Characterization of the role of a cycloartenol synthase gene (ZMCAS494) in *Zea mays* responses to drought stress

By

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KEYWORDS

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Zea mays

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Phytosterol signalling

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Antioxidant enzymatic activity

ROS signalling

Cycloartenol synthase gene expression

ABSTRACT

Characterization of the role of a cycloartenol synthase gene (ZMCAS494) in *Zea mays* responses to drought stress

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Plant Sterols are an important part of the membrane and their role is to control cell membrane fluidity and permeability. Several studies have indicated that plants that are mutant for genes that play a role in the sterol biosynthetic pathway show increased sensitivity to drought stress. Even though the exact molecular mechanism is unknown, such studies suggest that sterols do play a role in plant drought stress responses. To elucidate the mechanism through which sterols regulate *Zea mays* responses to water deficit, the effect of drought stress on phytosterol content and on expression of a cycloartenol synthase-like gene (ZMCAS494) was investigated. The effect of inhibition of sterol biosynthesis, using terbinafine, on maize reactive oxygen species production and on activity of the superoxide dismutase, ascorbate peroxidase and glutathione peroxidase enzymes was also investigated. Quantitative polymerase chain reaction (qPCR) analysis of the expression of ZMCAS494 indicated a change in gene expression in response to drought. Inhibition of phytosterols synthesis resulted in altered ascorbate peroxidase activity, glutathione peroxidase activity as well as superoxide dismutase activity in roots and leaves of maize. The ROS content of maize leaves was increased by inhibition of phytosterols synthesis. Data obtained in this study suggests a relationship between sterol biosynthesis, sterol content and reactive oxygen species signalling.

DECLARATION

I declare that "*Characterization of the role of a cycloartenol synthase gene (ZMCAS494) in Zea mays responses to drought stress*" is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name..... Date.....

Signed.....

AIMS OF THE STUDY

- To examine the extent of water stress on maize leaves and roots by measuring the relative water content, fresh weight and length.
- To determine the effects of water deficit on ROS content and antioxidant enzyme activity of maize leaves and roots
- To determine the effect of drought on the expression of a sterol biosynthesis gene.
- To determine effect of drought stress on sterol content in maize roots and leaves
- To examine the impact of changes in sterol content on maize ROS production as well as sterol synthesis inhibition on ROS production and antioxidant enzyme activities.

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LIST OF ABBREVIATIONS

ABA – Abscisic acid

APX- Ascorbate peroxidase

ASC- Ascorbic Acid

BLS- Bureau for Labour Statistics

BR's- Brassinosteroids

CAT- Catalase

CAS- Cycloartenol synthase

CO_2 - Carbon dioxide

DMAPP- Dimethylalkyl diphosphate

EDTA- Ethylenediaminetetraacetic acid

ETC- Electron transport chain

FPP- Farnesyl pyrophosphate

FAO- Food and Agricultural Organisation

GDP- Gross domestic product

GSH- Glutathione oxidised

GSSG- Glutathione reduced

GPOX- Guaiacol peroxidase

GPX- Glutathione peroxidase

GR- Glutathione reductase

IPP- isopentenyl pyrophosphate

Kcal- Kilo calories

MAPKs- mitogen-activated protein kinases

MDA- malondialdehyde

MDHA- monodehydroascorbate (MDHA)

NAD(P)H- Reduced nicotinamide adenine dinucleotide phosphate

TEMED- N,N,N',N'-Tetramethylethylenediamine

OSC- Oxidosqualene Cyclase

PCD- Programmed cell dead

PCR- Polymerase chain reaction

PSI - Photosystem I

PSII- Photosystem II

PUFA- Polyunsaturated fatty acids

PVP- Polyvinylpyrolidone

RWC – Relative water content

ROS – Reactive oxygen species

SAFEX- South African Features Exchange

SADC- Southern African Development Community

SOD- Super oxide dismutase

SQE- Squalene epioxidase

SQS- Squalene cyclase

TCA- Trichloroacetic acid

TFBS-Transcription factor binding sites

UN- United Nations

USA-United States of America

USDA- United States Department of Agriculture

XOD- Xanthine oxidase

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CHAPTER 1: LITERITURE REVIEW

1.1 Introduction

Together with other economy driving sectors, agriculture contributes significantly to the South African economy. Changes in the environment that are not conducive to agricultural practises tend to impact negatively on the economy. Insufficient water availability as a result of drought, mismanagement of natural water resources as well as a drastically growing world population is an example of one such change and it affects agricultural crop productivity negatively. Therefore, there rises a need for research that seeks ways to combat crop losses as a result of water unavailability. This study will investigate the role of phytosterols in responses of *Zea mays* to drought stress.

Zea mays, commonly referred to as maize, is a monoecious grass that belongs to the *Poaceae* family (Strable & Scanlon, 2009). It is amongst the top 3 most widely produced cereal crops in the world (Verheye, 2010). Maize cobs and kernels are rich sources of nutrients and starch (Ranum, Peña-Rosas, & Garcia-Casal, 2014; Strable Josh & Scanlon Michael J., 2009). Kernels are used as food and can be ground to make maize meal and flour (Australian Government Office of the Gene Technology, 2008). Aside from it being a staple food source in various regions of the world, maize is also used for ethanol production (Ranum et al., 2014). Forage and silage from maize are used as animal feed (Australian Government Office of the Gene Technology, 2008). Its many uses enable the crop to contribute a lot to the agricultural economy of Southern Africa. Owing to its large and diverse genome as well as a relatively short generation time, maize is also suitable model organism for plant genetics (Strable & Scanlon, 2009).

Maize requires specific environmental conditions for optimum growth and development. Environmental parameters such as temperature, radiant energy, soil structure and aeration, nutrient supply, biotic parameters as well as water supply or availability act together to influence maize growth and development (Strable & Scanlon, 2009). Maize changes its physiology and biochemical processes as means to survive unfavourable changes in these environmental parameters. Such changes usually result in yield and productivity losses. When crop plants that are of agricultural importance, such as wheat, rice and maize are affected by drought, food security and food supply become threatened. An improved understanding of the biochemical and genetic responses to drought can lead to the development of plants that are able to tolerate drought without yield or productivity losses.

Plants respond to water deficit through changes in gene expression, protein expression and production of metabolic compounds (Bray, 2001). These changes occur as a result of activation of various signalling pathways (Bray 2001; Xu et al., 2010). An improved understanding of such pathways is required in order to understand how plants respond to environmental stresses such as drought. Reactive oxygen species (ROS) are highly reactive oxygen-containing compounds that are produced by plants during normal metabolism (Gill & Tuteja 2010; Bailey-Serres & Mittler 2006; Karuppanapandian et al., 2011; Mittler 2002). They include hydrogen peroxide (H_2O_2), superoxide radical (O_2^-) and the hydroxyl radical (OH^-). They function as stress signalling molecules and are detrimental to plants when produced at high levels(Gill & Tuteja 2010; Bailey-Serres & Mittler 2006; Karuppanapandian et al., 2011; Xu et al., 2010; Baxter et al., 2014).

Drought and other abiotic stresses cause an overproduction of ROS (Karuppanapandian et al., 2011; Mittler, 2002). To combat this, plants produce metabolites that detoxify ROS and enzymes that catalyse ROS detoxification reactions. Ascorbate peroxidase (APX), glutathione

peroxidase (GPX) and superoxide dismutase (SOD) are some of the enzymes responsible for ROS scavenging (Gill & Tuteja 2010; Bailey-Serres & Mittler 2006; Karuppanapandian et al., 2011; Xu et al., 2010; Baxter et al., 2014). Understanding the mechanisms and pathways that affect ROS signalling will result in an improved understanding of crop responses to stress and development of strategies to improve plant stress tolerance.

In plants, ROS signalling is affected by various other pathways and signalling molecules such as nitric oxide production (Baxter et al., 2014; Carvalho 2008; Pei et al., 2000; Lin et al., 2009). Recently, there have been developments on the role of plant sterols in drought stress responses and efforts to elucidate the role of sterols in drought stress responses have resulted in a relationship between phytosterols and ROS being proposed (Xia et al., 2009; Posé et al., 2009a). Plant sterols are an important part of the membrane and their role is to control cell membrane fluidity and permeability (Piironen et al., 2000; JECFA 2008; Ohyama et al., 2009; Corey et al., 1993). Several studies have indicated that plants that are mutant for genes that play a role in the sterol biosynthetic pathway show increased sensitivity to drought stress and changes in ROS production (Clause 2002; Schaller 2003; Vriet et al.,2012; Hasan et al., 2011; Elmore & Coaker 2011; Lin et al., 2009). This study aims to elucidate the involvement of sterols in regulating *Zea mays* responses to water deficit and the effect of changes in sterol content on ROS production and detoxification.

This chapter will give an overview of drought as a concept and its effect on plant growth and development. The effect of drought maize productivity with specific reference to South Africa, will be discussed. The role of ROS in plant stress responses and in plant cell signalling will be discussed. Literature on phytosterol biosynthesis, their roles in plant growth and development will be reviewed. The role of phytosterols in plant stress signalling and their effect on ROS production will also be reviewed.

1.2 Importance of Maize

Maize is believed to have originated in central Mexico 7000 years ago from a wild grass. (Ranum et al., 2014). It is one of the top three largely produced cereal crops globally and is cultivated in many different parts of the world with the United States, China, and Brazil being the top three maize-producing countries, producing approximately 563 of the 717 million metric tons/year. (Ranum et al., 2014; Verheye, 2010). According to the Food and Agricultural Organization (FAO) of the United Nations (UN) report, global maize production in 2015, which makes up 80% of the world's coarse grains output, was estimated at 1007 million tonnes. Production of maize in The United States of America (USA), the world's largest producer, for 2015 was estimated at 345 million tonnes(United States Department of Agriculture 2016). In Southern Africa, drought conditions at the beginning of 2015 resulted in a sharp yield reduction. Dry weather conditions resulted in reduced outputs in most countries of the Southern African region compared with the 2014 levels. On average, the region's 2015 output was estimated at 20.5 million tonnes. South Africa, the region's largest producer, experienced a 30% reduction, leaving production estimates at 10.5 million tonnes (Bureau for Food and Agricultural Policy, 2015). The crop is largely and widely produced because of its many economic and research benefits.

Maize contains about 72% starch, 10% protein, and 4% fat, supplying an energy density of 365 Kcal/taking, making it a major source of nutrients in many regions of the world (Ranum et al., 2014), especially in developing countries and the Southern African region. Maize consumption in this region ranges between 52 and 328 g per person per day (Ranum et al., 2014). Maize can be processed into a variety of food products, including starch, sweeteners, oil and beverages. It can also be used as animal feed. This makes it an agricultural commodity that contributes greatly to the economy, especially that of developing countries in the Southern African

Development Community (SADC) region. Maize accounts for 14% of agricultural gross domestic product (GDP) of Zimbabwe. The crop is grown by over 90% of Zimbabwean subsistence farmers and its contribution to the GDP is an underestimation of its actual contribution to the country's economy (Zimbabwe Multi Donor Trust Fund, 2010) . Maize accounts for about 12 % of the agricultural gross domestic product (GDP) in Kenya and similar contribution to the agricultural economy of other SADC countries (Kang'ethe, 2011). According to an economic review issued by the Department of Agriculture, Forestry and Fisheries, income from maize was R27 525 million, 14.5 % higher than the previous year and almost 50% of the gross income from all field agricultural crops in South Africa in 2014 (Department of Agriculture Forestry and Fisheries, 2014).

Developing countries rely more on maize as a food source while countries with better developed economies process the crop to produce industrial products such as glue, industrial alcohol, and fuel ethanol (Ranum et al.,2014). For the past 10 years, the use of maize for fuel production has significantly increased, accounting for approximately 40% of the maize production in the United States (Ranum et al., 2014). As the ethanol industry absorbs a larger share of the maize crop, higher prices for maize will strengthen demand competition and could affect maize prices for animal and human consumption (Ranum et al., 2014). Low production costs, along with the high consumption of maize, especially in countries where micronutrient deficiencies are common public health problems, make this food staple an ideal food vehicle for fortification (Strable & Scanlon , 2009).

Several factors affect maize production and thus result in decreased production in different regions of the world. The Southern African region is the world's biggest consumer of maize and yet its production estimates are quite low. The region relies on maize imports from countries such as Brazil and America to meet its high demands. Consequently, maize and maize

product prices are much higher than they would be if the region produced enough maize to meet its demands. Global production seems high (1007 million tonnes in 2015) yet when compared to global consumption of or demand (USDA, 2015) it becomes clear that the world needs to produce more maize in order to maximise the gap, to prevent future crop shortfalls. Maize prices have increased significantly over the past few years. This and the thin gap between demand and supply indicate that there is a problem with global maize production. Factors that affect maize crop productivity have to be studied extensively and understood in order to find strategies to improve global maize yields and protect the crop.

Various abiotic and biotic environmental stresses have a major impact on maize productivity in the field. Abiotic stresses such as drought, salinity, extreme temperatures, chemical toxicity and oxidative stress are serious threats to agriculture and result in the deterioration of the environment and major crop losses. According to Boyer (1982), abiotic stress reduces average yields for most major crop plants by more than 50% and maize yields can be reduced by almost 60%. Drought, caused by climate change and water shortages as a result of socio-economic factors such as over-population and mismanagement of water resources is the main factor limiting crop production (Golbashy et al., 2010). Drought is a major limitation to maize crop productivity in developing countries and an occasional cause of losses of yields in developed ones (Ceccarelli & Grando, 1996).

1.3 The effects of drought on maize and implications for food security

1.3.1 Drought Definition

Drought refers to a prolonged period of water deficit, that has adverse effects on plants, animal and human lives. It results from prolonged periods of abnormally low rainfall and climatic changes such as extensive heat and reduced precipitation. The extent to which it occurs and its effect differs between regions of the earth. Long periods of water deficit are caused by natural climate changes but they also result from the impact of humans on the environment, such as overpopulation, industrial activity and mismanagement/use of water resources.

Drought affects various economic and social sectors differently and as a consequence, varying definitions have been developed by range of disciplines, based on its impact on the economy, socially and on the environment. Meteorological drought refers to the extent of dryness or the extent of lack of precipitation in comparison to a defined average amount of precipitation over a period of time (Wilhite and Glantz, 1985). Climate conditions that result in lack of precipitation vary between regions of the world. Meteorological drought is believed to be region-specific.

Hydrological drought occurs when there is insufficient water both above and below the earth surface in a specific region such that day to day operations are affected.(Hisdal, 2000; Mniki, 2009; Olaleye, 2010). Socio-economic drought occurs when the demand for water in an economy or society exceeds the supply due to a combination of climate-dependant water deficit as well as socio-economic factors such as a decline in the economy, overpopulation and mismanagement of water resources (Olaleye, 2010). Agricultural drought occurs when there is

deficiency of water in the soil, such that needs of a particular crop are not met. Agricultural drought affects plant growth and development and thus crop yield. This study focuses on maize responses to drought, therefore, drought in the context of agriculture.

1.3.2 Impacts of drought events on maize crop yields and production

Drought exerts severe pressure on agricultural economies of developing countries like South Africa as it affects yield and productivity of agriculturally important crops. When agricultural productivity is affected, various sectors of the economy, livestock and human lives become affected negatively. Maize, for example, is sensitive to variations in rainfall, as an extended dry period can result in lower grain formation and a decreased maize yield (Clay et al., 2003) When staple foods such as maize become affected by droughts, food security becomes an issue of concern as much of the hunger of the world is related to highly variable rainfall, especially in rural areas (UNDP-BCPR, 2005). Supply of foods made from agricultural crops decreases during drought events and as a result, food prices increase (Austin, 2008). The effects of drought are felt differently by different countries, with developing countries like South Africa suffering the consequences more than first world countries.

Until recently, the drought during 1991/92 was known to be one of the worst drought events South Africa has ever experienced due to its effect of food security (Vogel, 1995). Rural areas suffered a poor supply of food, as a consequence of extreme food price increases and soil degradation which lead to an extended reduction in crop productivity (Vogel et al.,2000). The drought had a very damaging impact on commercial agriculture through a reduction in rain-dependent crop yield and reduced availability of water for irrigation. South Africa needs 6.5 million tons of maize yearly, yet the crop estimate for 1992 was 2.4 million tons. Maize had to

be imported to meet the country's demand. Import costs were approximately R2.2 billion and food prices had to increase in order to cover import costs.

In 2015, the maize producing regions experienced remarkably challenging weather conditions (figure 1.1) causing yields to decrease by approximately 30%, with the greatest impact in the Free State and North West provinces, where more white maize is traditionally produced. Maize output was about 14.3-million tonnes in 2015. This is expected to shrink to 9.8-million tonnes in 2016, a 31% drop. On 30 September 2015, the mark-to-market price for white maize to be delivered in October 2015 amounted to R3 159/tons compared to R1 761/tons (up by 79%) the same time in 2014. For yellow maize, the price amounted to R2 924/tons compared to R1 770/tons (up by 65%) the same time the previous year. Figure 1.2 illustrates maize price trends according to the South African Features Exchange (SAFEX) as well as yellow and white maize production reduction between 2014 and 2015.

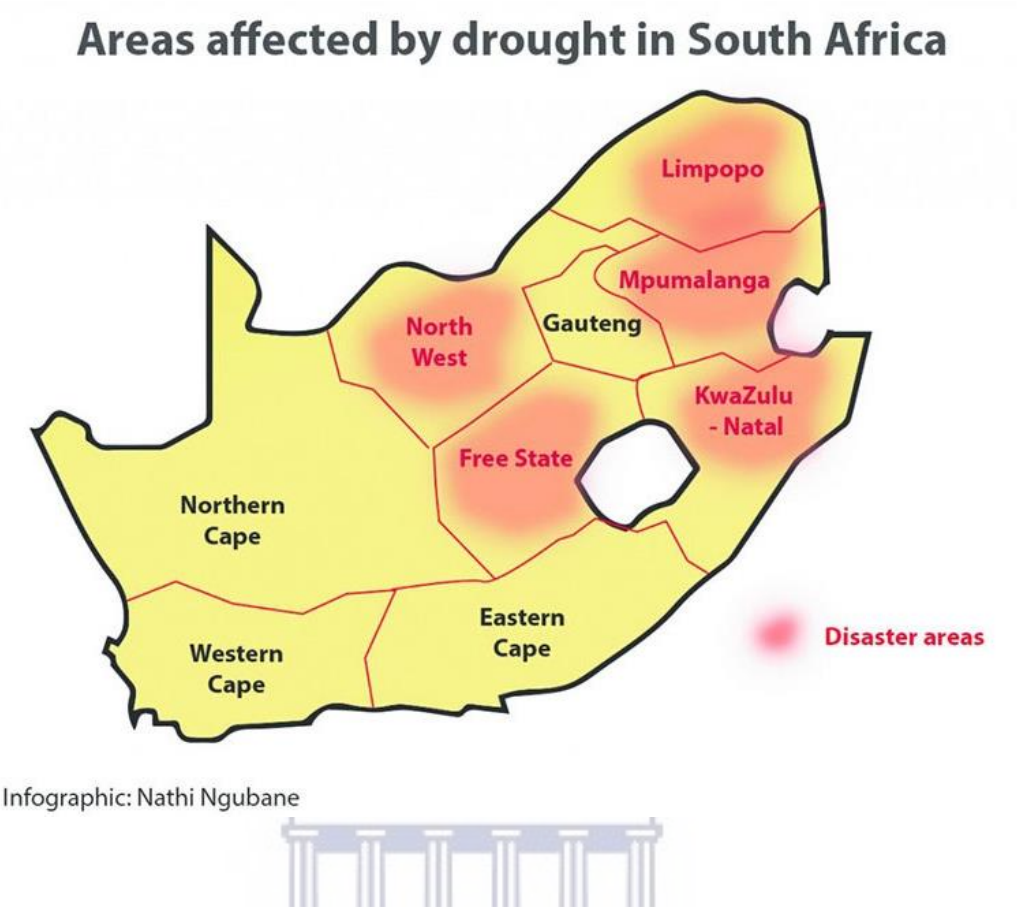


Figure 1.1: Areas affected by drought in 2015 in South Africa. Recent drought events mostly affected the maize producing regions of South Africa. (Tau, 2016)

In 2012, the worst drought since the 1950s had affected 84% of maize growth in the United States (USDA, 2012a). In July, the USDA declared more than 1000 USA counties to be drought disaster areas, and maize prices increased by nearly 23% (USDA, 2012b). U.S. export prices for maize soared nearly 128 percent above the 20-year historical average, as measured by the Bureau of Labour Statistics (BLS) monthly export price index. About 40 % of U.S. maize is for ethanol production. Maize growth reductions during the drought meant that there wasn't sufficient maize production to meet the demand for ethanol production and this had dire effects on the US economy.

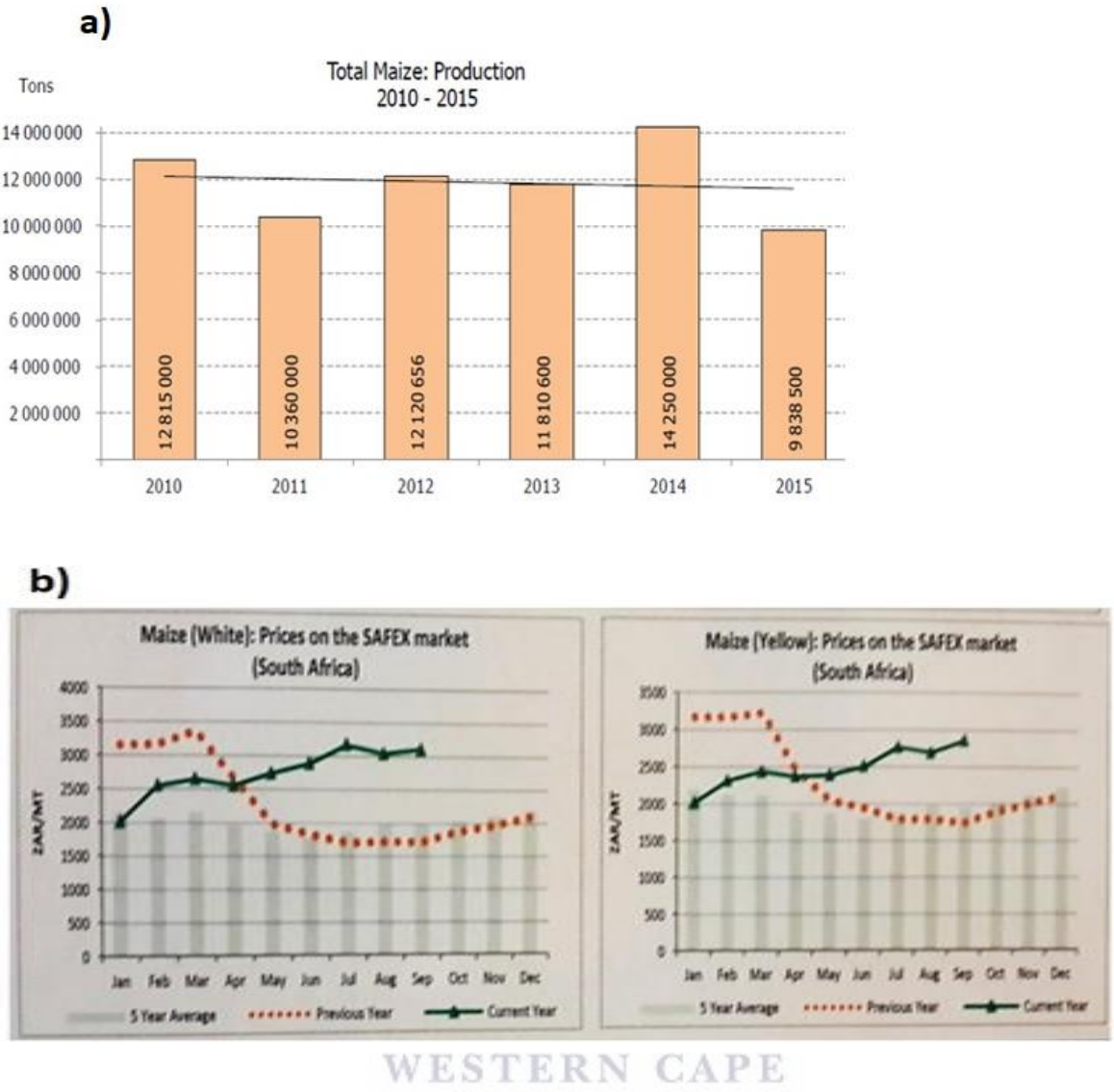


Figure 1.2: Total maize production between the years 2010 and 2015 in South Africa (a) and maize prices according SAFEX between 2014 and 2015(b).

During drought events, the area planted for agriculturally important crops such as maize is reduced and as a result, maize productivity gets reduced. For commercial farmers, low crop yields indicate that income will be lower and there will be no need for casual workers during harvest. Employment in the agricultural sector becomes scarce and the general rate of unemployment increases as a result of this. The gap between maize supply and demand

increases during drought periods, forcing reliance on imports of the crop and its products and thus causing an increase in food prices. Reduced crop yields also result in decreased availability of food for livestock. Farmers then resort to slaughtering livestock and selling meat at cheap prices. During drought periods, meat becomes the only affordable form of food. This however, results in meat shortages in the long run and an eventual increase in meat prices. Challenges such as these can be prevented by developing maize varieties that can better tolerate drought stress. For development of such plants, the effects of drought on maize and methods that plants use to tolerate and survive drought need to be studied.

1.3.3 Maize responses to drought

Plants under natural and agricultural conditions are exposed to stress constantly and maize is no exception. Drought limits plant growth and field crops production more than any other environmental stresses (Zhu, 2002). Drought tolerance of plants can be characterized by growth responses, changes in water relations of tissues exposed to low water potential, an increase of ions in tissues and stomatal conductance of leaves (Dash & Mohanty, 2001). Metabolite content as well as gene expression are also affected by drought stress. These responses occur as a consequence of a complex combination of signalling pathways. Understanding how plants respond to drought stress and elucidation of these signalling pathways is required in order to know how drought tolerance comes about and to develop crop plants that are able to tolerate and grow under drought conditions such that yields are not affected. In this section, cellular signalling in response to drought will be discussed.

Plants deal with water stress through various complex mechanisms. They either escape drought by completing their life cycle before severe stress occurs, or avoid drought by enhancing their capacity to absorb and retain water. Plants also tolerate drought by improving osmotic

adjustment ability and modifying cell wall elasticity to maintain turgidity. Drought resistance through altering the metabolic pathways responsible for life survival under severe abiotic stress (e.g., increased antioxidant metabolism) also occurs as a means to deal with stress in plants. These responses occur as a result of a series of morphological, physiological, biochemical and molecular changes (Anjum et al., 2011) in plants. Drought stress causes stomatal closure and reduced transpiration rates, a reduction in the water potential of plant tissues, reduction in photosynthesis and growth inhibition, increase in abscisic acid (ABA) levels , proline, mannitol, sorbitol, formation of radical scavenging compounds (ascorbate, glutathione, α-tocopherol etc.) and synthesis of new proteins and mRNAs (Yordanov et al., 2003).

Changes in relative water content (RWC) occur in plants as a result of changes in tissue growth, tissue turgor, stomatal conductance and cell damage (Yamasaki & Dillenburg, 1999). Relative water content measures the plant's water content and is used to determine the extent of water loss from tissue as a result of water deficit. A study conducted by Li-ping et al. (2006) to determine the effect of water stress on relative water content showed that severe water stress causes a significant decrease in RWC of maize leaves during the tasseling and silking stages of development. This decrease was linked to disrupted membrane permeability as a result of cell membrane damage caused by drought stress. This was demonstrated by measuring malondialdehyde (MDA) levels in leaves of water deficit-stressed plants versus well-watered plants. Levels of MDA were lower in well-watered plants compared to water-stressed plants. MDA is a product of lipid peroxidation, a cell damaging process that leads to programmed cell death and occurs as a result of oxidative damage caused by abiotic stresses such as drought (Li-ping et al., 2006).

Oxidative damage of cellular components in plants occurs as a result of excessive production of ROS in plants that are experiencing severe drought stress. One of the physiological responses to drought is stomatal closure, which reduces the amount of carbon dioxide (CO_2) entering

plant tissue. When this occurs, the ratio of CO_2 to oxygen in plants is altered, resulting in disruption in photosynthesis and an imbalance in the amount of CO_2 and oxygen produced. As a result, excessive oxygen (O_2) is prone to reduction and highly reactive versions of oxygen are produced. (Gill & Tuteja 2010; Carvalho 2008; Park et al., 2007).

1.4 Reactive oxygen species

As sessile organisms, plants have evolved various mechanisms to cope with drought stress. One such mechanism is the ability to close the stomata in order to reduce the rate of photosynthesis. Stomatal closure limits the amount of CO_2 entering the leaf guard cells and thus disrupts photosynthesis. As a consequence, the amount of oxygen inside the plant accumulates and eventually causes excessive reduction of oxygen and production of ROS. These (ROS) are moderately reduced forms of O_2 that result from reduction of O_2 to form singlet oxygen (O_2^1), the superoxide radical (O_2^-), the hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2).

One of the major sites for ROS synthesis in plants is the thylakoid membrane-bound primary electron acceptor in the chloroplast. Here, superoxide radicals are continuously produced during photosynthesis as a result of the partial reduction or energy transfer to oxygen (Gill & Tuteja, 2010). Superoxide radicals are usually the first ROS produced and may trigger the generation of other ROS including $^{\cdot}OH$ and $^1O_2$, which result in oxidative damage to membrane lipids and a general weakening of the cellular structure. The reaction of superoxide radicals with iron (Fe^{3+}) produces reduced iron (Fe^{2+}), which can reduce H_2O_2 to O_2^- and OH^- (Gill & Tuteja, 2010). According to (Krieger-Liszkay, 2005), $^1O_2$ is another ROS that is generated during photosynthesis but its formation is not a result of electron transfer to oxygen but rather insufficient energy dissipation during photosynthesis, which leads to the formation of the

chlorophyll triplet state. The chlorophyll triplet state is a lower energy state which has a longer half-life and can react with $3O_2$ to generate $^1O_2$, which has been proven to severely damage both photosystem I (PSI) and photosystem II (PSII) and the photosynthetic apparatus in general. $^1O_2$ is considerably damaging to plants because of its ability to oxidize a wide range of biological molecules and react with proteins, nucleic acids and lipids. $^1O_2$ is also thought to be responsible for light-mediated loss of PSI activity, which leads to cell death (Krieger-Liszkay et al., 2008).

Generated as a result of the univalent reduction of oxygen, H_2O_2 is a moderately reactive oxygen species with a longer half-life (1 ms) as compared to other ROS (2-4 μs). H_2O_2 causes oxidative damage, when present at high concentrations, by inactivating enzymes via oxidation of the thiol groups (Tewari, Kumar, & Sharma, 2006). Aside from its destructive capabilities, H_2O_2 also acts as a signalling molecule when it is present at homeostatically favoured concentrations and is a regulator of various physiological processes including senescence, photorespiration and photosynthesis. The use of H_2O_2 as a signalling and regulatory molecule can be attributed to its relatively long half-life and high permeability across membranes (Gill & Tuteja, 2010; Quan et al., 2008).

The most reactive ROS is ˙OH which can be generated from O_2^- and H_2O_2 by the Fenton reaction in the presence of iron (Gill & Tuteja, 2010).˙OH can react with all biological molecules and there is no characterized enzymatic mechanism that can be used to keep ˙OH concentrations in a steady state equilibrium, as a result increased concentrations of ˙OH always result in cell death (Gill & Tuteja, 2010).

Three organelles have been identified in plants to be the main sources of ROS generation. These are the chloroplast, the peroxisome and the mitochondria (Sharma et al., 2012). As a

result of this, photosynthetic plants are prone to oxidative damage because of the presence of the chloroplasts in their cells.

1.4.1 ROS Production in the Chloroplast

Photosynthesis is the process by which plants capture light energy from the sun and use it to produce triose phosphate and release oxygen and it occurs in the chloroplast. The chloroplast is structured to allow for optimal light harvesting as it contains the thylakoid membrane system which is highly organised and houses all the components required for the capturing of light (Pfannschmidt, 2003). Due to the nature of the chloroplast's function, $^1O_2$ is generated as a natural by-product of photosynthesis and it is produced by PS II even in conditions of low light. There are various routes for $^1O_2$ production in the chloroplast, as depicted in Figure 1.3. $^1O_2$ can be produced during photosynthesis when oxygen accepts electrons that have been passed through the photosystem (Gill & Tuteja, 2010), alternatively when the electron transport chain becomes overloaded the flow of electrons may be partially diverted from ferredoxin to O_2 (Rinalducci et al., 2008).

Electron leakage at the acceptor side of PS II is another means of $^1O_2$ generation. Here, electrons that have leaked from the PS II are accepted by O_2 and $^1O_2$ is produced. Superoxide radicals are also generated in the chloroplast, this however only occurs under conditions of high light intensity and insufficient CO_2 supply (Triantaphylides et al., 2008). Dismutation of O_2^- by SOD in the thylakoid membrane then leads to the generation of H_2O_2 and O_2. The generated H_2O_2 is then detoxified to produce H_2O by the anti-oxidant activity of APX. Although the chloroplast is a significant site for ROS production under stress conditions, certain plants have evolved mechanisms to use this to their advantage by linking chloroplast-generated ROS to the hypersensitive response (Mur et al., 2008)

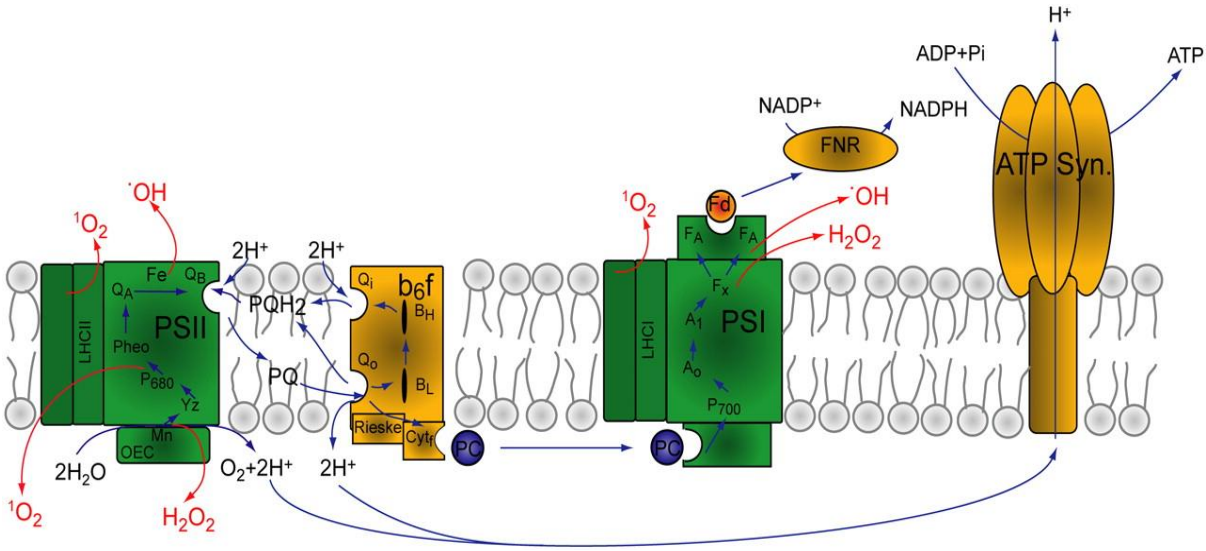


Figure 1.3 Schematic representation of the electron transport system in the thylakoid membrane showing three possible sites of ROS formation. Electron transfer reactions are indicated by blue arrows and ROS-generating reactions are indicated by red arrows. The two photosystems (PSI and PSII) are the major sites for production of ROS in chloroplasts. Exposure to light causes transfer of electrons towards O_2 and as a result superoxide (O_2^-) is produced. A membrane attached SOD near PSI converts O_2^- and forms H_2O_2. Excited chlorophyll in its triplet state at the light harvesting complexes (LHCI and II) can also generate singlet oxygen ($^1O_2$) when the electron transport chain is over reduced. Electrons are transferred from PSII to cytochrome b6f through plastoquinol molecules (PQ and PQH_2). Cytochrome b6f contains four b-type cytochromes (bH, bL, b6, and heme x), one c-type cytochrome f, and one 2Fe-2S cluster. Cytochrome b6f is the major proton pump that creates the potential gradient that is utilized by ATP synthase to produce ATP. Plastocyanin (PC), a Cu protein, transfers electrons from cytochrome b6f to PSI. In PSI, three 4Fe-4S clusters (FX, FA, and FB) participate in the electron transfer chain. Electrons from PSI are transferred to ferredoxin (Fd), a 4Fe-4S soluble protein. Figure adapted from Shcolnick & Keren (2006).

1.4.2 ROS production in the peroxisome

Peroxisomes are small, spherical organelles that are enveloped by a single lipid bilayer membrane. Their metabolism produces O_2^- as a natural by-product. These organelles function in the degradation of branched amino acids via photo morphogenesis, the biosynthesis of jasmonic acid and auxin as well as the production of glycine betaine (Gill & Tuteja, 2010; Hu, 2007). According to Corpas et al. (2001), there are two main sites for ROS production in the

peroxisome, the first of these sites being the organelle matrix where xanthine oxidases are responsible for the generation of superoxide radicals. The superoxide produced in the organelle matrix then becomes the precursor for the generation of H_2O_2 and O_2 by superoxide dismutase which scavenges the superoxide. Removal of H_2O_2 from the system is then catalysed by catalase under conditions of steady state equilibrium. The second site of ROS generation in the peroxisome is the peroxisomal membrane which houses an NAD (P) H-dependent O_2^- production site in the form of a small electron transport chain (ETC) that uses oxygen as an electron acceptor. Direct production of H_2O_2 also occurs at the peroxisome, via the photorespiratory glycolate oxidase reaction, where carbon is fixed to be used in the oxygenation of ribulose-1, 5-bisphosphate (Hofmann, 2011; Willekens et al., 1997).

Peroxisomal H_2O_2 generation may also occur as a result of fatty acid β-oxidation, which is the metabolism of triacylglycerols composed of mainly long chain fatty acid and are found mostly in storage organs such as endosperms and cotyledons. H_2O_2 generation by fatty acid β-oxidation occurs mainly in glyoxysomes, which are specialized peroxisomes dedicated to the degradation of fatty acid (Hayashi et al., 2002). Production of superoxide radicals and the functioning of antioxidant systems occur at two sites in the peroxisome. The first site is the organelle's matrix where the oxidation of xanthine by xanthine oxidase (XOD) liberates uric acid and O_2^- . The second site of ROS production is in the peroxisomal membrane where a small ETC composed of three membrane polypeptides namely PMP29, Cytochrome b and monodehydroascorbate reductase (MDAR) leads to production of O_2^-.

1.4.3 ROS production in plant mitochondria

Mitochondria are the organelles responsible for the production of energy in plants cells. They contain ETC components which enable them to produce energy and drive processes such as respiration. Mitochondria in plants are considered to be a major site for ROS generation as the ETC in the mitochondria contains electrons with enough free energy to facilitate the reduction of oxygen (Rhoad et al., 2006).

Due to the presence of the ETC, ROS are produced in mitochondria under normal respiratory conditions. However, this ROS production becomes intensified in response to biotic and abiotic stress conditions. The main sites of ROS production in mitochondria are complex I and complex III. These complexes generate a small amount of O_2^- as a side product of electron transport during oxidative phosphorylation (Camello- Almaraz et al., 2006). In complex I, O_2^- is released to the matrix and it is released to both the matrix and the intermembrane space in complex III. Cycling of the electron acceptor ubiquinone-which can donate electrons to molecular oxygen in both the internal and the external face of the inner mitochondrial membrane-in complex III results in formation of O_2^-. Once O_2^- is generated, it is then dismutated by SOD to form H_2O_2. The ROS production pathway in mitochondria is illustrated in Figure 1.3.3.

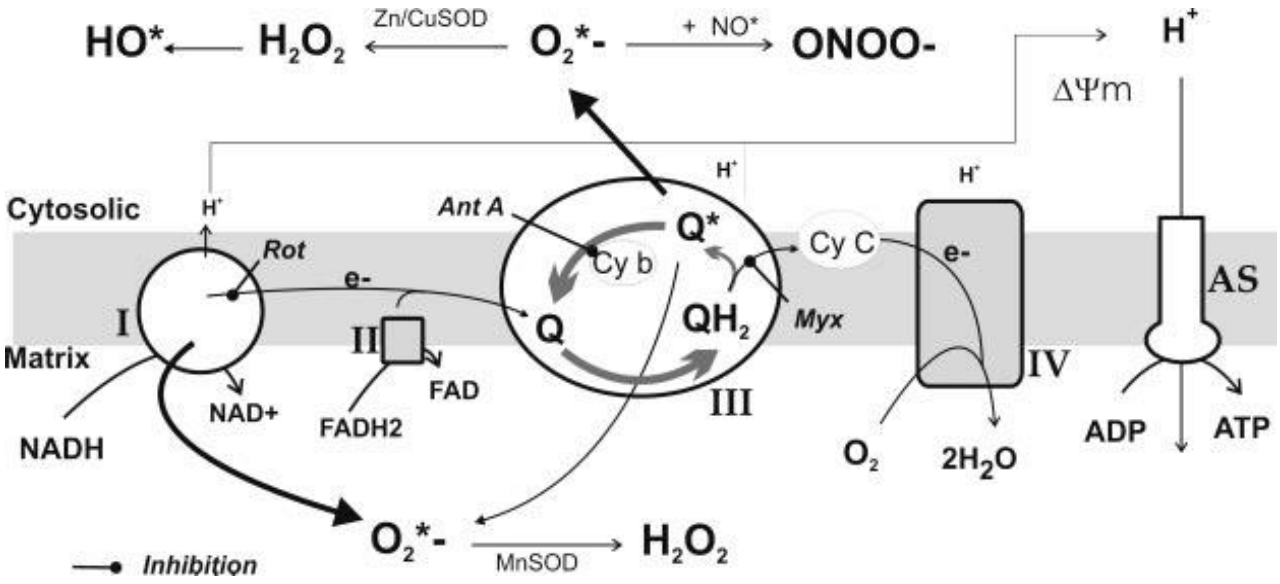


Figure 1.4 Production of ROS in mitochondria. Substrates from metabolic pathways provide electrons to complex I and II of the ETC. In complex III ubuquinone (Q) gains electrons and forms ubiquinol (QH_2). Ubiquinol then transfers an electron to cytochrome c (Cy C). The resulting semiubiquinone (Q*) is oxidized back to ubiquinone by cytochrome b (Cy b), and can also transfer electrons to oxygen to form O_2^-. In complex I NADPH is oxidised to NAD+ and the electron is transferred to oxygen to form O_2^-. The figure only represents the inner mitochondria. SOD: superoxide dismutase; H2O2: hydrogen peroxide; ONOO-: peroxynitrite; NO*: nitric oxide; Ψ_m: mitochondrial potential; AS: ATP synthase. Figure adapted from (Camello-Almaraz et al. 2006)

It is estimated that between 1-5% of oxygen consumed by the mitochondria results in the production of H_2O_2 (Møller, 2001). Accumulation of H_2O_2 is especially dangerous as these molecules can react with reduced Fe^{2+} and Cu^+ to generate the highly reactive $OH^{\cdot}$. Due to the uncharged nature of H_2O_2 molecules, they are able to travel across membranes, leading to the peroxidation of the polyunsaturated fatty acids (PUFA) that make up the mitochondrial and cell membranes. Peroxidation of the membrane PUFA leads to the formation of cytotoxic lipid aldehydes, alkenals and hydroxyalkenals. These lipid peroxidation products then go on to react with proteins, nucleic acids and other lipid (Améras et al., 2003). Due to the effect of abiotic stress on plant cell bioenergetics, plant mitochondria use a system of energy dissipation to alleviate generation of ROS. The use of an energy dissipation system in plant mitochondria was confirmed in a study done by (Pastore et al., 2007), which showed that the dissipation of

energy by durum wheat mitochondria lowered the levels of ROS production in the organelle under abiotic stress conditions.

Production of ROS occurs in mitochondria, peroxisomes and chloroplasts occurs at tolerable levels under optimal plant growth conditions. Drought stress causes an increase in the rate at which ROS are produced. When present at high levels, ROS are toxic to plants because they react with proteins, nucleic acids and lipids to cause serious damage to cells, eventually leading to cell death. A study conducted by (Yang et al., 2015) to investigate differences in drought responses of different maize genotypes showed that levels of O_2^- and H_2O_2 increased in all maize genotypes in response to drought.

1.5 The Effects of ROS on Plant Cells

1.5.1 Lipid peroxidation

It has been established that various abiotic stresses causes plants to over-produce ROS, which are known to attack PUFA and result in lipid peroxidation. This phenomenon is described by (Gill & Tuteja, 2010) as the most damaging process that occurs in living organisms. The process of lipid peroxidation occurs in three distinctive stages: initiation, propagation and termination. The initiation of lipid peroxidation occurs when a hydrogen atom.

1.5.2 Protein oxidation

Initiation of covalent protein modification by ROS or the by-products of oxidative stress is known as protein oxidation (Ghezzi & Bonetto, 2003). Protein oxidation occurs when a number of amino acids in a protein are oxidised, giving rise to free carbonyl groups, which leads to the inhibition of or altered enzyme activity. Particular amino acids; such as Arg, His, Lys, Pro, Thr and Trp; are usually targets for protein oxidation, which causes the proteins to

become more susceptible to proteolytic attack (Møller et al., 2007). Amino acids that contain sulphur and thiol groups, such as Cys and Met, are likely targets for ROS (Hancock et al., 2006). It has be shown that protein oxidation occurs shortly after ROS concentrations have exceeded antioxidant capacity and this causes a decrease in cellular ATP production and subsequently cell death (Stadtman & Levine, 2000).

1.5.3 DNA damage

The plant genome is stable, yet it is still vulnerable to the effects of genotoxic stress caused by biotic and abiotic stresses (Narendra et al., 2009). The damage suffered by DNA as a result of the activity of ROS is referred to as spontaneous DNA damage. The reactive molecules responsible for the damage of DNA are $OH^{\cdot}$ and $O_2{}^1$. The majority of DNA damage can be attributed to the reaction of $OH^{\cdot}$ with DNA, as it reacts with the purine and pyrimidine bases as well as the deoxyribose backbone, although $O_2{}^1$ is not as reactive with DNA it still causes damage by reacting with guanine (Gill & Tuteja, 2010). Damage to DNA by ROS occurs through base deletion and modifications (alkylation and oxidation) as well as formation of pyrimidine dimers, cross-links and strand breaks (Tuteja et al.,2001).

Damage to DNA by ROS has seriously detrimental effects on plants as it causes a reduction in protein synthesis, damage to the cell membrane as well as photosynthetic proteins resulting in dire effects on plant growth and development. The negative effects of ROS on plants are reduced by action of antioxidant enzyme systems that plants have evolved. Accumulation of ROS is dependent on the balance between ROS production and ROS scavenging. Plants have ROS scavenging mechanisms to reduce ROS content in plants during stress.

1.6 Plant antioxidant mechanism used to maintain ROS equilibrium

Normal plant metabolism results in ROS production and ROS have roles in plants as signalling molecules (Gill & Tuteja, 2010). However excessive ROS production as a result of various abiotic stresses can cause serious damage to plant cells and result in a reduction in growth and development. Accumulation of ROS is dependent on the balance between ROS production and ROS scavenging. Plants have thus evolved mechanisms to regulate production as well as levels of ROS. The levels of ROS are strictly controlled by a network of antioxidant enzymes which cooperate to systematically detoxify ROS (Miller, Shulaev, & Mittler, 2008). The ascorbate-glutathione cycle plays an important role in the detoxification ROS (Miller et al., 2008). In this cycle, superoxide dismutase (SOD) disproportionates O_2^- into H_2O_2, which in turn is converted by ascorbate peroxidase (APX) into H_2O and O_2, using Ascorbate (ASC) as a substrate, as depicted in figure 1.5.

In order to provide a steady supply of ASC to the peroxidase, ascorbate is regenerated from monodehydroascorbate (MDHA) (the product of the ROS detoxification reaction) by monodehydroascorbate reductase (MDHAR) through an NAD(P)H-dependent reaction or by reduced ferredoxin (Miller et al., 2010). When MDHA is not reduced back into ASC rapidly enough, it gets converted spontaneously to dehydroascorbate. The dehydroascorbate is then reduced to ASC by dehydroascorbate reductase, this is accomplished by using reduced glutathione generated from the reduction of oxidised glutathione (glutathione disulphide) by glutathione reductase in a NAD(P)H-dependent reaction (Gill & Tuteja, 2010).

The H_2O_2 generated as a result of the scavenging of O_2^- by SOD may also be scavenged by glutathione peroxidase (GPX), which uses two molecules of glutathione to disproportionate

H_2O_2 and forms glutathione disulphide (GSSG) (Gill & Tuteja, 2010). The GSSG is then reduced back into 2 molecules of glutathione by the NAD (P) H-dependent action of glutathione reductase. Along with SOD, APX and GPX, other enzymatic antioxidants include catalase (CAT) and guaiacol peroxidase (GPOX) which are scavengers of H_2O_2, as well as glutathione reductase (GR), Monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) which do not directly detoxify ROS but play a supporting role by regenerating the substrates required for detoxification to occur (Gill & Tuteja, 2010). Plants also have non-enzymatic systems for reducing damage as a result of elevated ROS levels.

Glutathione are two of the most abundant non-enzymatic antioxidants present in plants and are found in their reduced and oxidized form in all plant tissues and are concentrated in cellular compartments like the chloroplast and the mitochondria (Jiménez et al., 1998). They are important in the detoxification of ROS as they are the substrates used by APX and GPX respectively. Figure 1.5 illustrates the antioxidant system in which ASC and glutathione are used as substrates to detoxify H_2O_2. Other non-enzymatic antioxidants include proline, which according to (Chen & Dickman, 2005), is a potent antioxidant and can potentially inhibit programmed cell death (PCD). α-Tocopherol (vitamin E) is a lipid soluble non-enzymatic antioxidant which is a scavenger of ROS and lipid radicals (Holländer-Czytko et al., 2005).

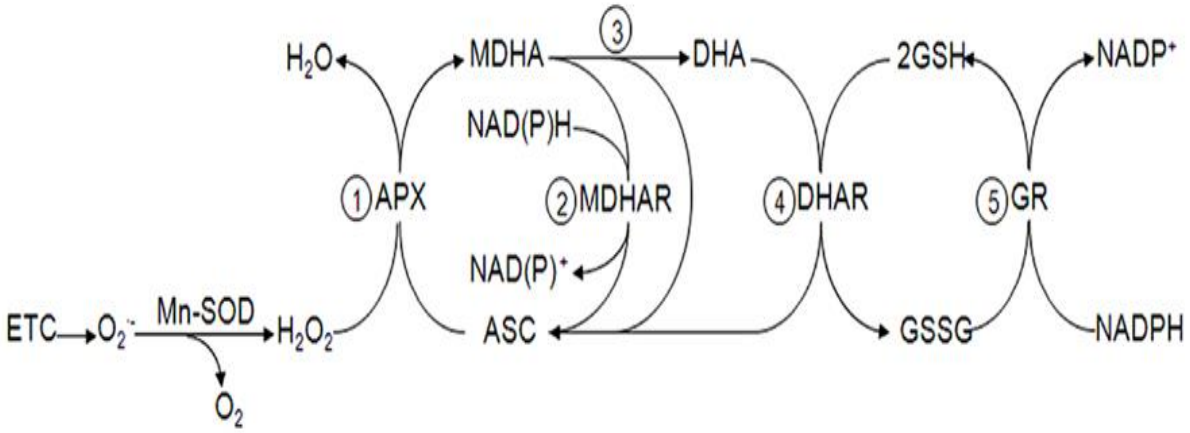


Figure 1.5 The antioxidant system used to detoxify ROS in plants. (1) Hydrogen peroxide in is reduced by APX at the expense of ASC to produce MDHA. (2) MDHA is then reduced to ASC by MDHAR using NAD(P)H as a substrate or (3) disproportionated to DHA and ASC. (4) DHAR reduces DHA using GSH as electron donor. (5) GSH is regenerated by GR using NAD(P)H. (Figure adapted from Lázaro et al. (2013))

The balance between ROS production and scavenging is disturbed when plants undergo drought stress. Production of ROS gets enhanced due to stomatal closure and limitation on CO_2 fixation. The increase in ROS production has detrimental effects on plants if not kept under control. However, plants are able to sense the increase production in ROS as a signal that triggers stress response pathways and enable the plant to acclimatise to changes in the environment. Mechanisms for ROS production and scavenging require cells to have ROS-specific sensors that are able to detect the levels of ROS in cells and in various organelles in order for plants to determine whether to produce or scavenge ROS. It has been shown that ROS can modulate the activity of specific transcription factors, thereby directly affecting gene expression (Jain et al., 2013; Kunsch & Medford, 1999; Miller et al., 2010). Products generated by ROS activity are also able to act as second messengers that trigger biological responses. For example, polyunsaturated fatty acids are a preferred target of ROS attack, and several lipid oxidation products are biologically active in that they can change gene expression (Sharma et

al., 2012). ROS can also modulate gene expression by changing the cellular redox state (Apel & Hirt, 2004; Foyer, 2005; Kunsch & Medford, 1999).

The ability of ROS to diffuse over short distances, and their size, make ROS suitable as signalling molecules. However, ROS such as O_2^-, O_2^1 and $HO^{\cdot}$ have relatively short half-lives and are thus not stable enough to diffuse between cell organelles and transmit signals (Baxter et al., 2014; Gill & Tuteja, 2010; Slesak et al., 2007). This then leaves H_2O_2 as one major ROS used by plants as a signalling molecule (Gill & Tuteja, 2010; Slesak et al., 2007). This ROS is produced from O_2^- and other ROS and thus its levels in the cells usually represent the relative rate of production of ROS. H_2O_2 has a long half-life which makes it able to diffuse between various cell organelles and it can also be metabolised by the antioxidant system, enabling it to be activated and deactivated as a signal.

Some of the downstream events modulated by H_2O_2 are calcium mobilisation, protein phosphorylation and gene expression. It has been shown that H_2O_2 induces an increase in $[Ca^{2+}]$ in the cytoplasm by the activation of hyperpolarization-dependent Ca^{2+}-permeable channels in the plasma membrane of Arabidopsis guard cells (Pitzschke et al., 2006) This form of H_2O_2 signalling results in stomatal closure and is induced by increased levels of ABA due to drought stress. As shown in Figure 1.6, accumulation of ROS occurs in response to ABA and induces stomatal closure via activation of plasma membrane ion channels.

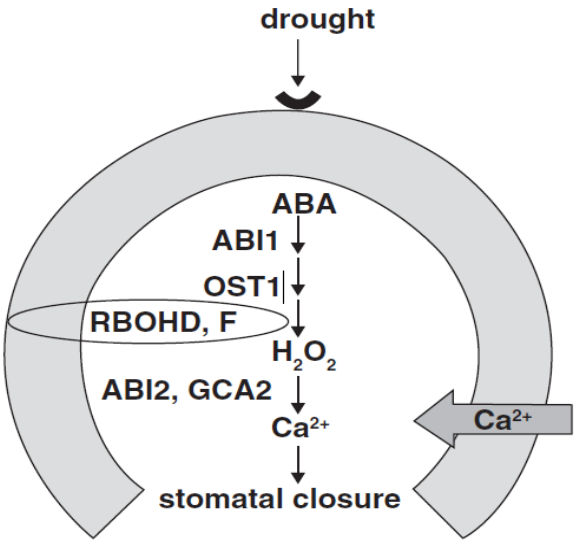


Figure 1.6 ABA-induced stomatal closure. Drought results in increased ABA levels, which leads to elevated cytoplasmic Ca2+ ion concentrations and thus stomatal closure. (Figure adapted from Pitzschke et al. (2006))

Several reports have also shown that H_2O_2 induces mitogen-activated protein kinases (MAPKs), which are in turn implicated in several signal transduction cascades that control gene expression. Ma et al. 2012 showed that H_2O_2 induced the production of NO, which then enhanced expression of ZmCCaMK, a gene responsible for regulation of the expression of antioxidant enzymes in maize. H_2O_2 has also been shown to induce expression of the ZmMPK5, a MAPK protein involved in phase II expression of four maize NADPH-oxidase genes (Lin et al., 2009).

Signalling downstream of ROS production has been extensively studied and is fairly understood. As depicted in Figure 1.6, ROS signalling involves production of plant stress hormones, modification of translated proteins as well as induction of MAPK pathways which then result in changes in expression of genes that are involved in production of antioxidant enzymes, as well as stress responses such as PCD (Pei et al., 2000). ROS are also able to act

as secondary messengers in hormone signalling pathways and there are thus feedback interactions between ROS production and plant hormone signalling. Pathways that lead to production and scavenging of ROS are also fairly understood. Brassinosteroids and other phytosterols have been implicated in processes involving ROS signalling (Posé et al., 2009b; Schaller, 2003; Vriet et al., 2012; Xia et al., 2009)

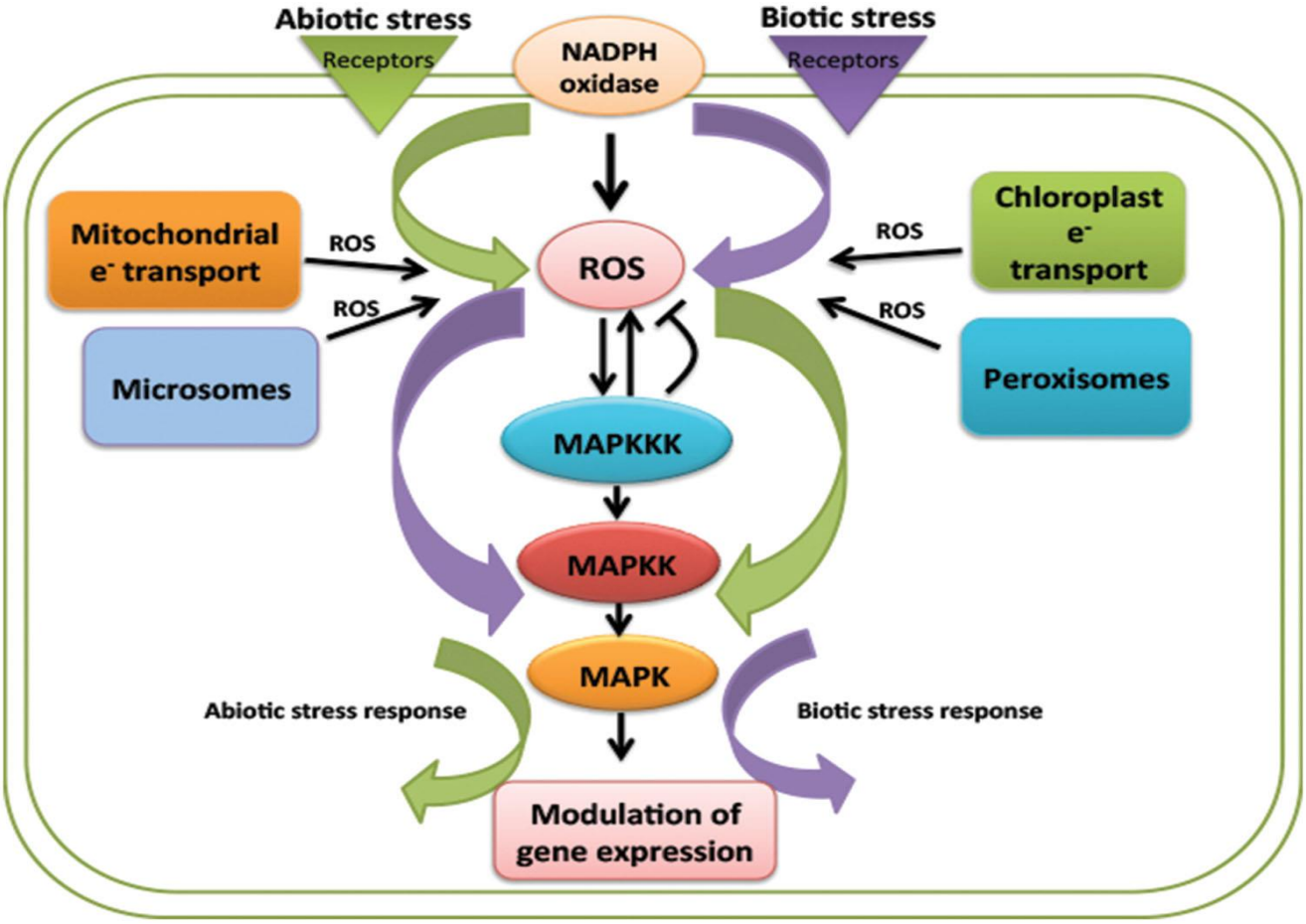


Figure 1.7 A representation of ROS regulation of mitogen activated protein kinases (MAPK) signalling pathway. Upon receiving stress signals, plants respond by over producing ROS in various ROS producing organelles. ROS production activates MAPK cascades which in turn result changes in gene expression. Figure adapted from Jalmi & Sinha 2015.

1.8 Phytosterols

Plant sterols, better known as phytosterols, include over 250 compounds found in land and marine plant species. Phytosterols are amphiphilic and are an integral part of the plasma membrane. They are found in the plasma membrane, the outer membrane of mitochondria as well as the endoplasmic reticulum. The most common phytosterols are campesterol, sitosterol and stigmasterol (Piironen et al., 2000). Sterols such as campesterol are precursors of oxidized plant steroids acting as growth hormones collectively named brassinosteroids (Schaller, 2003). The basic structure of plant sterols is similar to that of cholesterol but differs in the side chain. Sitosterol and stigmasterol have an ester at carbon-24 and campesterol has a methyl group (Piironen et al., 2000).

Phytosterols are made up of a tetracyclic cyclopenta[a]phenanthrene ring and a long flexible side chain at carbon 17. The four rings (A, B, C, and D) have *trans* ring junctures, forming a flat α system. The side chain has two methyl groups at carbon 18 and 19 that are angular to the ring structure and occur above the plane. Sterols create planar surfaces at both the top and the bottom, which allow for multiple hydrophobic interactions between the sterol nucleus and the membrane matrix. Plant sterols can be divided on structural and biosynthetic grounds into 4-desmethyl sterols, 4-amonomethyl sterols and 4,4-dimethyl sterols (Piironen et al., 2000). The 4, 4-dimethyl sterols and 4-α-minomethyl sterols are precursors of sterols and are found at lower levels than 4-desmethyl sterols (Hartmann & Pierre, 1987). The 4-desmethyl sterols may be categorized into D5 -sterols, D7 -sterols and D 5,7-sterols according to the position and number of double bonds in the B ring (Piironen et al., 2000) . Most plant sterols, e.g. campesterol and sitosterol, have a D5 bond and an additional one-carbon or two-carbon substituent in the side chain at C-24.

Plant sterols are steroid alcohols whose biological function and chemical nature resembles that of cholesterol (Spernath et al., 2003). Plant sterols and brassinosteroids exert a wide range of biological activities. Phytosterols are an integral part of the membrane lipid bilayer, and are important for its permeability and fluidity, they have also been shown to regulate the activity of membrane-bound proteins (Hartmann, 1998). Brassinosteroids are essential for plant growth, reproduction, and responses to various abiotic and biotic stresses. Given the role of phytosterols in plant growth and development, understanding their biosynthetic and signalling pathways offers exciting potentials for enhancing crop tolerance to biotic and abiotic stress.

1.8.1 Phytosterol biosynthetic pathway

Phytosterols are derived from squalene and form a group of triterpenes. Triterpenes are a class of chemical compounds composed of three terpene units with the molecular formula $C_{30}H_{48}$ and are biochemical precursors of plant metabolites such as sterols (James & Dubery, 2009). Squalene is a tripterpenoid which functions as an intermediate in sterol biosynthesis (Posé et al., 2009a). All triterpenoids are derived from the isoprenoid biosynthetic pathway, with isopentenyl pyrophosphate (IPP) produced during the mevalonic biosynthetic pathway being their precursor (James & Dubery, 2009; Lange & Croteau, 1999). In maize, however, it has been shown that IPP may be produced by a carbon flux from amino acids and sugars that are products of pathways other than the mevalonic pathway (Guo et al., 1995). This suggests that phytosterols may be products of not just the normal triterpenoid production pathway in plants but may be produced from other pathways. These pathways, however, have not yet been elucidated.

Figure 1.8 illustrates the biosynthetic pathway of sterols via the mevalonic pathway. During phytosterol production via the mevalonic pathway, mevalonic acid is produced from acetyl co-

enzyme A (Piironen et al., 2000). IPP produced from mevalonic acid is converted to dimethylalkyl diphosphate (DMAPP), DMAPP then gets converted to farnesyl pyrophosphate (FPP) by FPP synthetase (Piironen et al., 2000). Production of FPP is the branch point for either synthesis of sterols or sesquiterpenes. The pathway committed to sterol biosynthesis consists of 30 enzyme-catalysed reactions that occur in plant membranes and begins when FPP gets converted to squalene via activity of squalene synthetase (Piironen et al., 2000).

Squalene epoxidase (SQEs) catalyzes the conversion of squalene to 2, 3-oxidosqualene. Arabidopsis mutants defective in SQE activity have abnormal cell elongation (Rasbery et al., 2007) , root epidermal cell polarity, polar root hair tip growth and response of stomata to drought stress (Posé et al., 2009b). A mutation in *A. thaliana* sqe1 has also been shown to cause mis-localization of the polarly localized ROOT-HAIR DEFECTIVE2 (RHD2) protein required for the local production of ROS at the tip of root hairs, suggesting that sterol composition in membranes affects ROS signalling during root polar tip growth (Posé et al., 2009b)

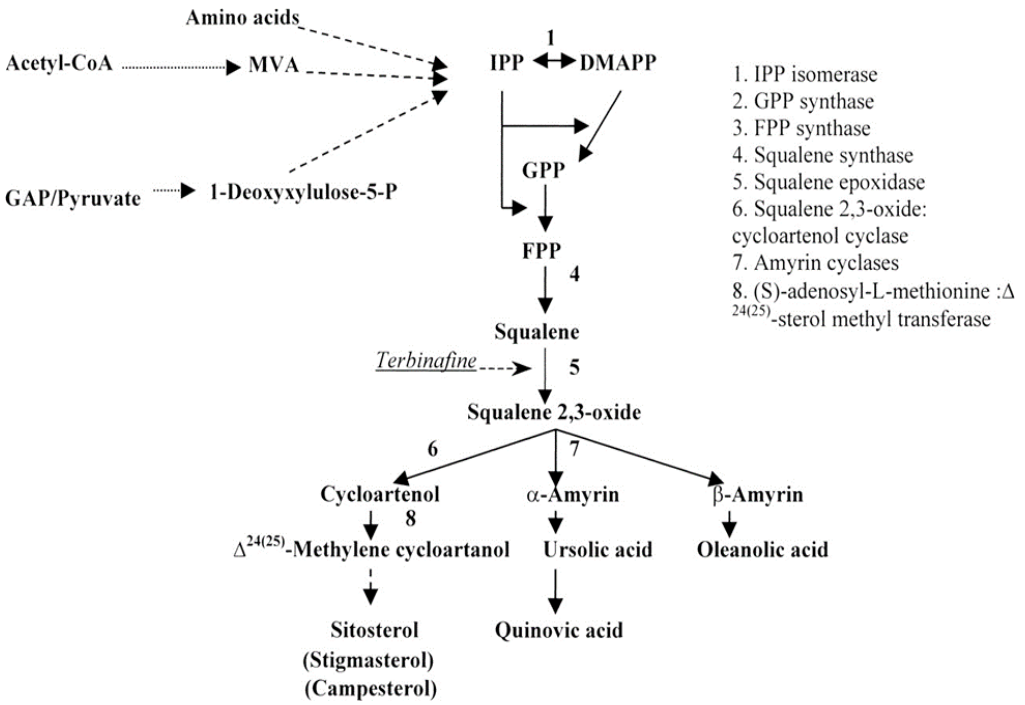


Figure 1.8 General overview of the sterol biosynthetic pathway in plants. Isopentyl pyrophosphate (IPP) generated from the mevanolic acid (MVA) pathway gets converted to FPP. Squalene is the synthesized from farnesyl pyrophosphate (FPP) in a reaction catalysed by squalene synthase. Squalene oxide is synthesized via cyclization of squalene by squalene epoxidase. Squalene oxide is used as a substrate for synthesis of the first set of sterol, cycloartenol through activity of cycloartenol synthase. Figure adapted from Flores-Sánchez et al. (2002)

Subsequent to 2, 3 oxidosqualene production is cyclization of this compound by an enzyme known as cycloartenol synthase, to form the sterol cyloartenol. Until recently, it was thought that plants solely synthesize sterols via the cycloartenol pathway, in contrast to fungi and mammals which convert oxidosqualene to lanosterol for the production of ergosterol and cholesterol, respectively. However, recent loss-of-function and gain-of-function studies of the Arabidopsis LANOSTEROL SYNTHASE1 (LAS1) gene, combined with substrate feeding studies and subsequent metabolite detection, revealed that plants do produce lanosterol and use it for production of subsequent sterols (Suzuki et al., 2006). Lanosterol has also been identified in *Panax ginseng* (Suzuki et al., 2006) and *Lotus japonicus* (Sawai et al., 2006) using

a yeast expression system. The presence of both lanosterol and cycloartenol in plants suggests that cyclization of 2, 3 oxidosqualene can either be performed by lanosterol synthase or cycloartenol synthase.

Once cycloartenol is produced, the pathway fully commits to production of sterols such as campesterol, sitosterol and stigmasterol. Campesterol is further used to produce the steroid hormones brassinosteroids. Mutations that affect various stages or steps in the phytosterol biosynthetic pathway affect various aspects of plant growth and development, suggesting that plant sterols have significant biological functions in plants.

1.8.2 Biological function of phytosterols.

Sterols regulate the fluidity of membranes and probably play a role in the adaptation of membranes to temperature. A free hydroxyl group in free sterols is an important factor enabling its specific interactions with phospholipids and proteins in membranes. Steryl glycosides and acylated steryl glycosides occur concurrently with free sterols in various membranes with the carbohydrate group oriented in the aqueous region (Moreau et al., 2002). In addition, sterols participate in the control of membrane-associated metabolic processes. Sterols are known to also play a significant role in cellular differentiation and proliferation. Sterol synthesis occurs actively following the germination of the seed and then gradually decreases as seed germination completes, suggesting that they provide a reservoir for growth of new cells (Guo et al., 1995). Steryl esters occur in soluble form in plant lipoprotein complexes and this suggests that they have a role in storage and transport of metabolites and other cell components. It has been noted that plant sterols modulate the activity of ATPase in the membrane of maize roots (Grandmougin-Ferjani et al., 1997). Cholesterol and stigmasterol stimulate the export of H^+ at low concentrations(Onyeike et al., 2010), whereas all other sterols act as inhibitors, suggesting

a function similar to that of cholesterol in mammalian cells. In this regard they may function in the same way as cholesterol can in activating the Na^+/K^+-ATPase of animal cells (Yeagle, Young, & Rice, 1988). Specific sterol molecules may also participate in some signal transduction events as is the case for cholesterol in mammalian cells (Hartmann, 1998).

Brassinosteroids (BRs) are a group of naturally occurring plant steroids that are important for various cellular and physiological processes, including stem elongation, pollen tube growth, leaf bending and epinasty, root growth, ethylene biosynthesis, proton pump activity, xylem differentiation, photosynthesis and gene expression. Both sterols and BRs are known to have an impact on vascular tissue development. Vascular tissues are responsible for movement of water and nutrients, they provide mechanical strength and are thus important for plant growth and development.

Mutants that are defective in brassinosteroid production generally display abnormal patterns in vascular differentiation that are characterized by over-proliferation of phloem cells and under-proliferation of xylem cells (Caño-Delgado et al., 2004; Caño-Delgado, Lee, & Demura, 2010). Consistent with an involvement of BRs in xylem development, treatment with the BR biosynthetic inhibitor brassinazole (Brz) hampers the development of secondary xylem in *Lipidium sativum* (Nagata, Asami, & Yoshida, 2001). Brassinosteroids have been also been shown to play a role in plant stress tolerance. Brassinosteroids have been shown to enhance water stress tolerance by reducing oxidative stress caused by drought in maize (Zhang et al., 2011). Exogenous application of brassionosteroids was also shown to increase H_2O_2 production in cucumber, suggesting the role of sterols in modifying production of ROS and in ROS signalling (Cui et al., 2011).

Treatment of Arabidopsis plants with brassinosteroids and studies of the Arabidopsis *det2* mutant reveal a brassinosteroid function in plant responses to oxidative stress (Bajguz &

Hayat, 2009; Hasan et al., 2011; Xia et al., 2009). The *det2* mutant is not affected by conditions of low O_2. This enhanced oxidative stress resistance has been associated with an increase in SOD activity and in catalase transcript levels (Cao et al., 2005). By contrast, BR levels positively correlated with an increased tolerance to photooxidative stress in cucumber plants treated with 24-epibrassinolide (EBL) and Brz (Xia et al., 2009). Most probably, exogenously applied brassinosteroids contribute to stress tolerance via production of antioxidants that protect cells from damage. The activity of the antioxidant defence machinery of two tomato cultivars exposed brassinosteroids treatment improved significantly (Hasan et al., 2011).

In a study conducted by Posé et al (2009) an *Arabidopsis* mutant characterized by its high sensitivity to drought stress was identified. This mutant had a mutation in a gene that encodes SQE, an enzyme responsible for conversion of squalene into 2, 3-oxidosqualene during sterol biosynthesis. Gas Chromatography-Mass Spectrometry analysis of mutant plants indicated that these plants had an altered sterol profile in roots. This study suggested a role of plant sterols in ROS production in *A. thaliana* roots through localisation of NADPH oxidase. It is the first and only study where sterols other than brassinosteroids have been directly linked to ROS production. These studies suggest that sterols play a role in plant drought stress responses as well as in ROS production and signalling.

CHAPTER 2: MATERIALS AND METHODS

2.1 *In silico* characterization of ZMCAS 494

The genomic and amino acid sequences of ZmCAS494 (accession number grmzm2g065494) were obtained from the phytozome (http://www.phytozome.org) database by using homology searches (BLAST) based on the *Arabidopsis thaliana* cycloartenol synthase 1 (CAS1) sequence (At2g07050). The molecular weight of the protein was predicted by using the compute pI/Mw tool found on the Expasy website (http://web.expasy.org/compute_pi/). The amino acid sequence of ZMCAS494 was then aligned with the *Arabidopsis thaliana CAS1* (At2g07050), *Nicotiana tabacum CAS1* (KM452913) and *Oryza sativa CAS* (AK121211) using the Clustal Omega multiple sequence alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalo/). Subcellular localization of ZMCAS494 was predicted using the TargetP topology prediction tool available from http://www.cbs.dtu.dk/services/TargetP/. Drought-responsive transcription factor binding sites (TFBS) were predicted using the PlantPAN promoter analysis program (version 2.0) available on http://plantpan2.itps.ncku.edu.tw/promoter.php. The *Zea Mays*, *Arabidopsis thaliana* and *Oryza sativa* databases were used for TFBS prediction.

2.2 Plant growth

2.2.1 Seed germination

Seeds of the maize cultivar CAP9001 were imbibed in 10 mM $CaSO_4$ for 16 hours and then allowed to germinate for 48 hours between sheets of moist paper towel placed in a dark container at room temperature.

2.2.2 Growth

The growth medium was Promix Organic (Windell Hydroponics) supplemented with nutrient solution (1mM K_2SO_4, 2 mM $MgSO_4$, 5 mM $CaCl_2$, 5 mM KNO_3, 10 mM NH_4NO_3, 1 mM K_2HPO_4 buffer at pH 6.0, 5 µM H_3BO_3, 5 µM $MnSO_4$, 1 µM $ZnSO_4$, 1 µM $CuSO_4$, 2 µM Na_2MoO_4, 1 µM $CoSO_4$, 100 µM Fe-NaEDTA and 10 mM 2-(N-Morpholino)ethanesulfonic acid (MES) at pH 6.0 (containing 50 µM Terbinafine for sterol synthesis inhibition treatments). Promix Organic is a mixture of perlite and coco peat and it has the ability to retain moisture while supplying enough air to the root system and to seedlings. Moisture from fresh growth medium was removed by drying at 80ºC for 48 hours. Water was added to the medium such that the water potential was -0.03 Mpa for control plants and sterol synthesis inhibitor treated plants but -0.21 Mpa for water-deprived plants. Plants were grown at 25°C under a 16/8 hour light/dark cycle at a photosynthetic photon flux density of 400 µmol.m^{-2}.s^{-1}during the light phase. When radicles were 3-5 mm long, seeds were then transferred to the growth medium in 20 cm diameter acrylic tubes with a height on 100 cm.

2.2.3 Harvesting and measurement of growth parameters

Harvesting was performed after 10 days, when plants reached the V1 stage of vegetative growth. The fresh, dry and turgid weights of roots and leaves were measured. The relative water content of leaves and roots for water deprived plants was measured using the formula described by Claussen (2005) . Root and shoot lengths of water-deprived plants were also measured and recorded. Roots and leaves were then cut from the rest of the plants, frozen in liquid nitrogen and ground using mortar and pestle. Frozen ground tissue was stored at – 80°C and used for subsequent experiments.

2.4 Measurement of H_2O_2content and lipid peroxidation

2.4.1 H_2O_2 content

The amount of H_2O_2 was determined using the method described in Velikova et al. (2000) with minor modifications. Plant tissue was weighed and homogenized in 500 µl 0.5 M trichloroacetic acid (TCA) (Sigma-Aldrich) to extract H_2O_2. A 200 µl reaction mixture containing 50 µl of the TCA extract, 50 µl 0.5M KI and 100 µl M 5mM K_2HPO_4 (pH 5.0) was prepared. The mixture was incubated at room temperature for 20 minutes and absorbance was measured at 390 nm using a FLUOstar Omega Multi-Mode Microplate Reader with CCD-based Spectrometer (BMG LABTECH).

2.4.2 Lipid peroxidation

MDA content was measured and used as an indication of lipid peroxidation. An adaptation of the method described by Buege & Aust (1978) was used. Plant tissue was weighed and homogenized in 500 µl 0.5 M TCA. The extract (100 µl) was mixed with 400 µl 0.5% thiobarbituric acid (TBA) in 20% TCA. The mixture was incubated at 95ºC for 30 minutes and then cooled on ice for 5 minutes. Absorbance at 532 nm and 600 nm of the supernatant was measured. The concentration of MDA was determined using the molar extinction coefficient of 155 $mM^{-1}cm^{-1}$. These experiments were repeated three times.

2.5 Determination of antioxidant enzyme activities

2.5.1 Superoxide dismutase (SOD) activity

Protein extracts were obtained from leaf and root tissue. Tissue (150 mg) was weighed and homogenized in 500 µl of buffer [40 mM K_2HPO_4, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 5% (w/v) polyvinylpyrolidone (PVP) molecular weight = 40 000]. Native polyacrylamide gels (12%) were prepared and equal amounts of protein extracts from leaves (40 µg) and roots (10 µg) were loaded on gels. Activity of all SOD isoforms was determined by staining with 0.5 mM riboflavin and 2.5 mM nitroblue tetrazolium, as described by Dewir et al. (2006). Densitometry was measured and used to estimate SOD activity for each sample.

2.5.2 Ascorbate Peroxidase (APX) activity

Detection of the activity of APX isoforms was achieved using an adaptation of the method described by Dewir et al. (2006). Native gels were equilibrated with running buffer containing 2 mM ascorbate for 30 minutes at 4°C. Following electrophoresis, gels were incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM ascorbate for 20 minutes. Incubation insolutions containing 50 mM potassium phosphate buffer (pH 7.8), 4 mM ascorbate and 2 mM H_2O_2 was then performed for 20 min. The gels were then stained with asolution of 50 mM potassium phosphate buffer (pH 7.8) containing 16 mM N,N,N′,N′-Tetramethylethylenediamine (TEMED) and 2.5 mM NBT for 10-20 minutes using gentle shaking in the presence of light.

2.5.3 Glutathione peroxidise (GPX) activity

Activity of GPX isoforms was determined using a method similar to that used for APX activity except that ascorbate was replaced with glutathione. Native gels were equilibrated with running buffer containing 2 mM glutathione for 30 minutes at 4°C. Following electrophoresis, gels

were incubated in 50 mM potassium phosphate buffer (pH 7.0) containing 2 mM glutathione for 20 minutes. Incubation in solutions containing 50 mM potassium phosphate buffer (pH 7.8), 4 mM glutathione and 2 mM H_2O_2 was then performed for 20 min. The gels were then stained with a solution of 50 mM potassium phosphate buffer (pH 7.8) containing 16 mM N,N,N′,N′-Tetramethylethylenediamine (TEMED) and 2.5 mM NBT for 10-20 minutes using gentle shaking in the presence of light.

2.6 Measurement of ZMCAS494 gene expression

2.6.1 RNA extraction and cDNA synthesis

ZMCAS494 is a soybean gene that likely encodes a cycloartenol synthase. Differences in ZMCAS494 expression in treated versus control plants were determined using both quantitative and semi-quantitative polymerase chain reaction (PCR). Total RNA was extracted using the ZR Plant RNA MiniPrep kit (Zymo Research, USA) as per manufacturer's instructions. The RNA was quantified using absorbance at 260 nm, its quality determined using gel electrophoresis and purity was determined by measuring the $A_{260/280}$ and $A_{260/280}$ ratios. Two micrograms of total RNA were treated with RNase-free DNase I (Thermo Fisher Scientific) according to the manufacturer's instructions. First strand cDNA synthesis was then performed using the ProtoScript® First Strand cDNA Synthesis Kit (New England Biolabs) on 300 ng of the DNase-treated RNA extracted from leaves and roots of treated (drought and terbinafine) and untreated plants. An oligo $(dT)_{18}$ primer (Thermo Fisher Scientific) was used to generate the cDNA.

2.6.2 Semi-quantitative PCR

For amplification of ZMCAS494, the following cycling conditions were used: 95ºC for 1 minute, 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 minute and 72°C for 5 minutes,

30 amplification cycles. To detect changes in gene expression, an equal volume of PCR product with 1X GelRed™ nucleic acid stain (Biotium, USA) was loaded onto a 1% agarose gel and gel electrophoresis was then performed at 70V for 2 hours. Stained gels were photographed (AlphaImage90 2200 system) and analyzed using the Spot Denso Tool (AlphaEase FC imaging software V4, Alpha Innotech Corporation). Differences in gene expression were detected by measuring densitometry on gel images. Maize β-tubulin was used as an internal control. The following cycling conditions were used for amplification of beta-tubulin: 95°C for 1 minute, 95°C for 30 seconds, 63°C for 30 seconds, 72°C for 1 minute and 72°C for 5 minutes, 30 amplification cycles. The reaction mixture, for amplification of both β-tubulin and ZMCAS494 was composed as shown in table 2.1. The primers listed in Table 2.2 were used for both semi qPCR and qPCR analysis.

Table 2.1 Reaction composition for semi-quantitative and quantitative real-time PCR for detection of ZMCAS494 expression.

Reactant	Amount
cDNA products from the reverse transcription reaction	2µl
10X Hot Start PCR Buffer	1X
$MgCl_2$	1.5 mM
deoxynucleotide triphosphates (dNTPs)	0.2 mM
Forward Primer	0.5 µM
Reverse Primer	0.5 µM
Maxima Hot Start Taq DNA Polymerase (Thermo Fisher Scientific)	1.25 Units
Total Reaction Volume	**25 µl**

Table 2.2 PCR primers used in semi-quantitative and quantitative real-time PCR for detection of ZMCAS494 expression.

Gene	Origin	Primer sequences (5′-3′)	Product size (bp)
ZMCAS494	*Zea Mays*	F : TGGTATGGCTCTTGGGCTGTTT R: CCCAACCAGTGTTCGCTGCAT	228
18s rRNA	*Zea Mays*	F : CCATCCCTCCGTAGTTAGCTTCT R: CCTGTCGGCCAAGGCTATATAC	152
β - tubulin	*Zea Mays*	F: AGCCCGATGGCACCATGCCCAGTGATACCT R: AACACCAAGAATCCCTGCAGCCCAGTGC	343

2.6.3 Quantitative PCR

Amplification was done using the Luminaris HiGreen qPCR Master Mix (Thermo Fisher Scientific). Both β-tubulin and 18s rRNA were used an internal controls but analysis was done using 18s rRNA. The cycling conditions for 18s rRNA were the same as those used for ZMCAS494 amplification. The cycling parameters for β-tubulin were 95°C for 5 minutes, 95°C for 10 seconds, 63°C for 10 seconds, 72°C for 10 seconds, 45 amplification cycles. A melting curve analysis was performed to ensure that detected fluorescence wasn't a result of amplification of non-specific production or excessive primer dimerization. The conditions for the melting curve were as follows: 95°C for 5 seconds, 58°C for 1 minute. Analysis of qPCR data was performed as described in (Livak & Schmittgen, 2001). The efficiency of

amplification of 18s rRNA and ZMCAS494 was determined and used to calculate the expression ratio. Gene amplification was performed three times and the mean Cp values were determined and used to assign expression levels.

2.7 Determination of plant sterol content

2.7.1 Phytosterol extraction

The method described by Gas-Pascual et al. (2014),with minor modifications, was used to extract phytosterols. Plant samples (100 mg) were saponified for one hour in 1ml of 6% potassium hydroxide methanolic solution at 80ºC. After saponification, 500 ml of n-hexane was added to the reaction mixture to extract the unsaponifiable fraction. The mixture was mixed by vortecing and left to separate at room temperature for 5 minutes. The soluble layer (300 µl) was transferred to a separate centrifuge tube and the liquid evaporated to dryness at 40°C. The dried extract was derivatized in 40 µl N, O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 100µl of pyridine at 70°C for 1 hour. A known amount of betuline-2, 3-diacetate, cycloartenol and lanosterol was added to the extracts before derivatization. External cycloartenol and lanosterol standards were prepared the same way as the samples for calibration.

2.7.2 Gas- Chromatography Mass Spectrophotometry analysis

Compounds were identified by GC-MS using a 6890N gas chromatograph (Agilent) equipped with a DB-225 MS column (J&W 122-2232 model ; 30 m length;250 µm diameter;0.25 µm thickness) and coupled to a CTCPAL mass analyser (Agilent). The injection volume was 1 µl, using a 10 µl syringe. The temperature program of ovens was a gradual 7°C/min increase from 200 ºC to 325 ºC. The inlet to the column was set to splitless mode, at an initial temperature of 250 ºC, 131.1 kPa pressure and the purge flow was 50.0 mL/min. The flow rate at the column was set 1.2 mL/min (constant flow), the pressure at 131.2 kPa and the speed at

42 cm/sec. Sterols were unequivocally identified by coincidental retention time and identical EI-MS spectra at 70 eV like reference compounds as described in Rahier & Benveniste (1989).

2.7 Statistical analyses

For growth parameter measurements, 10 plants were used for each treatment (well-watered/field capacity, water deprivation/drought and terbinafine) and each experiment was repeated three times. For molecular biology/biochemical analyses experiments, 10 plants were used for each treatment and the samples from each treatment were pooled into one sample per treatment; each experiment was repeated three times. Analysis of variance (ANOVA) was used to determine the statistical significance of the differences in the means for the treatments, based on the Tukey–Kramer test at 5% level of significance, using the GraphPad Prism 5.03 software.

CHAPTER 3: RESULTS

3.1 Maize physiological, morphological and biochemical responses to water deficit

3.1.1 Water deprivation results in reduced biomass and stunted growth

Drought stress affects various aspects of plant growth and development. Water deficit has been shown to result in stunted growth and reduced biomass in maize (Cakir, 2004). The effect of water deprivation on shoot and root weight as well as shoot and root lengths on CAP9001 seedlings was determined in order to evaluate the effect of water stress on plant growth. The results are depicted in figure 3.1. Roots of water deprived plants were 4 times shorter than those of well-watered plants. The weight of water deprived roots was half of the weight of well-watered roots. A similar trend was observed in shoots, however the reduction in shoot length was only 2 times.

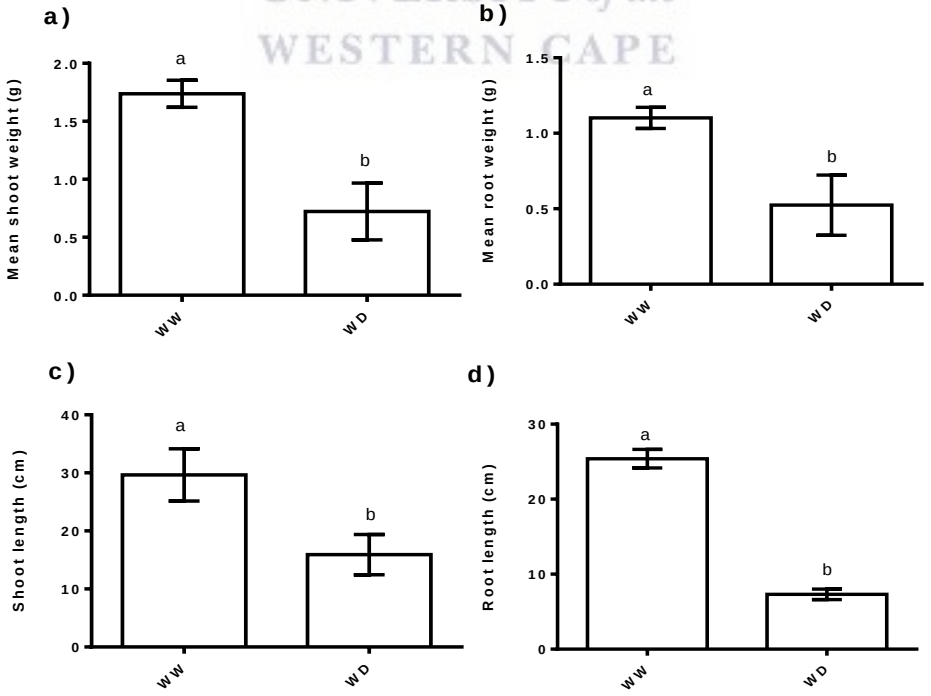


Figure 3.1 The effect of water deprivation on a) shoot weight b)root weight ,c)shoot length ,d)root length of maize at the V1 stage of vegetative growth. Values are means ± SE of 8 plants from three independent experiments, $p \leq 0.05$. WW: well watered, WD: water deprived.

Relative water content (RWC) is a measure of plant water status and a comparison of RWC of watered versus water deprived plants gives a good indication of the extent of cellular water deficit/stress as a consequence of drought. The relative water content of roots and leaves of plants grown under conditions of water deficit was measured and compared to that of plants grown in medium containing sufficient water. The RWC of well-watered leaves was 85% and that of water deprived leaves was 50%. Root relative water content of water deprived plants was 48% and that of well-watered plants was 86%. The results are shown in the graph on Figure 3.2 (b).

One of many biochemical changes that plants go through as a consequence of water stress is over production of ROS or oxidative stress. Overproduction of ROS such that the redox homeostasis of plants is disturbed results in lipid peroxidation which eventually leads to programmed cell death. To evaluate the extent of oxidative stress as a result of water deprivation on maize plants, the effect of water deprivation on ROS production and on lipid peroxidation was determined.

The level of ROS production was determined by measuring the amount of H_2O_2 in well-watered and water deprived plants. H_2O_2 content in leaves and roots of well-watered and water deprived plants is shown on the graph in Figure 3.2 (d). Root H_2O_2 content in water deprived plants was 4.8 times higher than root H_2O_2 content of well-watered plants. In leaves, the H_2O_2 content of well-watered plants was 2.4 times lower than H_2O_2 content of water deprived plants. The extent of lipid peroxidation was determined by measuring the amount of MDA, a product of the degradation of polyunsaturated fatty acids by ROS. A 2 fold increase in MDA content in response to water stress was observed in leaves (Figure 3.2c). Root MDA content was 4 times lower in well-watered plants as compared to water deprived plants.

3.1.2 The effects of water deprivation on relative water content, H_2O_2 and MDA content

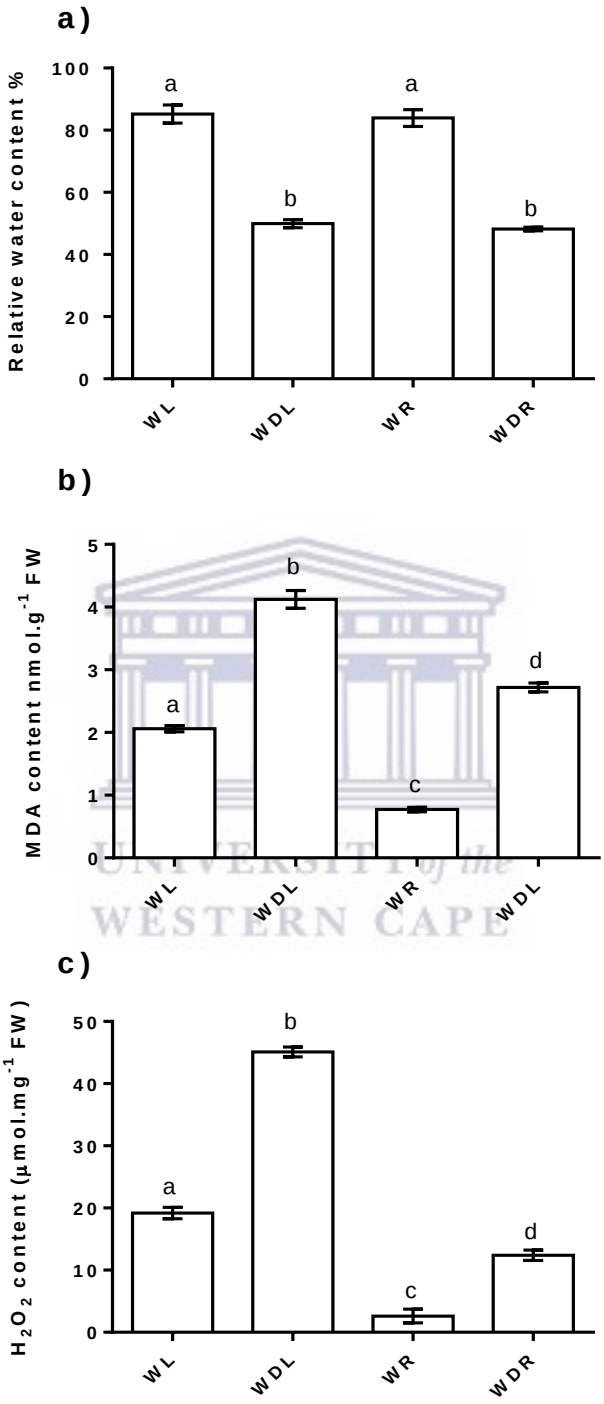


Figure 3.2 The effect of water deprivation on a) relative water content, b) lipid peroxidation and c) ROS production in maize leaves and roots at the V1 stage of vegetative growth. Malondialdehyde (MDA) content (b) and H_2O_2 content (c) were measured as indication of lipid peroxidation and ROS production, respectively. The bars represent mean values obtained from three biological replicates, error bars represent standard errors (SE) and the values indicated by different letters are significantly different at $p \leq 0.05$. WL: Watered leaves, WDL: water deprived leaves, WR: watered roots, WDR: water deprived roots

3.1.3 Drought stress increases antioxidant enzymatic activity in maize roots and leaves.

Drought stress is known to cause increases in ROS production in plants and, in response, plants increase their antioxidant enzyme activity to prevent oxidative damage caused by the increase in ROS. The effect of drought on antioxidant enzyme activity in maize was thus investigated. Maize tissue, from plants harvested at V1 was used to extract total protein and SOD, APX and GPX isoforms were detected using in-gel assays. Densitometry analysis was then used to determine the activity of identified isoforms. The enzymatic activity (for the respective antioxidant enzymes) of each isoform in the treatments was scored as an average of the relative pixel intensities from gels from three independent experiments and expressed in arbitrary units. A value of 1 was assigned to each isoform for well-watered tissue and the pixel intensity for each type of isoform in water deprived tissue was then expressed relative to the pixel intensity of the same isoform type in well-watered tissue.

Glutathione peroxidase and ascorbate peroxidase catalyse the conversion of lipid hydroperoxides and H_2O_2, into H_2O and O_2 using glutathione and ascorbate, as substrates, respectively. To investigate the effect of drought on the maize antioxidant system, GPX and APX isoforms in roots and leaves of water deprived plants were detected and their activity was compared to that of isoforms in well-watered plants.

Five GPX isoforms were identified in leaves and roots of both well-watered and water deprived plants (Figure 3.3a). Activities of GPX 1, 3 and 5 were significantly higher in water deprived leaves as compared to well-watered leaves (Table 3.1a) and thus the total GPX activity was increased in response to drought in leaves. It is noted though, that leaf GPX isoforms 1 to 3 did not resolve well and densitometry was extremely difficult for these isoforms and may not be an accurate representation of the changes that occur in response to drought. A similar trend was

observed for GPX activity in roots as in leaves (albeit much better resolution in roots than in leaves), except for GPX1 for which drought caused a small reduction in GPX activity in response to drought (Table 3.1a).

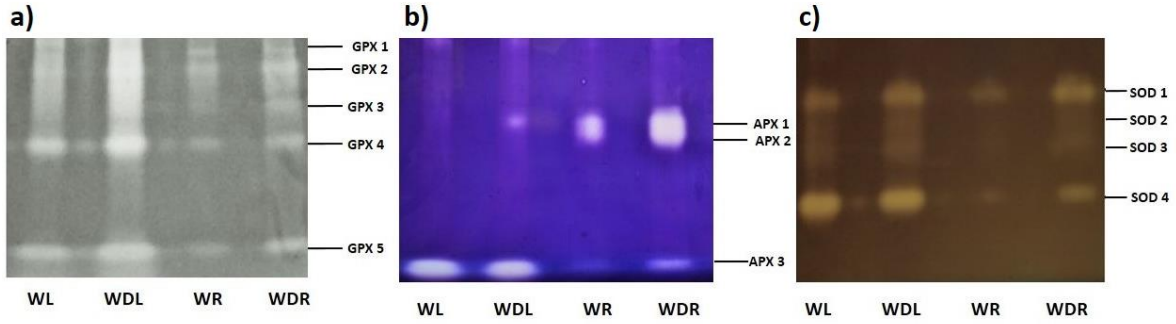


Figure 3.3 The effect of water deprivation on ROS detoxification enzymes in maize leaves and roots at the V_2 stage of vegetative growth. Native acrylamide gels (12%) were stained for the activity of a) GPX, b) APX and c) SOD isozymes using methods described by (Dewir et al., 2006). Equal amounts of protein from leaves (40 µg) and roots (10 µg) subjected to treatments were loaded on gels. WL: Watered leaves, WDL: water deprived leaves, WR: watered roots, WDR: water deprived roots.

Two APX isoforms were detected in leaves (Figure 3.2b). APX 1, had significantly increased activity and the activity of APX 3 did not change in response to drought (Table 3.1b). In roots, three APX isoforms were detected (Figure 3.2b). APX 2 (activity not detected in leaves) had the highest activity and the activity of all isoforms increased significantly in response to drought (Table 3.1b).

Superoxide dismutase catalyses partitioning of the $O_2^{\cdot-}$ radical into the less reactive ROS, H_2O_2. Super oxide dismutases represent the first line of plant defence against ROS among enzymes that defend plant cells against oxidative damage. In this study, four SOD isoforms were detected in both untreated and water deprived root and leaf tissues (Figure 3.3 c). The activities of all four SOD isoforms increased significantly in response to drought in leaves and thus the total activity of SOD in maize leaves increased in response to drought (Table 3.1c). Activities of all root SOD isoforms increased in response to drought.

Table 3.1 Relative enzymatic activity of maize GPX,APX and SOD isoforms in response to water deprivation

a)

Relative GPX Activity (Arbitrary units)	GPX isoforms	Samples			
		WL	WDL	WR	WDR
	GPX 1	1.0 ± 0.05[a]	1.25 ± 0.09[b]	1.0 ± 0.05[a]	0.92 ± 0.05[a]
	GPX 2	1.0 ± 0.05[a]	1.29 ± 0.04[b]	1.0 ± 0.05[a]	1.25 ± 0.24[a]
	GPX 3	1.0 ± 0.05[a]	1.11 ± 0.05[a]	1.0 ± 0.05[a]	1.19 ± 0.06[b]
	GPX 4	1.0 ± 0.05[a]	1.21 ± 0.13[b]	1.0 ± 0.05[a]	1.16 ± 0.03[b]
	GPX 5	1.0 ± 0.05[a]	1.34 ± 0.03[b]	1.0 ± 0.05[a]	1.17± 0.07[b]

b)

Relative APX Activity (Arbitrary units)	APX isoforms	Samples			
		WL	WDL	WR	WDR
	APX 1	1.0 ± 0.05[a]	1.92 ±0.12[b]	1.0 ± 0.05[a]	1.30 ± 0.04[b]
	APX 2	No activity	No activity	1.0 ± 0.05[a]	1.26 ± 0.02[b]
	APX 3	1.0 ± 0.05[a]	0.97 ± 0.01[a]	1.0 ± 0.05[a]	1.36± 0.07[b]

c)

Relative SOD Activity (Arbitrary units)	SOD isoforms	Samples			
		WL	WDL	WR	WDR
	SOD 1	1.0 ±0.05[a]	1.60±0.09[b]	1.0 ±0.05[a]	1.48 ±0.05[b]
	SOD 2	1.0 ±0.05[a]	1.47 ±0.04[b]	1.0 ±0.05[a]	1.36 ±0.24[b]
	SOD 3	1.0 ±0.05[a]	1.39 ±0.05[b]	1.0 ±0.05[a]	1.33 ±0.06[b]
	SOD 4	1.0 ±0.05[a]	1.02 ±0.13[a]	1.0 ±0.05[a]	1.87 ±0.03[b]

Data presented in this table are the means ± standard error of three biological replicates (n = 3). Means marked with different letters in the same row for the same isoform indicate statistically significant difference with p values ≤0.05 according to ANOVA analysis. WL: Watered leaves, WDL: water deprived leaves, WR: watered roots, WDR: water deprived roots.

3.2 The effect of water deficit on maize sterol content

Various studies have shown that exogenous application of brassinosteroids results in increased drought tolerance. Other major sterols such as campesterol and stigmasterol have been suggested to have roles in various plant stress responses. This study aims to establish if phytosterols participate in maize responses to water stress. As a means to achieve this aim, the effect of drought on sterol content was investigated. Sterols were extracted from roots and leaves of water deprived and well-watered plants. Identification and quantification of sterols was then performed using GC-MS. Cycloartenol, stigma sterol, β-sitosterol, campesterol and lanosterol were positively identified using the mass spectrum of available standards and their content could then be quantified.

Figures 3.4 and 3.5 depict the cycloartenol and lanosterol contents in response to drought, respectively. The amount of cycloartenol in water deprived leaves was statistically similar to the amount of cycloartenol in well-watered leaves. Water deprivation resulted in a two-fold reduction in cycloartenol content in roots. Lanosterol content was also not affected by water deprivation in leaves. However, the amount of lanosterol in water deprived roots was 2.3 times lower than that of well-watered roots. Water deprivation caused a reduction in stigmasterol, β-sitosterol and campesterol content in roots and no significant change in leaves (Table 3.2)

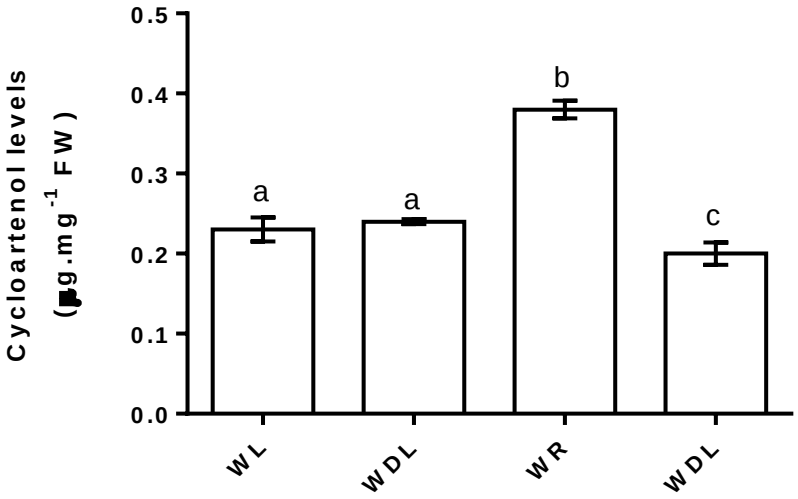


Figure 3.4 Cycloartenol content in response to water deprivation. The effect of drought on levels of cycloartenol in maize leaves and roots was measured. Sterols were extracted from plant tissue and quantified using GC-MS. WL: Watered leaves, WDL: water deprived leaves, WR: watered roots, WDR: water deprived roots. The bars represent mean values obtained from three biological replicates, error bars represent standard errors (SE) and the values indicated by different letters are significantly different at $p \leq 0.05$.

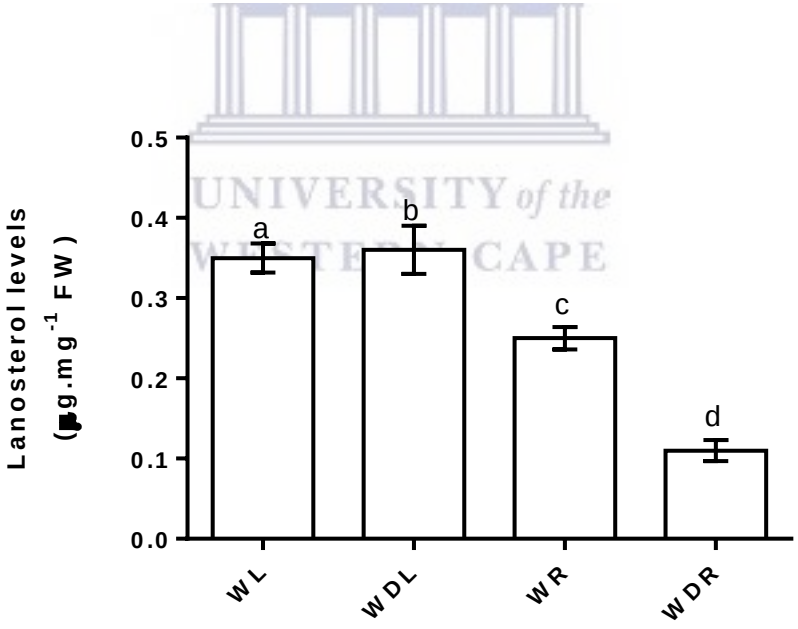


Figure 3.5 Lanosterol content in response to water deprivation. The effect of drought on levels of lanosterol in maize leaves and roots was measured. Sterols were extracted from plant tissue and quantified using GC-MS. WL: Watered leaves, WDL: water deprived leaves, WR: watered roots, WDR: water deprived roots. Data represents means from three independent biological replicates. Error bars represent SE, $p \leq 0.05$.

Table 3.2: Sitosterol, Stigmasterol and β-sitosterol content in response to sterol synthesis inhibition and water deprivation

	Control leaves	Water deprived leaves	Control roots	Water deprived roots
β-sitosterol	**0,35 ± 0.015[a]**	**0,36 ± 0.034[a]**	**0,25 ± 0.041[a]**	**0,20 ± 0.038[b]**
Campesterol	**0,43 ± 0.041[a]**	**0,42 ± 0.021[a]**	**0,30 ± 0.031[a]**	**0,26 ± 0.047[b]**
Stigmasterol	**0,32 ± 0.032[a]**	**0,30 ± 0.009[a]**	**0,28 ± 0.048[a]**	**0,23 ± 0.033[b]**

Data presented in this table are the means ± standard error of three biological replicates (n = 3). Means marked with different letters in the same row for the same isoform indicate statistically significant difference with p values ≤0.05 according to ANOVA analysis. WL: Watered leaves, WDL: water deprived leaves, WR: watered roots, WDR: water deprived roots.

3.3 The effect of inhibition of sterol synthesis on sterol content, ROS production and antioxidant enzyme activity.

3.3.1 Terbinafine reduces sterol content

Given that drought results in a reduction in sterol content in maize, the effect of inhibition of sterol biosynthesis through use of a well-established sterol biosynthesis inhibitor (terbinafine) on ROS production, lipid peroxidation as well as the activity of antioxidant enzymes, was investigated. Terbinafine is routinely used in medicine to treat mycoses since it is an inhibitor of fungal sterol biosynthesis and is thus fungicidal. Plants treated with sufficient amounts of terbinafine have been shown to have altered sterol content. To confirm that the biochemical changes observed in plants treated with terbinafine in this study were a result of changes in sterol content, sterols were extracted in roots and leaves of plants that were treated with 50 µM terbinafine and harvested at V1. Identification and quantification of sterols was then performed using GC-MS.

Cycloartenol, stigma sterol, β-sitosterol, campsterol and lanosterol were positively identified using the mass spectrum of available standards. Treatment with terbinafine resulted in significantly reduced cycloartenol (Figure 3.6), lanosterol (Figure 3.7), campesterol, β-sitosterol and stigmasterol content in both leaves and in roots (Table 3.3).

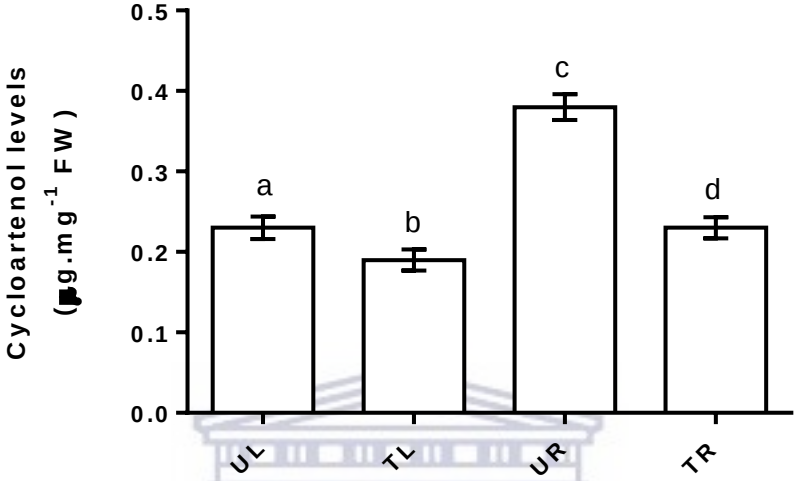


Figure 3.6 Cycloartenol content in response to sterol synthesis inhibition. The effect of sterol synthesis inhibition on levels of cycloartenol in maize leaves and roots was measured. Sterols were extracted from plant tissue and quantified using GC-MS. UL: Untreated leaves, TL: terbinafine treated leaves, UR: Untreated roots, TR: terbinafine treated roots. Error bars represent SE ($p \leq 0.05$) based on values obtained from three independent experiments.

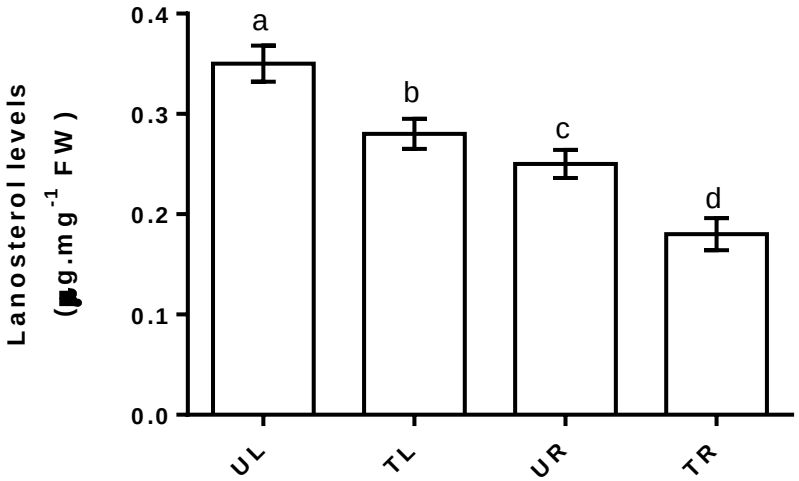


Figure 3.7 Lanosterol content in response to sterol synthesis inhibition. The effect of sterol synthesis inhibition on levels of lanosterol in maize leaves and roots was measured. Sterols were extracted from plant tissue and quantified using GC-MS. UL: Untreated leaves, TL: terbinafine treated leaves, UR: Untreated roots, TR: terbinafine treated roots. Error bars represent SE ($p \leq 0.5$) based on values obtained from three independent experiments.

Table 3.3: Sitosterol,Stigmasterol and β-sitosterol content in response to sterol synthesis

	Control leaves	Terbinafine treated leaves	Control roots	Terbinafine treated roots
β-sitosterol	**0,35 ± 0.021[a]**	**0,28 ± 0.038[b]**	**0,25 ± 0.018[a]**	**0,18 ± 0.018[b]**
Campesterol	**0,43 ± 0.035[a]**	**0,38 ± 0.018[b]**	**0,30 ± 0.013[a]**	**0,21 ± 0.037[b]**
Stigmasterol	**0,32 ± 0.025[a]**	**0,27 ± 0.050[b]**	**0,28 ± 0.048[a]**	**0,22 ± 0.048[b]**

The units of measurement are µg/mg of fresh weight. Data represents means from three independent biological replicates. Different letters mark statistically different values in a row based on SE ($p \leq 0.05$)

3.3.2 MDA and H_2O_2 increase in response to reduced sterol content in leaves

Given the role of phytosterols in plant growth and development as well as evidence of their involvement in the regulation of ROS production (Lindsey et al., 2003; Posé et al., 2009b; Schaller, 2003), the effect of sterol inhibition on H_2O_2 and MDA content was determined in order to evaluate the effect of changes in sterol content on ROS production and lipid peroxidation. Maize was grown in Promix containing 50 µM terbinafine. Plants were harvested at V1 and the amount of H_2O_2 and MDA present in roots and leaves of treated and untreated plants was measured. The results are shown in Figure 3.8. There were no significant differences in the root H_2O_2 content and root MDA content of treated and untreated plants. The MDA content of untreated leaves was 4 times lower than that of terbinafine treated leaves (Figure 3.8b). Treatment with terbinafine resulted in a 0.5 fold increase in H_2O_2 content in leaves (Figure 3.8a).

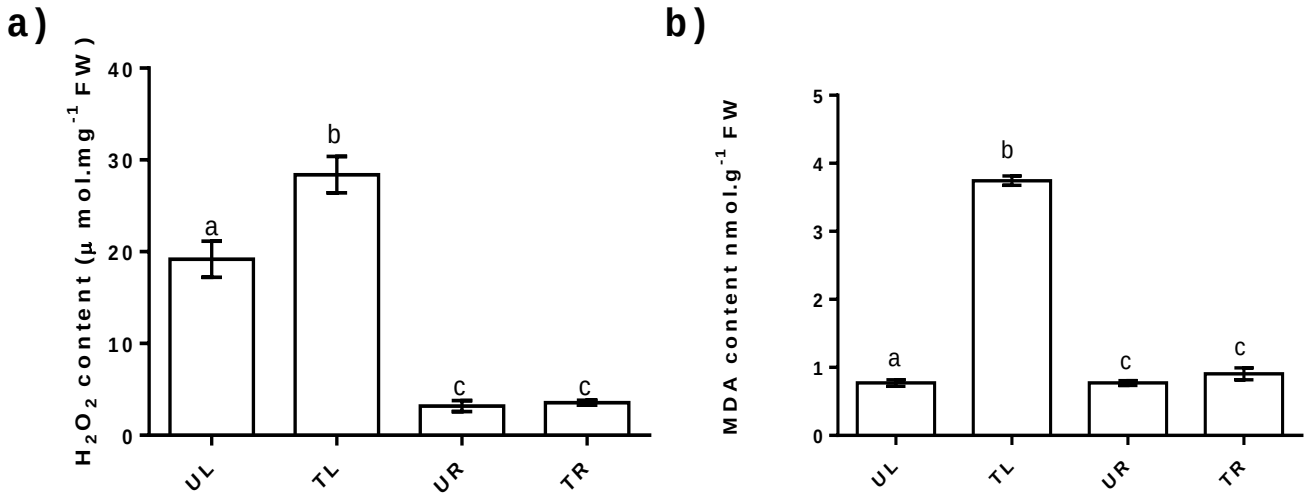


Figure 3.8 The effect of sterol synthesis inhibition on a) ROS production (H_2O_2 content) b) lipid peroxidation (MDA content) in maize leaves and roots at the V1 stage of vegetative growth. The bars represent mean values obtained from three biological replicates, error bars represent SE and the bars with different letters are significantly different at $p \leq 0.05$. UL: Untreated leaves, TL: terbinafine treated leaves, UR: Untreated roots, TR: terbinafine treated root

3.3.3 Inhibition of sterol synthesis changes antioxidant enzyme activity

Given that drought affected the sterol content, as shown in Section 2 of this chapter, the effect of sterol synthesis inhibition on the activity of antioxidant enzymes was investigated in order to elucidate the involvement of sterols in ROS signalling.

Six GPX isoforms were identified in both leaves and roots (Figure 3.9a). The activity of GPX 1 to 5 isoforms increased in response to sterol inhibition (Table 3.3) in leaves. In roots, the activity of three isoforms, GPX 1, 2 and 6 did not change and all the other isoforms had increased activity in response to terbinafine treatment, except GPX3 for which there was a slight decrease in activity.

Four APX isoforms were detected in roots and two were detected in leaves of both untreated and terbinafine treated plants (Figure 3.9b). There was no difference in the activities of APX 1 and APX 2 in roots in response to treatment and root APX3 and 4 activities did not change

significantly; whereas leaf APX 3 was reduced slightly increased (Table 3.3b). No change in APX 5 activity was observed in response to terbinafine in roots but there was a decrease in its activity in leaves.

Four SOD isoforms were detected in leaves of plants treated with terbinafine (Figure 3.9c) and the activity of two of these isoforms decreased in response to terbinafine treatment. Two isoforms were detected in roots, isoforms 2 and 3 found in leaves were not active in roots. The activity of SOD isoforms 1 and 4 in roots was increased by treatment with terbinafine whereas the activities of all SOD isoforms in leaves were decreased.

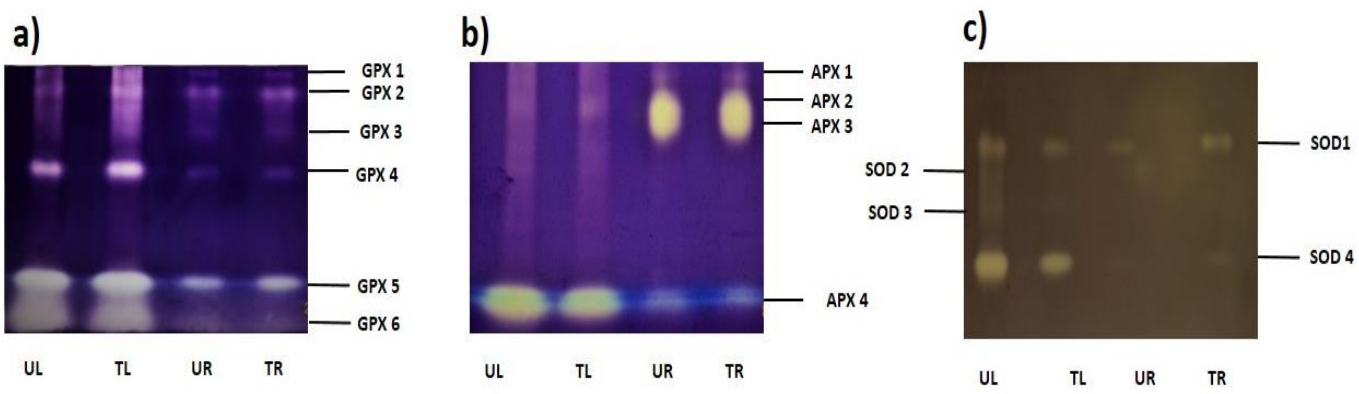


Figure 3.9 Effect of sterol synthesis inhibition on ROS detoxification enzymes in maize leaves and roots at the V1 stage of vegetative growth. Native gels (12%) were stained for the activity of a) GPX, b) APX and c) SOD isozymes using methods described by Dewir et al. (2006). Equal amounts of protein from leaves (40µg) and roots (10 µg) subjected to treatments were loaded on gels. UL: Untreated leaves, TL: terbinafine treated leaves, UR: untreated roots, TR: terbinafine treated roots.

Table 3.3 Relative enzymatic activity of maize GPX, APX and SOD isoforms in response to treatment with terbinafine

a)

Relative GPX Activity (Arbitrary units)	GPX isoforms	Samples			
		UL	TL	UR	TR
	GPX 1	1.0 ±0.05^{a}	3.38±0.20^{b}	1.0 ±0.05^{a}	1.06 ± 0.001^{a}
	GPX 2	1.0 ± 0.05^{a}	2.63 ± 0.02^{b}	1.0 ± 0.05^{a}	1.20 ± 0.06^{a}
	GPX 3	1.0 ± 0.05^{a}	2.71 ± 0.09^{b}	1.0 ± 0.05^{a}	1.15 ± 0.03^{b}
	GPX 4	1.0 ± 0.05^{a}	1.76 ± 0.01^{b}	1.0 ± 0.05^{a}	0.92 ± 0.03^{b}
	GPX 5	1.0 ± 0.05^{a}	1.31± 0.03^{b}	1.0 ± 0.05^{a}	1.12± 0.02^{b}
	GPX 6	1.0 ± 0.05^{a}	1.05± 0.04^{b}	1.± 0.05^{a}	0.98± 0.02^{b}

b)

Relative APX Activity (Arbitrary units)	APX isoforms	Samples			
		UL	TL	UR	TR
	APX 1	No activity	No activity	1.0 ± 0.05^{a}	0.98 ±0.004^{a}
	APX 2	No activity	No activity	1.0 ±0.05^{a}	1.26 ±0.12^{a}
	APX 3	1.0 ± 0.05^{a}	1.41 ± 0.08^{b}	1.0 ±0.05^{a}	0.97±0.005^{a}
	APX 4	No activity	No activity		
	APX 5	1.0 ± 0.05^{a}	0.90 ±0.007^{b}	1.0 ±0.05^{a}	0.94±0.03^{a}

c)

Relative SOD Activity (Arbitrary units)	SOD isoforms	Samples			
		UL	TL	UR	TR
	SOD 1	1.0 ±0.05^{a}	0.93±0.005^{b}	1.0 ±0.05^{a}	1.16 ±0.002^{b}
	SOD 2	1.0 ±0.05^{a}	0.57 ±0.02^{b}	No activity	No activity
	SOD 3	1.0 ±0.05^{a}	0.51 ±0.03^{b}	No activity	No activity
	SOD 4	1.0 ±0.05^{a}	0.72 ±0.01^{b}	1.0 ±0.05^{a}	1.19 ±0.05^{b}

Data presented in this table are the means ± standard error of three biological replicates (n = 3). Means marked with different letters in the same row for the same isoform indicate statistically significant difference with p values ≤ 0.05. UL: Untreated leaves, TL: terbinafine-treated leaves, UR: untreated roots, TR: terbinafine-treated roots.

3.4 The effect of drought on the expression of a candidate cycloartenol synthase gene (ZMCAS494)

3.4.1 *In silico* characterization of the candidate cycloartenol synthase gene (GRMZM2g065494)

To elucidate the mechanism with which the reduction in sterol content during water stress is achieved in this species, a maize candidate cycloartenol synthase gene was identified and functionally characterised. Drought responsive promoter elements were predicted and the effect of drought on expression of this gene was determined.

The protein sequence of ZMCAS494 (accession number, GRMZM2g065494) was obtained using the key word search on Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html). Characterized cycloartenol synthase sequences from *A. thaliana*, *O. sativa* and *N. tabacum* were obtained from GenPept (http://www.ncbi.nlm.nih.gov/). All these sequences were then aligned and DCTAE motif found in cycloartenol synthase genes was identified (Figure 3.10). The terpene synthase signature was also identified. The maize candidate cycloartenol synthase gene ZMCAS494 had 97% similarity with *A. thaliana* CAS1, 98% similarity with *O. sativa* CAS and 99% similarity with *N. tabacum*. The terpene synthase signature occurs at amino acid 601 to 615 and the DCTAE motif was found at amino acid 801 to 805 on the ZMCAS494 sequence. The subcellular localization of a protein is closely related to its function (Jensen et al., 2002). For evidence of the function of ZMCAS494 in *Zea Mays*, the subcellular localization of the protein was predicted using the subcellular prediction programme for eukaryotic proteins. The program predicted ZMCAS494 localisation the presence of a mitochondrial transit peptide with a reliability class of 4. The result is depicted in figure 3.11.

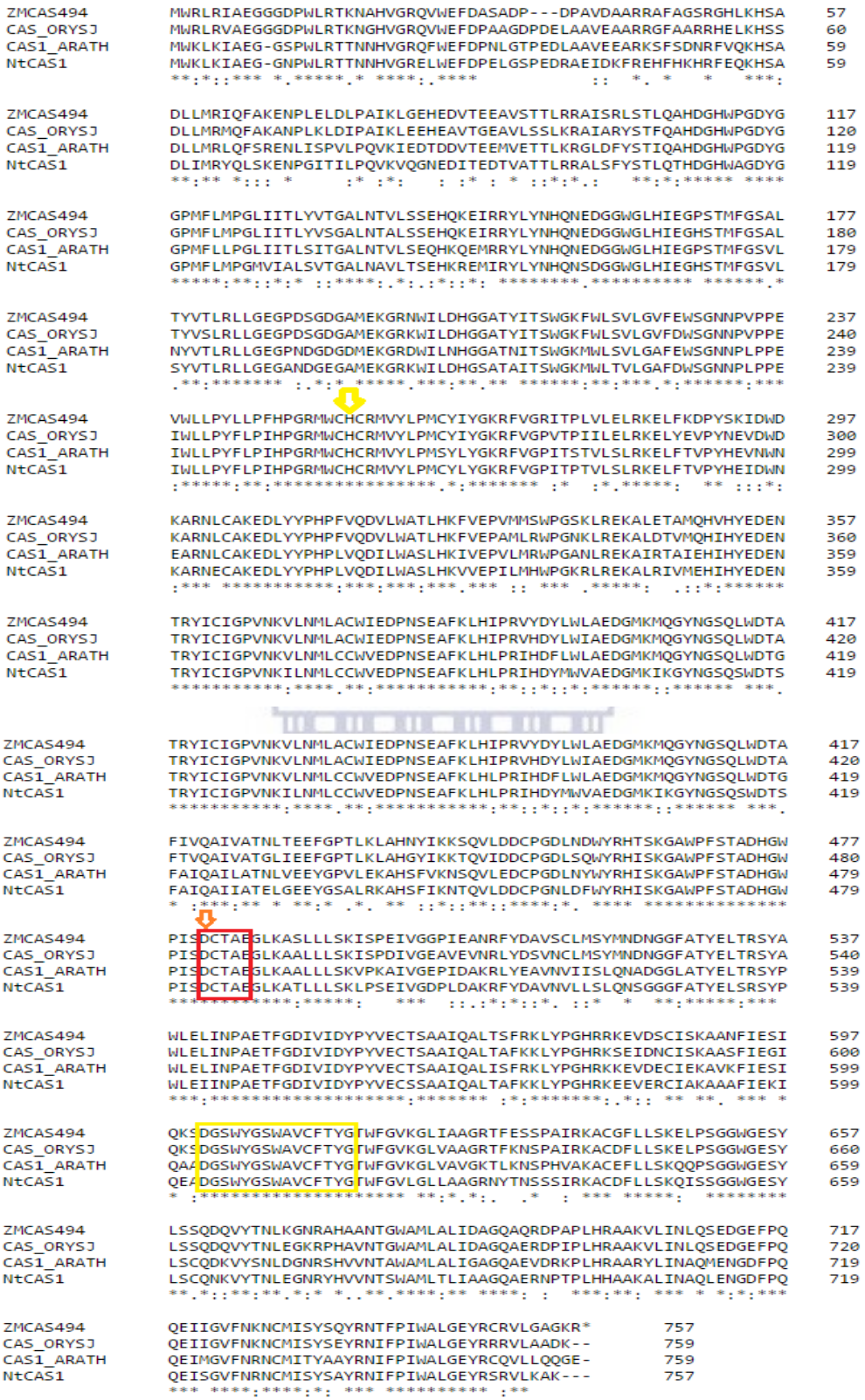
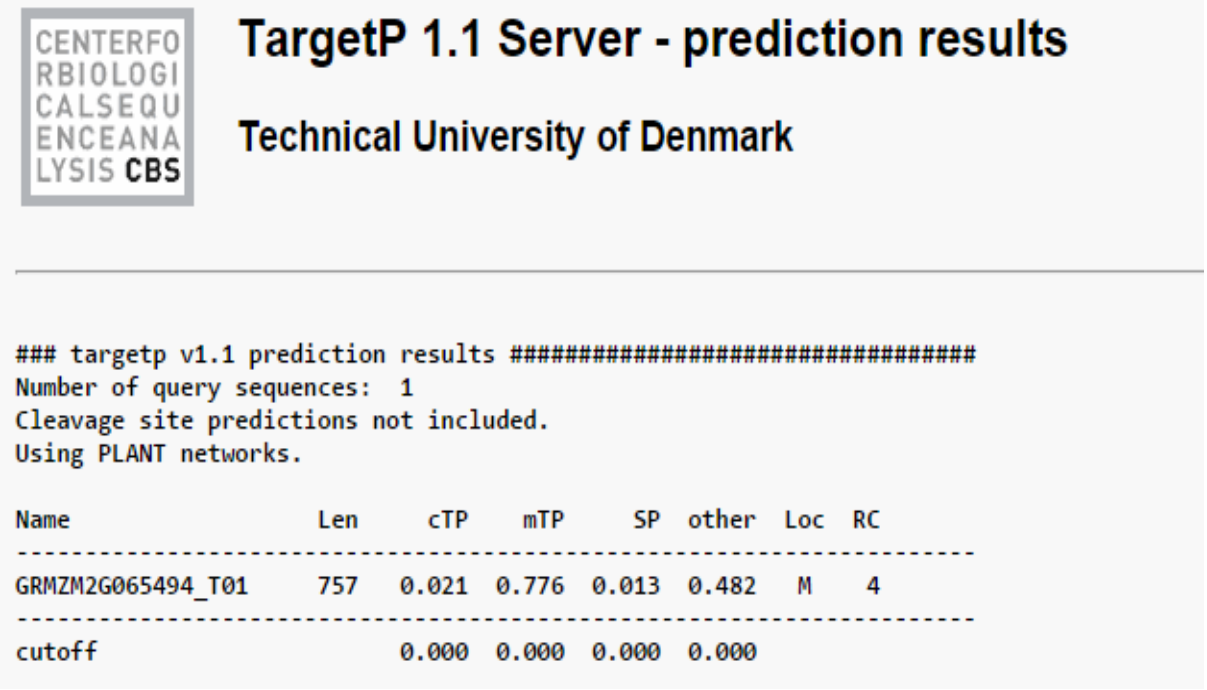


Figure 3.10 A Clustal Omega multiple sequence alignment of selected cycloartenol synthase (CAS) amino acid sequences. CAS_ORYSJ: *Oryza sativa;* CAS1_ARATH: *Aradipobsis thaliana*; NtCAS1: *Nicotiana tabacum*; ZMCAS494: *Zea mays*. The DCTAE motif is boxed in red and catalytic amino acid residues are marked with arrowheads (Tyr 410, His 477 and Ile 481 in *Arabidopsis thaliana* numbering). The terpene synthase signature DGSWyGsWAVcFtYG is boxed in yellow.



TargetP 1.1 Server - prediction results

Technical University of Denmark

```
### targetp v1.1 prediction results ###################################
Number of query sequences:  1
Cleavage site predictions not included.
Using PLANT networks.

Name                  Len     cTP    mTP     SP  other  Loc  RC
-----------------------------------------------------------------------
GRMZM2G065494_T01     757   0.021  0.776  0.013  0.482   M    4
-----------------------------------------------------------------------
cutoff                      0.000  0.000  0.000  0.000
```

Figure 3.11 Subcellular localization prediction results. The final scores for various subcellular site transit peptides (cTP: chloroplast transpeptide; mTP: mitochondrial transpeptide; SP: signal peptide), on which the final prediction is based are shown in the table. The scores are not really probabilities, and they do not necessarily add to one. However, the location with the highest score is the most likely a, and the relationship between the scores also known as the reliability class (RC) may be an indication of how certain the prediction is. Reliability class values are from 1 to 5, where 1 indicates the strongest prediction. Len: peptide length.

3.4.2 GRMZM2g065494 expression in response to treatment with terbinafine

The first step committed to the sterol branch of the isoprenoid pathway is catalysed by the squalene synthase (SQS), which mediates the reductive head-to head condensation of two molecules of FPP to form squalene via presqualene diphosphate (Piironen et al., 2000). Squalene is converted to by squalene epoxidase (SQE) to (*3S*)-2,3-oxidosqualene through a stereospecific reaction in the presence of molecular oxygen (Ohyama et al., 2009; Piironen et al., 2000).

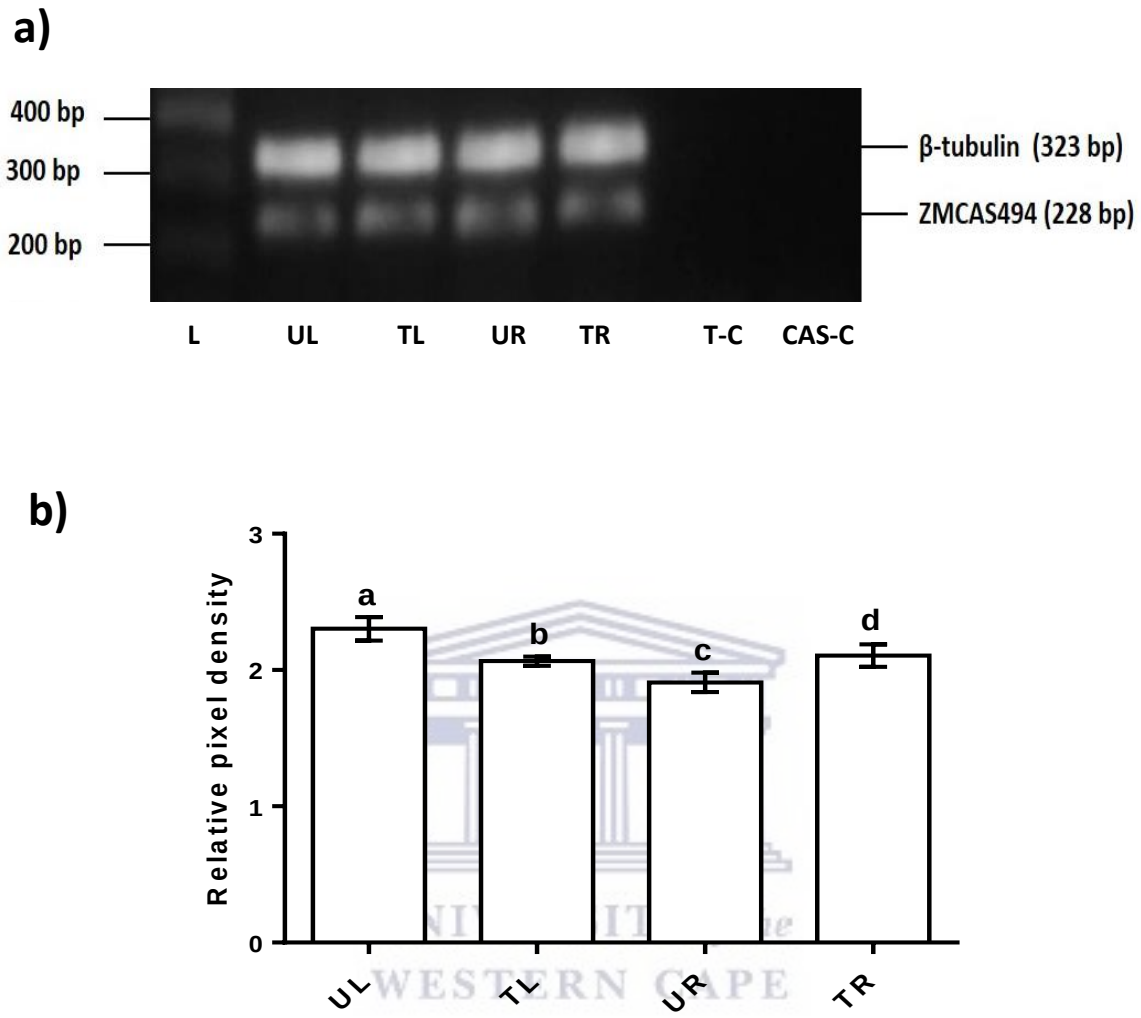


Figure 3.12 Gene expression of ZMCAS494 in response to treatment with terbinafine. The effect of the sterol synthesis inhibitor on mRNA levels of ZMCAS494 in maize leaves and roots at V1 was measured using semi-quantitative RT-PCR. The gene was amplified from cDNA synthesized from 300 ng mRNA in all samples. a) Gel electrophoresis of all PCR products was performed on a 1.5 % agarose gel at 80V. Maize β-tubulin was used as a reference gene. b) Gene expression was quantified by comparing pixel densities of bands formed by ZMCAS494 PCR products relative to β-tubulin products. Error bars represent SEMs based on three gel images and the values indicated by different letters are significantly different at $p \leq 0.05$. UL: Untreated leaves, TL: terbinafine treated leaves, UR: Untreated roots, TR: terbinafine treated roots, CAS-C: negative control using ZMCAS494 primers.

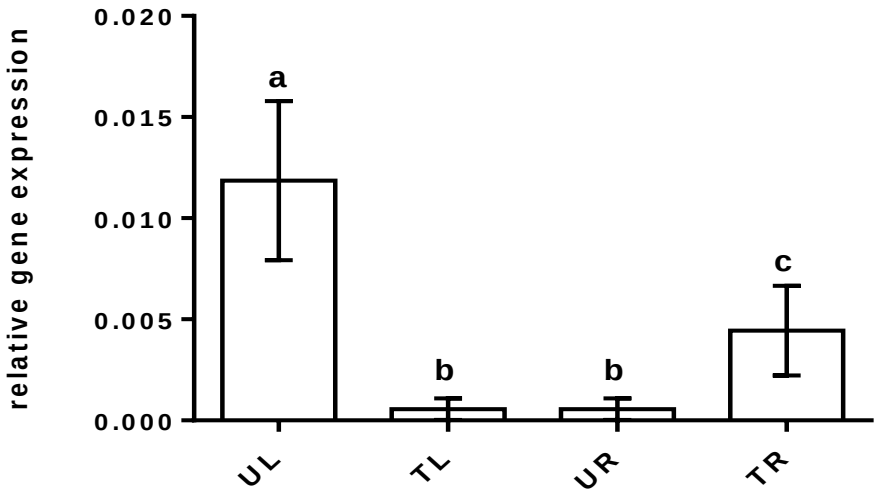


Figure 3.13 Measurement of gene expression of ZMCAS494 in response to treatment with terbinafine using relative qPCR. The effect of the sterol synthesis inhibitor on mRNA levels of ZMCAS494 in maize leaves and roots at V1 was measured using quantitative RT-PCR. The gene was amplified from cDNA synthesized from 300 ng mRNA in all samples. Relative gene expression was measured using the $2^{\Delta Ct}$ method described in Livak & Schmittgen (2001) and β tubulin was used as the internal control. Error bars represent SEMs based on three technical repeats and the values indicated by different letters are significantly different at $p \leq 0.05$. UL: Untreated leaves, TL: terbinafine treated leaves, UR: Untreated roots, TR: terbinafine treated root

Terbinafine is a chemical compound with the ability to inhibit fungal squalene epoxidase (SQE) and has been shown to have the same effect in plants (Gas-pascual et al., 2014; Wentzinger, Bach, & Hartmann, 2002). SQEs function in the sterol biosynthetic pathway is to catalase formation of oxidosqualene, the precursor of cycloartenol.

The ZMCAS494 gene is predicted to encode cycloartenol synthase, an enzyme whose function is to convert oxidosqualene to cycloartenol. Oxidosqualene is produced from squalene oxide by SQE. An SQE inhibitor would result in decreased or no production of oxidosqualene, the substrate used by cycloartenol synthase. To further confirm the predicted function of the ZMCAS494 gene in maize, the effect of treatment with terbinafine on gene expression of ZMCAS494 was determined using semi-quantitativr PCR as well as quantitative PCR. The

presence of terbinafine in the growth medium resulted in a reduction in ZMCAS494 mRNA levels in leaves and an increase in mRNA levels of the same gene in roots (Figure 3.11 and 3.12).

3.4.3 Identification of drought responsive promoter elements

The PlantPAN 2.0 (http://plantpan2.itps.ncku.edu.tw/search.php) promoter analysis programme was used to predict transcription factor binding sites (TFBS) from the sequence 1500 bp upstream of the start codon of the coding sequence of ZMCAS494. The programme was set to predict TFBS using the *Zea mays*, *A. thaliana* and *O. sativa* data bases and predicted TFBS with a score of above 0.75 were selected. Sites for transcription factors that are responsive to stress signals as well as those involved in regulation of sterol biosynthesis were identified and are listed in Table 3.1. Stress responsive promoter elements as well as those that are involved in regulation of sterol biosynthesis were identified and are listed in Table 3.1.

Binding sites that belong to the bZip , LEA ,bHLH ,AP2,ARF ,C2H2 transcription factor families were identified. The bZip family of transcription factors are bZIP proteins with a specific structure that allows them to control gene activity. Each bZIP protein has a leucine zipper region, the region contains leucine at specific sites (Glover et al.,1995). The leucines (or related amino acids) of two bZIP proteins interact with one another, bringing the proteins together into a structure called a dimer. Two copies of the same bZIP protein may bind to each other, or two different bZIP proteins may interact (Glover et al., 1995).

Table 3.5: Stress responsive and terpene biosynthesis transcription factor binding sites found in the sequence 1700 bp upstream of grmzm2g065494

Transcription Factors				Recognition sites		
gene ID	Family	Species	Function	Sequence	Position	Score
Abscisic acid responsive elements-binding factor 4 (ABRE4)	Basic Leucine Zipper (bZIP)	*Arabidopsis thaliana* *Zea mays* *Oryza sativa*	Functions as transcriptional activator in the ABA-inducible expression of rd29B. Binds specifically to the ABA-responsive element (ABRE) of the rd29B gene promoter.	ACACT	120 560 1029 1200 1287 1287	0.75 0.75 0.75 0.75 0.75 0.75
Embryonic abundant protein 1	Late embryonic abundant (LEA_5)	*Oryza sativa*	Regulation of gene expression of cytoplasm protectants during desiccation	CATGC	364 457	0.75 0.75
Transcription factor MYC2 (AtMYC2) Protein JASMONATE INSENSITIVE 1 Z-box binding factor 1 protein(ZBF1) RD22 binding protein 1 (RD22BP1) R-homologous Arabidopsis protein 1 (RAP-1) Basic helix-loop-helix protein 6 (AtbHLH6	Basic helix-loop-helix (bHLH)	*Arabidopsis thaliana*	Common transcription factor of light, ABA and JA signalling pathways In cooperation with MYB2 is involved in the regulation of ABA-inducible genes under drought stress conditions Positive regulator of lateral root formation Regulates sesquiterpene biosynthesis	CACNTG	1270	1

Transcription Factors				Recognition sites		
gene ID	Family	Species	Function	Sequence	Position	Score
ABSCISIC ACID-INSENSITIVE 3 (ABI3)	Apetala 2 (AP2); Ethylene responsive binding factor (ERF)	*Arabidopsis thaliana* *Zea mays* *Oryza sativa*	Participates in abscisic acid-regulated gene expression during seed development Also regulates the terpenoid biosynthetic process	GCATG	458	0.97
Dehydration-responsive element-binding protein 1D	Apetala 2 (AP2)	*Zea mays* *Oryza sativa*	response to salt stress, response to water stress, regulation of gibberellin biosynthetic process	CCGAC	1447	0.91
Protein SHINE 1 Integrase-type DNA-binding superfamily protein	Apetala 2 (AP2)	*Arabidopsis thaliana*	Promotes cuticle formation by inducing the expression of enzymes involved in wax biosynthesis Confers drought resistance Binds to the GCC-box pathogenesis-related promoter element May be involved in the regulation of gene expression by stress factors and by components of stress signal transduction pathways	CGCCG	1609 1542	0.95 0.97

Transcription Factors				Recognition sites		
gene ID	Family	Species	Function	Sequence	Position	Score
Dehydration-responsive element-binding protein 1F (Protein DREB1F)	Apetala 2 (AP2); Ethylene responsive binding factor (ERF)	*Arabidopsis thaliana*	mediates cold or dehydration-inducible transcription Binds to the GCC-box pathogenesis-related promoter element. May be involved in the regulation of gene expression by stress factors	AG]CCGAC	1449	1
Zinc finger protein (ZAT10) salt tolerance zinc finger (STZ)	C2H2	*Arabidopsis thaliana*	Transcriptional repressor involved in abiotic stress responses Can repress the stress responsive genes DREB1A and LTI78 May regulate the expression of the JA biosynthesis gene LOX3 and control the expression of TIFY10A/JAZ1, A key repressor in the JA signalling cascade.	ACACT	125 1199 1349	1 1 1

The paired bZIP proteins form two extended arms that grasp the DNA molecule, like a pair of chopsticks (Glover et al., 1995). When the proteins bind to DNA, they control the activity of genes that are involved in various cellular and developmental processes such as pathogen defence, light and stress signalling, seed maturation and flower development (Jakoby et al., 2002; Rian & Schrago, 2008). Five recognition sites for the abscisic responsive element binding factor 4 (ABRE 4) were identified in the promoter region of ZMCAS494. ABRE4 is found in *Zea mays*, *Arabidopsis thaliana* and in *Oryza sativa*. The transcription factor belongs to the bZip family and it activates transcription of abscisic acid responsive genes.

Two recognition sites for the late embryonic abundant protein 1 were identified 1336 and 1243 bp away from the start codon. Late embryonic abundant proteins (LEA proteins) as the name suggests, are highly expressed during the late period of embryo development in plants. They are mainly composed of hydrophilic amino acids ordered in repeated sequence (e.g. Gly and Lys), forming hyper-hydrophilicness and thermal stability (Hong-Bo et al., 2005). LEA proteins mainly play a role in dehydration tolerance as well as in whole-plant stress tolerance to drought, salt, and cold (Hong-Bo et al., 2005) .

The sequence, CACCT, occurs 430 bp from the transcription start site and is recognise by various bHLH proteins. These transcription factors are functionally diverse and are characterised by a region with two functionally different domains (Jones, 2004). The amino-terminal end of the region is the basic domain and is responsible for recognition of the promoter element. The carboxy-terminal end of the region is the helix loop helix domain and is responsible for formation of homo and heterodimeric complexes with other proteins (Jones, 2004). There are different classes of bHLH proteins and they recognise different promoter elements (Jones, 2004). The site identified in ZMCAS494 is recognised by transcription factor MYC2, JASMONATE INSENSITIVE 1,Z-box binding factor 1 protein, RD22 binding protein 1, R-homologous Arabidopsis protein 1 (RAP-1) and basic helix-loop-helix protein 6

(AtbHLH6). These proteins play a role in sesquiterpene biosynthesis, regulation of ABA-inducible genes under drought stress conditions as well as lateral root formation.

Zinc finger (Znf) domains are relatively small protein motifs which contain multiple finger-like protrusions that make tandem contacts with their target molecule. The Cystein2/Histidine 2 (C2H2) class of Znf proteins contain a short beta hairpin and an alpha helix (beta/beta/alpha structure), where a single zinc atom is held in place by Cys (2) His (2) residues in a tetrahedral array (Sakamoto, Araki, Meshi, & Iwabuchi, 2000). The characterized plant C2H2 zinc finger proteins are mainly involved in plant growth and development and the responses to environmental stresses. Recognition sites for two types of C2H2 zinc finger transcription factors was identified in the promoter sequence of ZMCAS494.

The APETALA 2/ethylene-responsive element binding factor (AP2/ERF) family is a large group of plant-specific transcription factors that are involved in plant abiotic stress responses by regulating gene expression (Mizoi, Shinozaki et al., 2012). They also play a role in primary and secondary metabolism, growth and developmental programs, as well as responses to environmental stimuli (Licausi et al., 2013).The AP2/ERF domain found in these proteins contains an N-terminal, three-strand β-sheet that recognises a target sequence, as well as a C-terminal α-helix (Mizoi et al., 2012; Ofuku, 1997). The AP2/ERF transcription are divided into the AP2, RAV, ERF and dehydration-responsive element-binding protein (DREB) subfamilies and each of these sub families recognise distinct promoter elements and have distinct roles in plants (Mizoi et al., 2012). Recognition sites for ABSCISIC ACID-INSENSITIVE 3 (ABI3), Dehydration-responsive element-binding protein 1D, SHINE 1 and Integrase-type DNA-binding protein were identified in the promoter regions of the sequence of ZMCAS494.

3.4.6 Drought affects GRMZM2g065494 gene expression

Cycloartenol synthase catalyses conversion of oxidosqualene to cycloartenol during sterol biosynthesis. Several studies have indicated that plants that are mutant for genes that play a role in the sterol biosynthetic pathway have increased sensitivity to drought stress and exogenous application or overexpression of plant brassinosteroids improves drought stress tolerance (Piironen et al., 2000; Posé et al., 2009b; Schaller, 2003). These studies suggest that sterols play a role in plant drought responses. In this study, drought has been shown to cause a reduction in sterol content. To determine whether ZMCAS494 plays a role in maize drought responses and to elucidate the mechanism by which sterol content is reduced in response to drought, the effect of drought on expression of ZMCAS494 in maize was investigated.

Maize plants were subjected to drought stress and the effect of water deprivation on mRNA levels of ZMCAS494 in maize leaves and roots at the V1 stage of development was determined using semi-quantitative (Figure 3.14) and quantitative PCR (Figure 3.15). Levels of ZMCAS494 mRNA were significantly reduced in maize roots in response to drought. Measurement of mRNA levels using quantitative PCR showed no significant changes in ZMCAS494 gene expression in leaves. Semi-quantitative PCR showed a slight decrease in mRNA levels in response to drought.

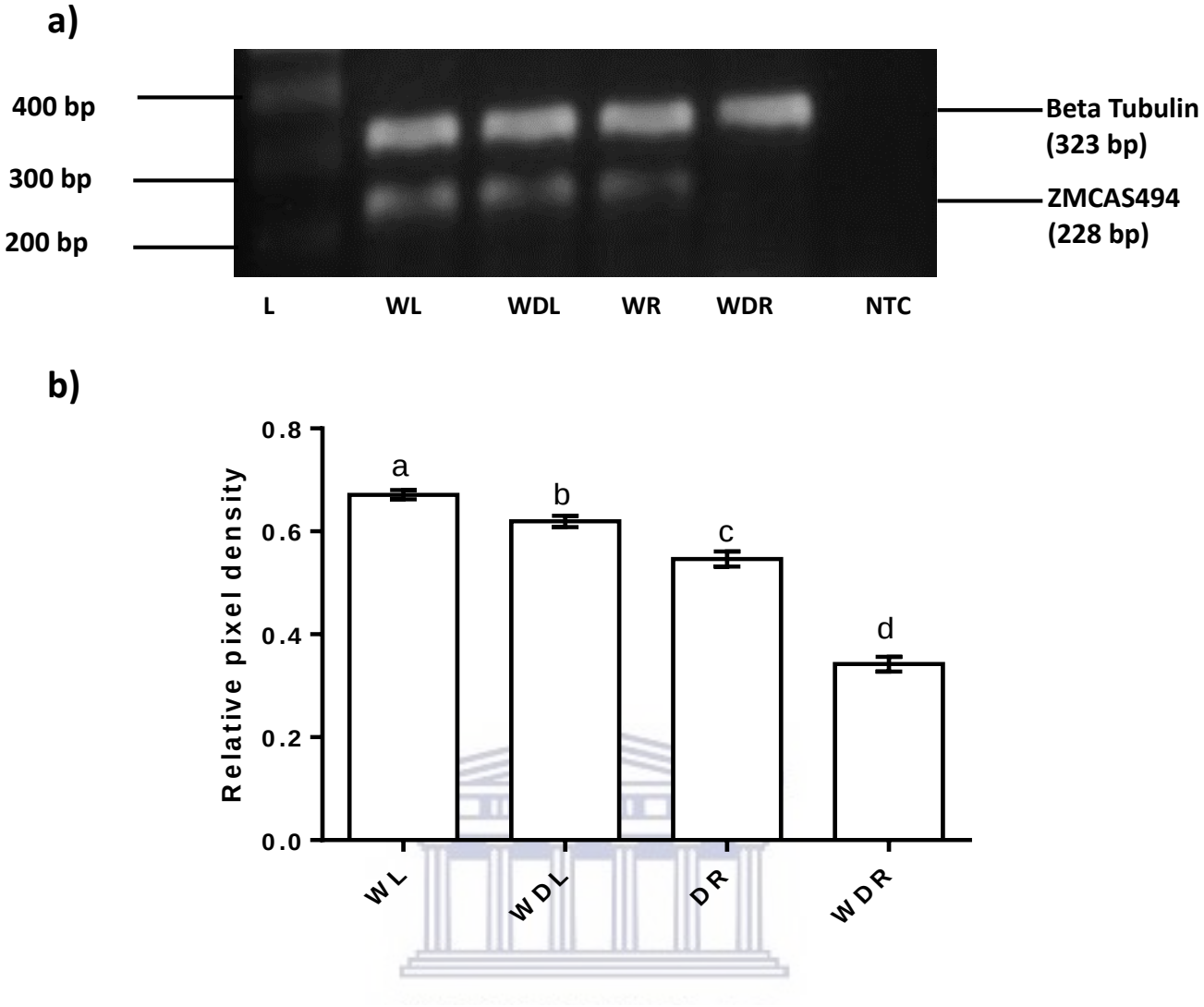


Figure 3.14 Relative expression of ZMCAS494 in response to water deprivation. The effect of water deprivation on mRNA levels of ZMCAS494 in maize leaves and roots at V1 was measured using semi-quantitative RT-PCR. The gene was amplified from cDNA synthesized from 300 ng mRNA in all samples. a) Gel electrophoresis of all PCR products was performed on a 1.5 % agarose gel at 80V. Maize β-tubulin was used as a reference gene. b) Gene expression was quantified by comparing pixel densities of bands formed by ZMCAS494 PCR products relative to β-tubulin products. Error bars represent SEMs based on three gel images. L – 100 bp DNA ladder, WL: Watered leaves, WDL: water deprived leaves, WR: watered roots, WDR: water deprived roots, NTC: No template control

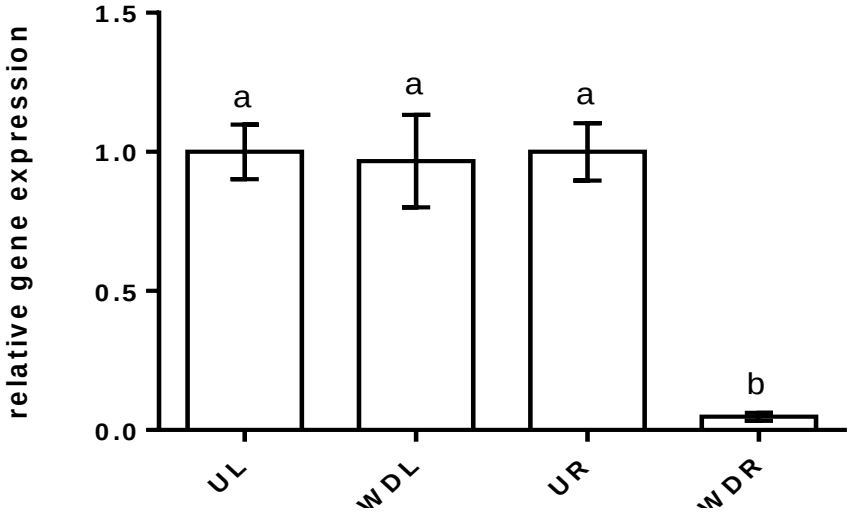


Figure 3.15 Measurement of gene expression of ZMCAS494 in response to water deprivation using relative qPCR. The effect of the water deprivation on mRNA levels of ZMCAS494 in maize leaves and roots at V1 was measured using quantitative RT-PCR. The gene was amplified from cDNA synthesized from 300 ng mRNA in all samples. Relative gene expression was measured using the 2ΔCt method Livak & Schmittgen (2001) and β- tubulin was used as the internal control. Error bars represent SEMs based on three technical repeats and the values indicated by different letters are significantly different at $p \leq 0.05$. WL: Watered leaves, WDL: water deprived leaves, WR: watered roots, WDR: water deprived roots.

CHAPTER 4: DISCUSSION AND CONCLUSION

The aim of this study was to investigate the involvement of phytosterols in *Zea mays* responses to drought stress. In order to achieve this aim, maize was subjected to drought and the effects of drought were determined to ensure that the responses observed were a result of water deficit stress. The water requirement of plants varies across tissues and the growth stages of the same species. Consequently, it was important that both the shoot and root tissues of maize plants that were at the same stage of development are analysed.

Growth is achieved through cell division, cell enlargement and differentiation, and involves genetic, physiological, ecological and morphological events and their complex interactions. The quality and quantity of plant growth depends on these events, which are affected by water deficit. Growth is characterised by component parameters which can be estimated by characteristics such as plant height and plant biomass (Aslam et al., 2015). It has been shown that drought stress during vegetative growth stages 1 to 5 (V1 to V5) reduces growth rate and thus adversely affects development at later stages (Aslam et al., 2015). For purposes of this study, plants were grown under water deficit conditions (water potential = -0.21MPa) and field capacity (water potential = -0.03 MPa) and harvested at V1.

To examine the extent of water deficit stress, the RWC of water-deprived plants was determined and compared to that of well-watered plants. Since the mid-80s, RWC has been used and known as a suitable measure for plant water status and can reliably indicate the balance between absorbed water and water consumed through transpiration (Arjenaki et al., 2012). The relative water content analysis in this study yielded similar results. The RWC of

leaves and roots was significantly reduced in response to water deprivation. Li-ping et al. (2006) investigated the effect of drought stress on maize RWC and discovered that severe drought stress reduces leaf RWC. Several other studies have been performed and indicate that drought results in a reduction in plant RWC (Good & Maclagan 1993; Valentović et al. 2006; Li-ping et al. 2006; Yamasaki & Dillenburg 1999; Khodarahmpour & Hamidi 2011).

The water status or the amount of water present in plant tissues is related to several physiological variables, such as leaf turgor, growth, stomatal conductance, transpiration, photosynthesis and respiration (Kramer & Boyer, 1995). A correlation between RWC, the transpiration rate as well as water uptake has been reported in plant tissues (Aslam et al., 2015). Reduced relative water content results in reduced transpiration-dependent cooling of leaves and thus an increase in leaf temperature. High leaf temperature under drought conditions inhibits enzymatic activity and reduces photosynthesis (Chaves et al., 2002). A reduction in relative water content as a result of water deprivation is thus an indication of water stress, reduced rate of photosynthesis and possible negative impact on metabolic processes and growth.

4.1 Drought slows down growth and causes a reduction in maize plant biomass

The impact of drought on growth of maize in this study was evaluated on the basis of changes in the length and fresh weight of shoots and roots. Plants that were grown under conditions of water deficit had a significantly shorter shoot length and fresh weight, which is a clear indication of reduced growth. This result is in agreement with the results of Avramova et al. (2015) and Avramova et al. (2016), where maize shoot growth was reduced as a consequence of inhibited cell growth and cell expansion caused by water stress. Shoot length and weight

was also reduced in *Prunus mongolica* (Guo et al., 2015), *A. thaliana* (Pandey et al., 2013) and *O. sativa* (Price et al., 2002) as a consequence of drought.

Growth is an increase in plant size and it is directly associated with an increase in the number of cells and cell size (Aslam et al., 2015). Growth impediment or slowing down of growth in plants suggests a reduction in the rate of cell division as well as cell size. Reduction of the rate of cell division also reduces leaf area, which is an adaptation strategy that reduces plant water requirements, allowing the plant to survive longer under conditions of water deficit (Yeo & Flowers, 2008). Following reduction in leaf area, light interception is reduced and this causes a decrease in biomass production (Delfine et al., 2001), hence the decrease in shoot fresh weight. It would have thus been useful to evaluate the effect of drought on cell number or cell size in this study in order to establish if the observed reduction in growth, consequent to water deficit, was a result of reduction in cell division (i.e. cell number) or cell size.

Roots are responsible for spatial and temporal water uptake and thus serve as detectors of soil water deficit in plants. Root length, root volume, root density and mass are characteristic structural traits and are reduced during severe water deficit (Aslam et al., 2015). Roots of *A.thaliana* plants grown in medium containing 40% PEG were shown to completely cease growth in a study conducted by Cao & Li (2014). Maize roots have also been shown to have reduced length and biomass in response to drought in several studies (Ashagre et al.,2014; Avramova et al., 2016; Bilgin et al., 2008). A reduction of root fresh weight and root length also occurred in response to water deficit in this study. This could have been due to a reduction in the rate of cell division or cell elongation. A reduction in root length occurs under conditions of severe water deficit and affects the ability of roots to absorb water and nutrients from the soil. Decreased water and nutrient uptake causes an increase of pH in the xylem tissue. This then transduces ABA mediated signals to the shoot tissue for prevention of water loss by stomatal closure (Wilkinson et al., 1998).

4.2 Drought results in oxidative stress in Zea mays

Several studies in maize (Li-ping et al., 2006; Yang et al., 2015) and other plant species have shown that the negative effects of drought on plant growth are, in part, caused by oxidative stress. Oxidative stress occurs as a result of increased accumulation of reactive oxygen species (ROS) such as $O_2^{\cdot-}$ that cause lipid peroxidation. In this study, drought resulted in an increase in H_2O_2 production in both leaves and roots, suggesting an increase in ROS production as a consequence of drought. The increase in ROS content resulted in lipid peroxidation, as indicated by the increase in MDA content. It can thus be concluded that drought stress caused an overproduction of ROS, causing destabilization of the cell membrane (due to lipid peroxidation.

As a survival strategy and means to combat oxidative stress, plants increase the activity of antioxidant enzymes such as SOD, APX, GPX as well as catalase. The enzymatic activity of SOD, APX and GPX in this study was higher in water deprived plants compared to well watered plants. This result is consistent with findings by Moussa & Abdel-Aziz (2008) and Li-ping et al. (2006). Moussa & Abdel-Aziz (2008) found that the activity of SOD and peroxidase enzymes in leaves of a drought sensitive and drought tolerant maize cultivar increase in response to drought. Li-ping et al. (2006) observed an increase in antioxidant enzyme activity in response to drought before the tasseling stage of maize development. Increased antioxidant enzyme activity during early developmental stages reduces the extent of oxidative stress and thus allows plants to continue growing, albeit slower than under normal conditions.

In roots, there was also an increase in the activity of most isoforms for the enzymes studied here. There was prominent increase in APX activity. An additional APX isoform, not present

in leaves was detected in roots. The increase in APX activity and the presence of the additional isoform would contribute to the reduction of oxidative stress.

4.3 Drought reduces sterol content in maize roots

Sterols are responsible for cell membrane fluidity and stability in plants. Aside from their functions in cell membranes, they are also suggested to play a role in plant abiotic stress responses, by either being involved in signalling pathways that combat water stress or having functions that reduce the effects of drought and other abiotic stresses on plants (Clause, 2002; Galea & Brown, 2009; Schaller, 2003; Vriet et al., 2012). Given the suggested role of sterols in plant stress responses, it would be reasonable to expect that abiotic stresses would cause changes in plant sterol content. Studies have been conducted to investigate the impact of various stresses on sterol content in plants. Roche et al. (2010) showed that the sterol content of sunflower seeds changes in response to high temperatures and suggested that sterols have an adaptive role against seed desiccation during high temperatures. In another study, conducted by Suh et al. (2013), the sterol content of *Lemna paucicostata* was altered when jasmonic acid and silver nitrate were added to plants, suggesting a role of phytosterols in reducing the negative effects of heavy metals on the plant. Recently, a role of phytosterols has been suggested *in* rice drought responses in a study by Kumar et al. (2015).

Given the data in literature, the role of phytosterols in *Zea mays* responses was determined by firstly examining the effect of drought on maize sterol content. There was no significant change in sterol content in leaves but a significant reduction in sterol content in roots was observed. These results conflicted with the findings of Kumar et al. (2015), where drought was shown to cause an increase in plant sterols. This can however be explained by genetic variation between maize and rice, which results in different biochemical responses to various

environmental conditions. Variation in sterol content based on genotype has been reported in various studies (Alignan et al., 2009; Nurmi et al., 2008).

Phytosterols participate in regulating cell membrane fluidity and permeability as well as in activation of membrane bound enzymes. It is thus reasonable to suggest that the decrease in sterol content seen in this study in response to drought justifies the damage to cell membranes, as demonstrated by lipid peroxidation. The reduction in sterol content caused by drought in maize roots possibly serves as a signal that triggers the activity of enzymes whose role is to strengthen cell membranes and prevent further cellular membrane damage. Antioxidant enzymes indirectly perform this function by reducing ROS content. Reduction in sterol content also possibly results in over production of ROS which then signals activation of antioxidant enzymes.

The effect of inhibition of sterol synthesis on ROS production as well as on the activity of antioxidant enzymes was investigated. The sterol content in plants treated with a sterol synthesis inhibitor was reduced in both leaves and roots, confirming that the inhibitor was effective.

In leaves, sterol synthesis inhibition resulted in significantly increased ROS production. This shows that sterol biosynthesis and regulation of ROS levels, mediated by antioxidant enzymes such as SOD and APX, form a signalling network that contributes to activities of antioxidant enzyme activity. Having established that overproduction of ROS causes damage to membrane lipids and given that there was no change in sterol content in response to drought in leaves, it is reasonable to suggest that a decrease in sterol content in leaves would likely not be favoured because it is detrimental to plant survival.

Reduction in sterol content caused by sterol synthesis inhibition had no effect on ROS production in roots, as suggested by the MDA and H_2O_2 content results. Inhibition of

phytosterol synthesis also had no impact on SOD and APX activity and this result was consistent with the fact that ROS content did not change. This suggests a possibility that regulation of sterol signalling in roots may differ from its regulation in leaves. Further study is required to elucidate the mechanisms by which sterol signalling in roots and leaves is regulated in relation to its role in the control of ROS accumulation.

4.4 Characterization of a maize cycloartenol synthase gene

To further elucidate the mechanism by which the reduction in sterol content in roots is achieved, a cycloartenol synthase gene (GRMZM2G065494) was identified and characterized *in silico*. The expression of the gene in response to sterol synthesis inhibition was also determined. When the candidate protein encoded by the identified gene sequence was aligned with cycloartenol synthase sequences from *A. thaliana*, *O. sativa* and *N. tabacum*, similarities above 90% were observed.

Identification of the DCTAE motif, which is well conserved among oxidosqualene cyclase (OSC) enzymes and is responsible for substrate protonation, as well as the terpene synthase signature sequence in the amino acid sequence of GRMZM2g065494 suggests that the gene encodes an OSC enzyme. These enzymes are responsible for oxidosqualene cyclization to produce sterols. In plants, oxidosqualene can be cyclized to form cycloartenol by cycloartenol synthase but recent studies have shown that oxidosqualene can also be cyclized to form lanosterol in plants. A gene encoding lanosterol synthases has been identified in various plant species. Cycloarternol synthases have a strict requirement for a His477 and I481 (*A.thaliana* numbering) in the vicinity of the conserved DCTAE motif implicated in substrate protonation, whereas all lanosterol synthase (LAS) proteins have a strict requirement for a V481. The catalytic His477 and I481 were also identified in the GRMZM2g045494 sequence, confirming that this gene encodes a cycloartenol synthase from maize.

Treatment with terbinafine resulted in increased expression of ZMCAS494 (GRMMZM2g04594) mRNA in roots and reduced expression in leaves. The change in expression of this gene in response to a sterol synthase inhibitor suggested that this gene in involved in sterol biosynthesis, further confirming its function as a cycloartenol synthase. Given the fact that the sterol content of roots was reduced in response to the same treatment, this result suggests a negative feedback mechanism that regulates production of sterols. Terbinafine inhibits synthesis of the substrate for cycloartenol synthase. Reduction of the substrate content as well as reduced cycloartenol content possibly signals up-regulation of gene expression of the cycloartenol synthase enzyme by activating transcriptional activators of ZMCAS494 in roots. In leaves, inhibition of cycloartenol substrate formation possibly signals up-regulation of the cycloartenol synthase gene expression. Further experiments that aim to elucidate the signalling pathway that regulates transcription of cycloartenol synthases in maize (e.g. quantification of oxidosqualene), would have to be performed in order validate this hypothesis.

4.5 ZMCAS494 gene expression is affected by drought stress

About 17 drought responsive TFBS in the region 1500 upstream of the transcription start site of ZMCAS494 were predicted. Recognition sites for the bZIP family of transcriptional factors, to which the ABRE4, belongs,were identified. The bZIP family controls the activity of genes that are involved in various cellular and developmental processes such as pathogen defence, light and stress signalling, seed maturation and flower development (Jakoby et al., 2002; Rian & Schrago, 2008) and ABRE4 activates expression of abscisic acid responsive genes. ABA is a hormone that is important for plant growth and development and plays a significant role in integrating various stress signals and controlling downstream stress responses. Recognition

sequences for transcription factors involved in the regulation of the expression of late embryogenesis abundant (LEA) proteins were also identified. LEA proteins, as the name suggests are expressed during late embryogenesis and are known to play a role in dehydration tolerance in plants. Identification of TFBS for these families of TF's suggested that ZMCAS494 is responsive to drought stress.

Expression of ZMCAS494 in response to drought was then determined using quantitative and semi-quantitative PCR. In leaves, ZMCAS494 mRNA expression did not change in response to drought and this result was consistent with the fact that sterol content in leaves did not change in response to drought. Root ZMCAS494 mRNA expression was reduced drastically in response to drought. Down-regulation of ZMCAS494 expression would result in reduced cycloartenol synthase and thus less activity of this enzyme in roots, reducing the rate of formation of the sterol synthase precursor, cycloartenol, hence the reduction in root sterol content in response to drought.

4.6 Conclusion

In conclusion, the study has shown that ZmCAS494 is a candidate cycloartenol synthase whose expression in roots is supressed during drought, resulting in low sterol content in maize roots. This reduced sterol content in roots in response to drought corresponds to increased ROS production and increased oxidative stress and may suggest a link between sterol biosynthesis and ROS production. However, there appears to be a much more complex interplay between sterol content and ROS content in relation to potential signalling of these molecules in the absence of drought since a decrease in root sterol content (achieved via use of a sterol biosynthesis inhibitor) did not alter ROS content in roots but did so in leaves. This may suggest

differences in ROS and sterol regulation in different organs and under different growth environments.

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