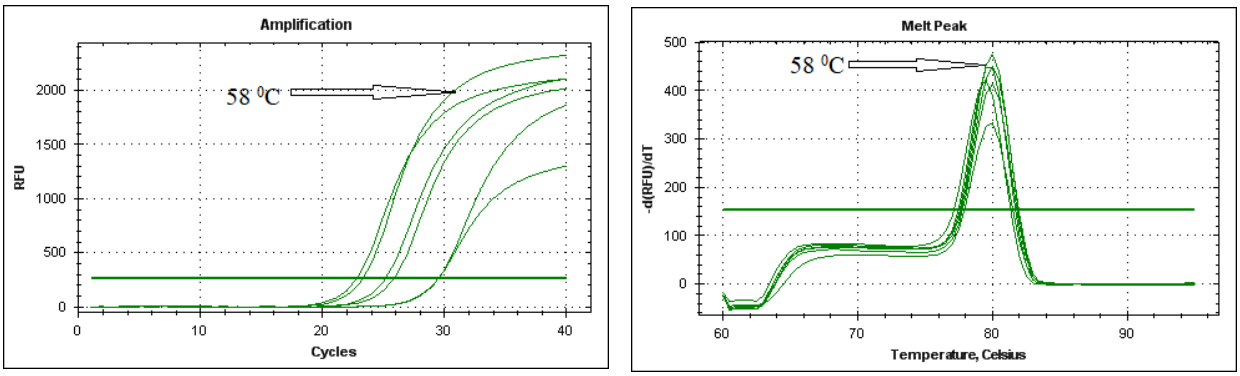


Figure 5.3: Data graphs from q-RT PCR for tubulin primer representing (A) quantification graph and (B) melt peak graph under temperature gradient (50 ^{0}C to 60 ^{0}C).

Table 5.16: Annealing temperatures of the primers

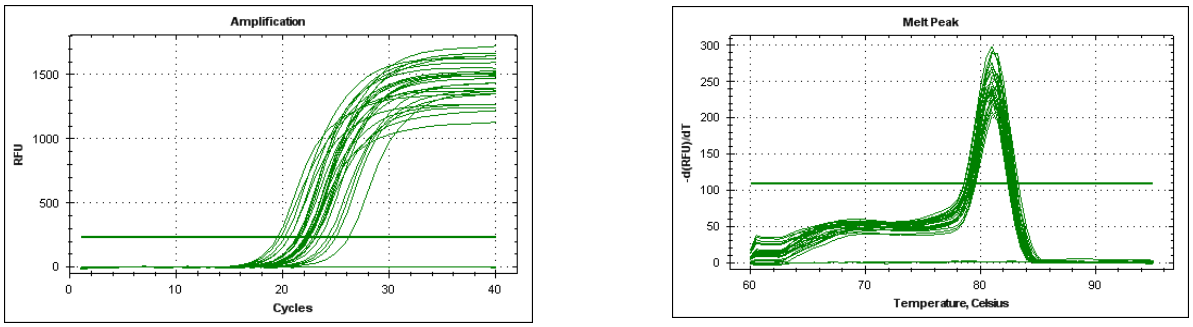
Primer	Annealing temperature
a-Tubulin F/R	58 ^{0}C
GAD1 F/R	60 ^{0}C
GAD2 F/R	60 ^{0}C

Ubiquitin F/R	60 ^{0}C
AOX1a F/R	60 ^{0}C
AOX1d F/R	60 ^{0}C
Ndb2 F/R	60 ^{0}C

5.3.4.0 Determine the accuracy of data received from q-RT PCR

Single peak in the (Figure B and D) demonstrate only one type of product formed in q-RT PCR that shows samples were free from genomic DNA contamination.

(A) (B)



(C) (D)

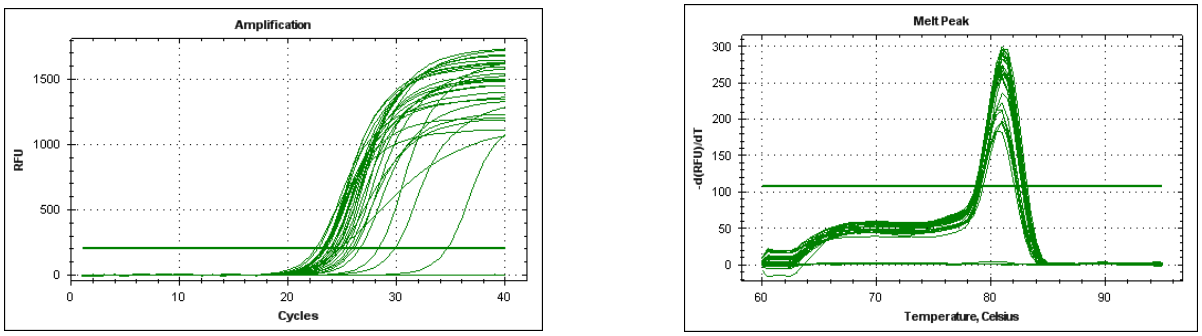
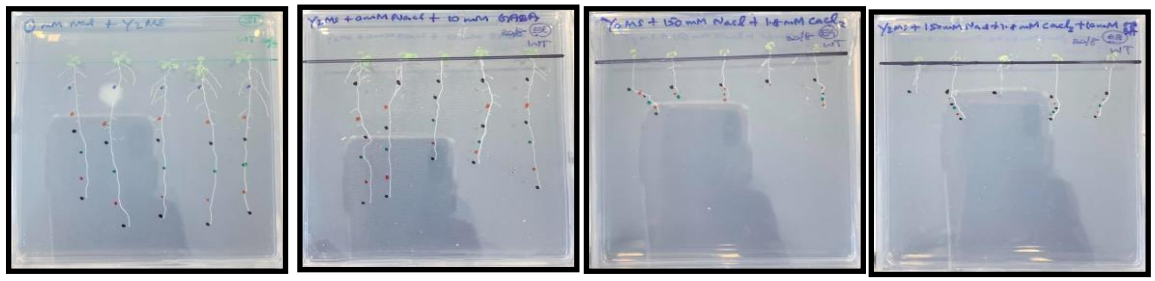
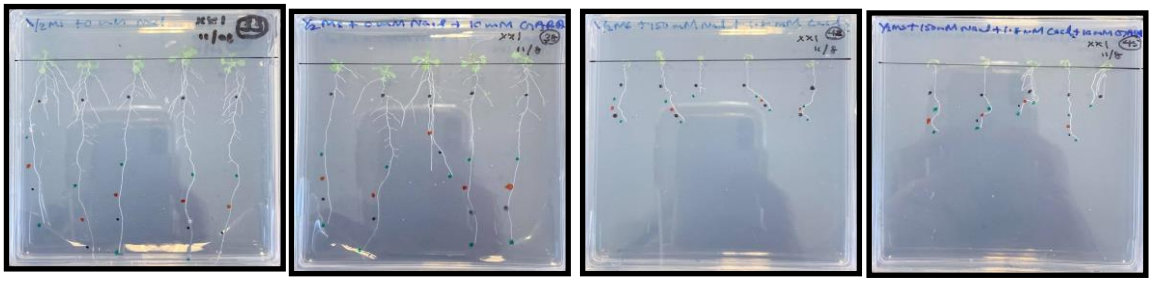


Figure 5.4: Represent quantification graphs of root (A) and shoot (C) and melt peaks of roots (B) and shoots (D) of *Ndb2* in control and salt treated samples of Arabidopsis thaliana WT and XX1.

5.4.0.0. Extra figures relevant for results



WT (C) WT (C+G) WT (S) WT (S+G)



XX1 (C) XX1 (C+G) XX1 (S) XX1 (S+G)

KO (C) KO (C+G) KO (S) KO (S+G)

Figure 5.5: Root growth of plants grown in plates under different treatments.

Arabidopsis thaliana wild type (WT), AOX1a over expressor (XX1) and aox1a knock out (KO) plants exposed to GABA, NaCl and combination of NaCl and GABA. Control (C), Control + GABA (C+G), Salt treated (S), Salt + GABA (S+G).

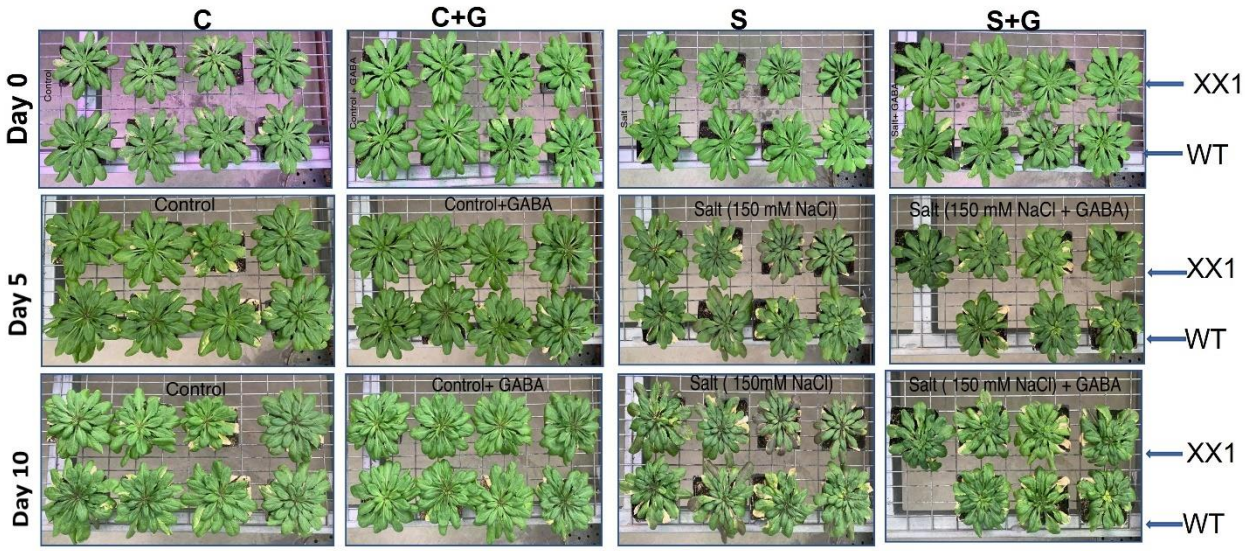
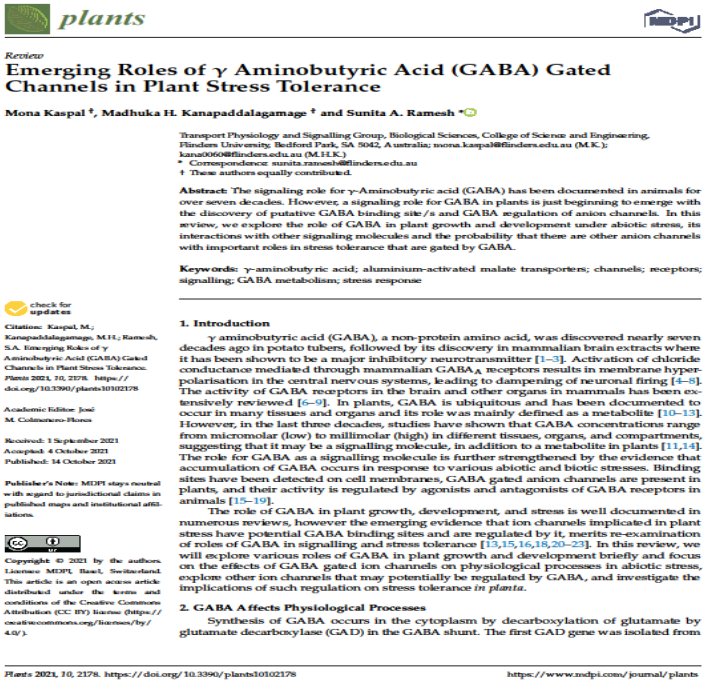


Figure 5.6: Plants subjected to exogenous GABA under control and salinity stress in soil. *Arabidopsis thaliana* wild type (WT), AOX1a over expressor (XX1) and aox1a knock out (KO) plants exposed to GABA, NaCl and combination of NaCl and GABA. Control (C), Control + GABA (C+G), Salt treated (S), Salt + GABA (S+G).

5.5.0.0. Publication

Review article on "Emerging Roles of γ Aminobutyric Acid (GABA) Gated Channels in Plant Stress Tolerance" (Mona Kaspal, Madhuka H Kanapaddalagamage, Sunita A Ramesh) in 'Plants".



plants

Review

Emerging Roles of γ Aminobutyric Acid (GABA) Gated Channels in Plant Stress Tolerance

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Abstract: The signaling role for γ-Aminobutyric acid (GABA) has been documented in animals for over seven decades. However, a signaling role for GABA in plants is just beginning to emerge with the discovery of putative GABA binding site/s and GABA regulation of anion channels. In this review, we explore the role of GABA in plant growth and development under abiotic stress, its interactions with other signaling molecules and the probability that there are other anion channels with important roles in stress tolerance that are gated by GABA.

Keywords: γ-aminobutyric acid; aluminium-activated malate transporters; channels; receptors; signalling; GABA metabolism; stress response

check for updates

Citation: Kaspal, M.; Kanapaddalagamage, M.H.; Ramesh, S.A. Emerging Roles of γ Aminobutyric Acid (GABA) Gated Channels in Plant Stress Tolerance. *Plants* 2021, *10*, 2178. https://doi.org/10.3390/plants10102178

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1. Introduction

γ aminobutyric acid (GABA), a non-protein amino acid, was discovered nearly seven decades ago in potato tubers, followed by its discovery in mammalian brain extracts where it has been shown to be a major inhibitory neurotransmitter [1–3]. Activation of chloride conductance mediated through mammalian $GABA_A$ receptors results in membrane hyperpolarisation in the central nervous systems, leading to dampening of neuronal firing [4–8]. The activity of GABA receptors in the brain and other organs in mammals has been extensively reviewed [6–9]. In plants, GABA is ubiquitous and has been documented to occur in many tissues and organs and its role was mainly defined as a metabolite [10–13]. However, in the last three decades, studies have shown that GABA concentrations range from micromolar (low) to millimolar (high) in different tissues, organs, and compartments, suggesting that it may be a signalling molecule, in addition to a metabolite in plants [11,14]. The role for GABA as a signalling molecule is further strengthened by the evidence that accumulation of GABA occurs in response to various abiotic and biotic stresses. Binding sites have been detected on cell membranes, GABA gated anion channels are present in plants, and their activity is regulated by agonists and antagonists of GABA receptors in animals [15–19].

The role of GABA in plant growth, development, and stress is well documented in numerous reviews, however the emerging evidence that ion channels implicated in plant stress have potential GABA binding sites and are regulated by it, merits re-examination of roles of GABA in signalling and stress tolerance [13,15,16,18,20–23]. In this review, we will explore various roles of GABA in plant growth and development briefly and focus on the effects of GABA gated ion channels on physiological processes in abiotic stress, explore other ion channels that may potentially be regulated by GABA, and investigate the implications of such regulation on stress tolerance *in planta*.

2. GABA Affects Physiological Processes

Synthesis of GABA occurs in the cytoplasm by decarboxylation of glutamate by glutamate decarboxylase (GAD) in the GABA shunt. The first GAD gene was isolated from