Dissertation

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Oral-examination:

Impact of drought stress on the sulfur assimilation pathway in *Zea mays*

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*This Thesis is dedicated to my parents, without their tremendous inspiration and encouragement it was not possible. I love you Mom and Dad, You gave me your wisdom to know when to turn away and when to charge ahead, you taught how to live right, to be gentle, to live day by day, to be patient and forgiving, to hope and to pray, to be proud of who I am and giving me the strength to always strive for better, no matter what. You are my rock and foundation. You are my angel in the darkness keeping the way ahead bright. You sheltered me all through the years, calmed my worries and my fears. Thanks to you, my hopes and dreams can never grow dim. "Allah took two pair of Angel wings, gentle loving hands, eyes that only see the good, a hearth that understands a smile to encourage, love that never ends…. He wrapped it up with tender care and called this gift as "**PARENTS**".*

Nisar Ahmad

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Summary

Maize is an important cereal crop that provides staple food to many populations. It is a major source of income for the farmers and is grown all over the world. Drought tolerance is the most important trait in maize, since limitation of water supply limits yield at most. The enhanced production of ROS during drought requires an increased GSH production for the efficient detoxification of ROS, thus the regulation of sulfur assimilation during drought is vital due to the dependency of GSH synthesis on the sulfur assimilation pathway. In this study I analysed the impact of drought on the sulfur assimilation pathway in maize.

Maize seedlings exposed to drought for 10 and 12 days were severely affected in leaf and root biomass due to a decrease in plant water content and caused elevated levels of H_2O_2. The drought-induced increase in the ROS formation altered the redox state of GSH pool towards a more oxidized state and indicated oxidative stress in leaves and roots of drought-treated plants compared to control. Moreover, induction of *GR* transcription in leaves and roots and an increase in GR activity in leaves under drought imply an important role of GR in ROS detoxification and maintaining reduced GSH during drought. The lower steady state level of thiols in leaves is a consequence of decreased rate of GSH biosynthesis during drought. A decrease in the sulfate contents was observed indicating low availability of sulfur in the shoot during drought. Accordingly, the up-regulation in the *Sultr1;1* and *Sultr4;1* that is responsible for sulfate efflux from the vacuole and a decrease in the steady state levels of sulfate most likely indicate sulfur-starved situation in leaves during drought. Moreover, the transcriptional up-regulation of more than two-fold in ATPS isoforms and an increase in steady state level of APS reflects that ATPS is also rate limiting and regulated by sulfur status during drought. A reduction in the incorporation of ^{35}S into cysteine and GSH suggests that drought limits the availability of sulfate to shoot, thus causing lower flux through the sulfur assimilation pathway into GSH.

On the other hand in roots, thiols, sulfate, APS and sulfide were increased relative to the control. A reduction in the incorporation of ^{35}S into cysteine and GSH during drought and the down-regulation of *Sultr4;1* indicate the storage of sulfate in the vacuole that might contribute to reduced flux into cysteine and GSH. A strong reduction was observed in the transport of labeled sulfate in the stem of drought stressed plants. This clearly indicates that drought limits the availability of sulfate to shoot, thereby causing the down regulation of sulfur assimilation pathway and ultimately elevated levels of H_2O_2 in leaves.

Zusammenfassung

Mais ist eine wichtige Nutzpflanze, die in vielen Ländern als Grundnahrungsmittel dient. Mais wird weltweit angebaut und ist vielerorts die Haupteinnahmequelle in der Landwirtschaft.
Trockenresistenz ist das wichtigste Züchtungsmerkmal in Mais, da Wasserknappheit der am stärksten limitierende Faktor für den Ernteertrag darstellt.
Die verstärkte Produktion von ROS während Trockenheit macht eine erhöhte GSH-Produktion zur effizienten Entgiftung von ROS nötig, weswegen die Regulation der Schwefelassimilation bei Trockenstress aufgrund der Abhängigkeit der GSH-Synthese vom Schwefelassimilations-Stoffwechselweg essentiell ist. In dieser Arbeit habe ich den Einfluss von Trockenheit auf den Schwefelassimilations-Pathway in Mais untersucht.
Maiskeimlinge, welche für zehn und zwölf Tage Trockenstress ausgesetzt waren, wiesen eine deutlich geringere Blatt- und Wurzelbiomasse auf, verursacht durch das Absinken des Wassergehalts, und einen erhöhten Gehalt an H_2O_2. Die durch Trockenheit erhöhte ROS-Bildung veränderte den Redoxstatus des GSH-Pools zu einem verstärkt oxidierten Zustand hin, was auf oxidativen Stress in Blättern und Wurzeln trockengestresster Pflanzen im Vergleich zu Kontrollpflanzen hindeutet. Desweiteren legten eine Induktion der Transkription von *GR* in Blättern und Wurzeln und ebenfalls erhöhte GR-Aktivität in Blättern bei Trockenstress eine wichtige Rolle der GR bei der Entgiftung von ROS und zur Aufrechterhaltung des Gehalts an reduziertem GSH nahe. Der konstant niedrigere Gehalt an Thiolen in Blättern ist eine Konsequenz der verminderten Bildung von GSH während Trockenheit. Ein Indiz für niedrige Schwefelverfügbarkeit im Spross während Trockenstress war ein konstant verringerter Sulfatgehalt. Entsprechend deuten ebenfalls die erhöhte Expression der Schwefeltransporter *Sultr1;1* und *Sultr4;1*, welcher für den vakuolären Export von Sulfat verantwortlich ist, auf Schwefelmangel in Blättern während Trockenheit hin. Desweiteren zeigten die mehr als zweifach erhöhte Expression der ATPS-Isoformen und eine Zunahme im APS-Gehalt, dass auch ATPS einen limitierenden Faktor darstellt, welcher während Trockenheit durch den Schwefelgehalt reguliert wird. Eine verringerte Einbaurate von ^{35}S in Cystein und GSH legt nahe, dass Trockenheit die Sulfat-Verfügbarkeit im Spross einschränkt, was zu einem verminderten Flux innerhalb des Schwefelassimilations-Pathways zu GSH führt.
Im Gegensatz hierzu war in Wurzeln der Gehalt an Thiolen, Sulfat, APS und Sulfid im Vergleich zur Kontrolle erhöht. Eine Verminderung der Einbaurate von ^{35}S in Cystein und GSH während Trockenheit und die gleichzeitig erniedrigte Expression von *Sultr4;1* deuten eine vermehrte vakuoläre Speicherung von Sulfat an. Dies könnte zur reduzierten Bildung von Cystein und GSH beitragen. Weiterhin wurde stark verminderter Transport von markiertem Sulfat im Spross trockengestresster Pflanzen gemessen. Dies zeigt deutlich, dass Trockenheit die Verfügbarkeit von Sulfat für den Spross einschränkt, woraus sich eine Herabregulation des Schwefelassimilations-Pathyways ergibt und letztendlich ein erhöhter Gehalt an H_2O_2 in Blättern.

1 Introduction

1.1 Drought stress, major yield limiting factor for crop plants

Crop plants are permanently exposed to various biotic and abiotic stresses during their life cycle. Drought, salinity and extreme temperature are the major abiotic stresses that cause a significant reduction in crop yield by more than 50% (Boyer, 1982; Bray *et al.,* 2000). This is likely caused by a series of negative stress responses that adversely affect plant growth, ranging from altered gene expression and cellular metabolism (Wang *et al.,* 2001a). Among abiotic stresses, water availability is the most crucial factor for crop productivity, contributing as same magnitude as all other environmental factors combined. Yield loss of approximately more than 24 million tons was reported annually due to drought stress in maize (Maiti *et al.,* 1996; Heisey and Edmeades, 1999).

Water is vital for plant growth and development that constitutes about 80-95% mass of growing tissues of plants. It is taken up from soil by roots, and transported through the xylem of the stem to leaves for various important processes such as photosynthesis and nutrients uptake. It is ultimately lost to the atmosphere during transpiration. The availability of water for plant on right quantity and quality is crucial and is dependent on and determined by natural rainfall or irrigation (Breda *et al.,* 1995; Chen *et al.,* 2010). Replenishing plants with water leads to a decrease in transpiration and uptake of water by roots (Breda *et al.,* 1995, Duursma *et al.,* 2008) and ultimately limits the contact between roots and soil to water movement under pronounced drought (Nobel and Cui, 1992; North and Nobel, 1997). Thus, roots are considered as the first sensor during water shortage and other rhizosphere stresses in plants (Jackson, 1997; Davies *et al.,* 2000).

Photosynthesis plays a vital role in plant performance and is affected by drought stress (Chaves *et al.,* 2003, 2009; Flexas *et al.,* 2004; Lawlor and Tezara, 2009). The early response of plant to water shortage is stomata closure that is important for plant water saving strategy (Maroco *et al.,* 1997; Chaves *et al.,* 2003; Chaves and Oliveira, 2004; David *et al.,* 2007). This causes a decline in the net carbon uptake under water stress by altering the allocation of photoassimilates to different plant parts, causing an increase in root to shoot ratio. As a result, shoot growth is inhibited while root growth is maximized to facilitate water uptake from the soil (Lambers, 1998; Sharp, 2002). This inhibition in shoot growth is an adaptive response of plant survival by extending the period of soil water availability under drought stress (Chapin, 1991; Neumann, 1995; Achard *et al.,* 2006). Also leaf or shoot expansion is decreased under water deficit (Boyer, 1970a;

Hsiao, 1973; Ben Haj Salah and Tardieu, 1997; Tardieu *et al.,* 1999, 2000), although the activity of Rubisco is maintained even at 50% relative water content and 75% stomata closure (Kaiser, 1987; Flexas *et al.,* 2006b). In C_4 plants, reduction in chlorophyll and protein contents was observed upon water stress (Du *et al.*, 1996; Foyer *et al.*, 1998; Marques da Silva and Arrabaça, 2004b; Carmo-Silva *et al.,* 2007). Accumulation of amino acids is suggested to be due to increased protein degradation during drought (Becker and Fock, 1986). In crop plants, a decrease in root growth rate was found under drought stress (Nayyar and Gupta, 2006). Many environmental stresses trigger the accumulation of proline in higher plant that helps to maintain membranes and protein structure (Rhodes *et al.,* 1999; Oztürk and Demir, 2002). It thus can be used for the selection of stress resistance genotype (Ashraf and Haris, 2004; Shao *et al.*, 2006). There are two ways that cause proline accumulation in plants: either by the activation of proline biosynthesis or by the inactivation of degradation of proline (Girija *et al.,* 2002). However, the accumulation of proline is only considered as a stress marker (Secenji *et al.,* 2010) but not an adaptive response to stress conditions (Hanson, 1979; Lutts *et al.,* 1999).

1.2 Drought stress, major contributor to formation of reactive oxygen species

Plants are continuously producing reactive oxygen species (ROS) at a very low level under normal growth conditions in chloroplasts, mitochondria and peroxisomes (Fig. 1). However abiotic stress including drought can increase the rate of ROS production which can cause oxidative damage (Iturbe-Ormaetxe *et al.,* 1998; Mittler *et al.,* 2002; Apel and Hirt 2004; Moller *et al.,* 2007; Takahashi and Murata, 2008; Gill and Tuteja, 2010). The early response of drought is recognised by roots due to a decrease in soil water status. Root to shoot signalling by small molecules such as abscisic acid (ABA) plays an important role in stomata closure in order to prevent water loss through transpiration (Zhang and Davies, 1987, 1991, Hartung *et al.,* 2002; Jiang and Hartung, 2007). Under drought stress, reduced CO_2 availability due to stomata closure has a direct or indirect negative effect on photosynthesis (Mittler, 2002). Specifically, excessive light energy in the form of electrons not required for carbon reduction may be redirected towards molecular oxygen, causing an accumulation of ROS at PS1 by the Mehler reaction (Asada, 2006; Miller, 2010). Under drought stress, approximately 50% leakage of photosynthetic electrons to the Mehler reaction was observed in wheat (Biehler and Fock, 1996). It has also been shown in sunflower that drought stress triggers an increase in the thylakoid membrane electron leakage to O_2 (Sgherri *et al.,* 1996). Also the peroxisome is a major compartment where the production rate of H_2O_2 and O_2 is high and can even be

enhanced under certain environmental conditions. Photorespiration is enhanced under reduced water availability due to a decrease in CO_2 to O_2 ratio in the mesophyll cells that account for over 70% of total H_2O_2 generation (Noctor *et al.*, 2002). Production of glycolate in chloroplast and oxidation by glycolate oxidase in peroxisomes contribute to the production of H_2O_2 during photorespiration (Noctor *et al.*, 2002; Karpinski *et al.*, 2003). β-oxidation, flavin oxidase pathway and the dismutation of superoxide radicals are other sources that generate H_2O_2 in peroxisomes (Corpas, *et al.*, 2001; Palma *et al.*, 2009). The amount of ROS generation in mitochondria is lower compared to chloroplast and peroxisomes (Foyer and Noctor, 2005; Rhoads *et al.*, 2006) and complex I and III of the electron transport chain are considered as the major sites for ROS production in mitochondria (Moller 2001; Rhoads *et al.*, 2006, Moller *et al.*, 2007). Enhanced mitochondrial respiration can lower ROS production during water stress by transferring reducing equivalents from the cytochrome electron transport system to uncoupling proteins or KCN-insensitive alternative oxidase (Norman *et al.*, 2004). The mitochondrial ATP synthesis also increases due to an increase in respiration rate under severe drought. This happens in order to compensate for the reduced ATP synthesis in chloroplast (Atkin and Macherel, 2009).

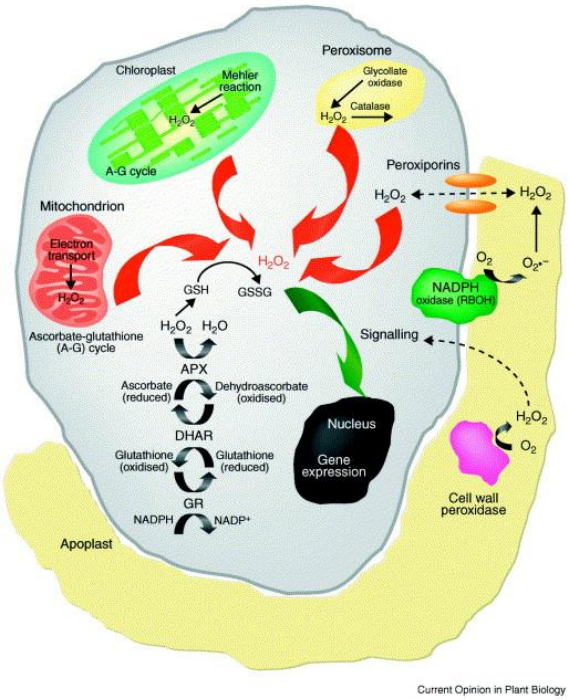


Fig. 1 Formation of H_2O_2 in the plant cell (Neill *et al.*, 2002)
H_2O_2 is generated in normal metabolism via the Mehler reaction in chloroplasts, electron transport in mitochondria and photorespiration in peroxisomes

1.3 Scavenging system that keeps ROS under tight control

Under optimum condition a balance between ROS production and scavenging prevails in the level of intracellular ROS. ROS play a dual role in response to stress. They function as important signalling molecules in stress response pathways by triggering stress defence/acclimation mechanisms. The signalling role of ROS is well documented, such as defence against pathogens to prevent oxidative burst, regulation of root hair development, stomata closure and regulation of gene expression (Mittler, 2002; Kwak *et al.,* 2003; Overmyer *et al.,* 2003).

However, when reaching a certain level, ROS become deleterious and can cause programmed cell death, initiating damages to membranes and other important cellular components including proteins, lipids and sugars (Dat *et al.,* 2000; Mittler, 2002; Gechev *et al.,* 2006).

Plants have well established enzymatic and non-enzymatic defence systems that function in an efficient way in order to keep the ROS level at minimum level during stress. Superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR) and metabolites from the glutathione-ascorbate cycle together play an important role in ROS scavenging mechanism (Bowler *et al.,* 1992; Willekens *et al.,* 1997, Noctor and Foyer, 1998). The production of superoxide can efficiently be dismutated by SOD to H_2O_2, thus acts as a first line of defence against ROS (Bowler *et al.,* 1992). SOD activity is induced upon drought stress in many crop species including wheat, pea, bean, rice and olive trees (Badiani *et al.,* 1990; Mittler and Zilinskas, 1994; Turkan *et al.,* 2005; Sharma and Dubey, 2005) and its over-expression shows an enhanced tolerance towards oxidative stress (Perl *et al.,* 1993; Sen Gupta *et al.,* 1993; Van Camp *et al.,* 1996; Basu *et al.,* 2001; Alscher *et al.,* 2002). CATs localized mainly in peroxisomes and are able to detoxify H_2O_2 that are produced during photorespiration (Mittler *et al.,* 2004; Vandenabeele *et al.,* 2004). In contrast to catalase, detoxification of H_2O_2 by APX in the ascorbate-glutathione cycle needs a stable reductant, such as ascorbate and GSH, which can move from one organelle to another easily (Levine *et al.,* 1994). In this cycle, H_2O_2 is reduced to H_2O via ascorbate and GSH and as a result oxidized glutathione (GSSG) is formed (Noctor and Foyer, 1998) (Fig. 2). PEG-imposed mild water stress in detached leaves of maize showed significant increase in APX activity due to an elevated level of ROS production (Jiang and Zhang, 2002).

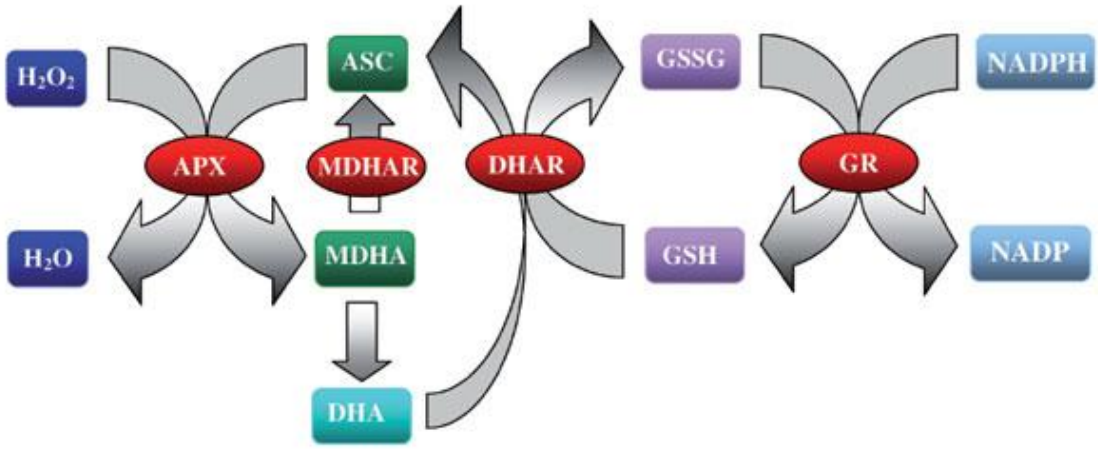


Fig. 2 Detoxification of ROS via glutathione-ascorbate cycle (Saruhan *et al.*, 2009)
ASC: Ascorbate; APX: Ascorbate peroxidase; GSH Reduced glutathione; GSSG: Oxidized glutathione; GR: Glutathione reductase; DHA Dehydroascorbate; DHAR: Dehydroascorbale reductase; MDHA: Monodehydroascorbale MDHAR: Monodehyroascorbale reductase.

1.4 Glutathione, an important player in stress response

GSH is the most abundant non-protein thiol in cells of prokaryotes and eukaryotes which play an important role in various cellular processes (Fig. 3). Under normal conditions, leaves typically maintain a high GSH: GSSG ratios of at least 20:1 (e.g. Mhamdi *et al.*, 2010a), although cytosol and vacuole have a higher and lower ratio, respectively. The accumulation of GSSG in cytosol might be important in oxidative stress response (Meyer *et al.*, 2007; Queval *et al.*, 2011). Under stress, the elevated concentration of GSH can trigger the gene expression that gives ability to the cell to counteract with oxidation of GSH (Pasqualini *et al.*, 2001; Ruiz and Blumwald, 2002; Freeman *et al.*, 2004). This elevation in GSH is an important process in signal transduction and defence against ROS that induces the genes related to GSH biosynthesis (Secenji *et al.*, 2010). This change in the redox state makes glutathione status an important marker for oxidative stress in response to increased intracellular H_2O_2 production.

1.4.1 Compartmentation of GSH biosynthesis

GSH biosynthesis takes place in two ATP dependent steps (Rennenberg, 1982; Meister, 1988; Noctor *et al.*, 2002; Mullineaux and Rausch, 2005). In the first step, L-cysteine links with γ-carboxyl group of L-glutamate to form γ-EC by GSH1 (GSH1, GCS, GCL; EC 6.3.2.2). In the second step, glycine is added to γ-EC by GSH2 (GSH2, GS; EC 6.3.2.3) to form GSH. In Arabidopsis, each of the enzymes in GSH biosynthesis is encoded by a single copy gene (May and Leaver, 1994; Ullman *et al.*, 1996). GSH biosynthesis takes place in plastids and cytosol. Localization study in Arabidopsis revealed the presence of GSH1 exclusively in plastids, whereas GSH2 is found both in

plastids and cytosol (Wachter *et al.,* 2005). In contrast to Arabidopsis, both enzymes of GSH biosynthesis were detected in chloroplast and cytosol by immunohistochemistry in maize, indicating species-specific differences (Gomez *et al.,* 2004). The activity of γ-ECS in wheat is strongly associated with chloroplasts (Noctor *et al.,* 2002). Knock out of GSH1 is embryo lethal (Cairns *et al.,* 2006) while GSH2 show a lethal phenotype at the seedling stage (Pasternak *et al.,* 2008). GSH1 activity is rate limiting in GSH biosynthesis and is feedback inhibited by GSH (Jez *et al.,* 2004). One of the specific inhibitors of GSH1 is buthionine sulphoximine (BSO) that has been used to deplete GSH (Griffith and Meister, 1979). Feedback inhibition is not considered as a major control feature in GSH biosynthesis (Meyer *et al.,* 2001), although GSH1 and GSH2 are inhibited by GSH (Jez and Cahoon, 2004; Jez *et al.,* 2004). GSH1 is regulated at transcriptional, translational and post-translational levels in Arabidopsis (May *et al.,* 1998; Xiang and Oliver, 1998). *GSH1* and *GSH2* mRNA abundance and the steady state level of GSH were increased in response to jasmonic acid (JA) and heavy metals treatment, light and some stress conditions such as drought and certain pathogens, although the induction of these genes were not affected by treatment with GSH and H_2O_2 (Schäfer *et al.,* 1997, 1998; Xiang and Oliver, 1998; Sung *et al.,* 2009).

1.4.2 Physiological role of GSH

Glutathione forms a conjugate with xenobiotics and some metabolites such as anthocyanines by glutathione S-transferases (GST). These compounds are subsequently transported into the vacuole. Thus the formation of GSH-conjugate plays an important role in the detoxification of organic compounds (Marrs, 1996). GSH in combination with GSH reductase and catalase are essential to the detoxification of ROS in plants via a glutathione-ascorbate cycle (Asada, 1999; Mhamdi *et al.,* 2010). ROS such as H_2O_2 can oxidize GSH to GSSG. The role of GSH in plant development is well described by the phenotypic analysis of glutathione-deficient mutants (Vernoux *et al.,* 2000; Cairns *et al.,* 2006; Reichheld *et al.,* 2007; Frottin *et al.,* 2009; Bashandy *et al.,* 2010). The *rml1* mutant caries a mutation in the *GSH1* gene and shows a strong phenotype with no proper root development and has only residual amounts of GSH (Vernoux *et al.,* 2000). GSH is an important precursor of phytochelatins that are produced in response to cadmium and other heavy metal. GSH1 allelic mutant *cad2* is sensitive to low concentration of cadmium and has about 30% less GSH level than wild type due to the impairment of GSH biosynthesis in this mutant (Howden *et al.,* 1995; Cobbett *et al.,* 1998). The Arabidopsis *pad2* is a camalexin-deficient mutant that has less GSH contents compared to *cad2* and the

expression gene of *GSH1* is affected in this mutant. A down-regulation in Pathogenesis Related 1 (PR1), a marker gene in salicylic acid (SA) signalling pathway and is controlled by nonexpressor of PR genes (NPR1) (Mou *et al.*, 2003), was also observed in *pad2* (Roetschi *et al.*, 2001), which may account for its enhanced susceptibility to various pathogens (Ferrari *et al.*, 2003; Parisy *et al.*, 2007). Recently, it has been shown that GSH acts as a precursor of the thiazole ring in the camalexin synthesis (Böttcher *et al.*, 2009; Su *et al.*, 2011). The germination of pollen and pollen tube growth is also dependent on GSH synthesis in Arabidopsis (Zechmann *et al.*, 2011). GSH is also important in triggering the expression of various genes essential to plant defense and ROS detoxification (Roetschi *et al.*, 2001; Ball *et al.*, 2004). For example, cytosolic ascorbate peroxidase 2 (APX2) is a stress-induced gene that is up regulated upon a decrease in GSH level (Ball *et al.*, 2004). The alteration in a less negative E_{GSH} due to electron drain from GSH pool under oxidative stress is considered as an important signal that leads to changes in cell metabolism and ultimately to adaptation to stress condition (May *et al.*, 1998).

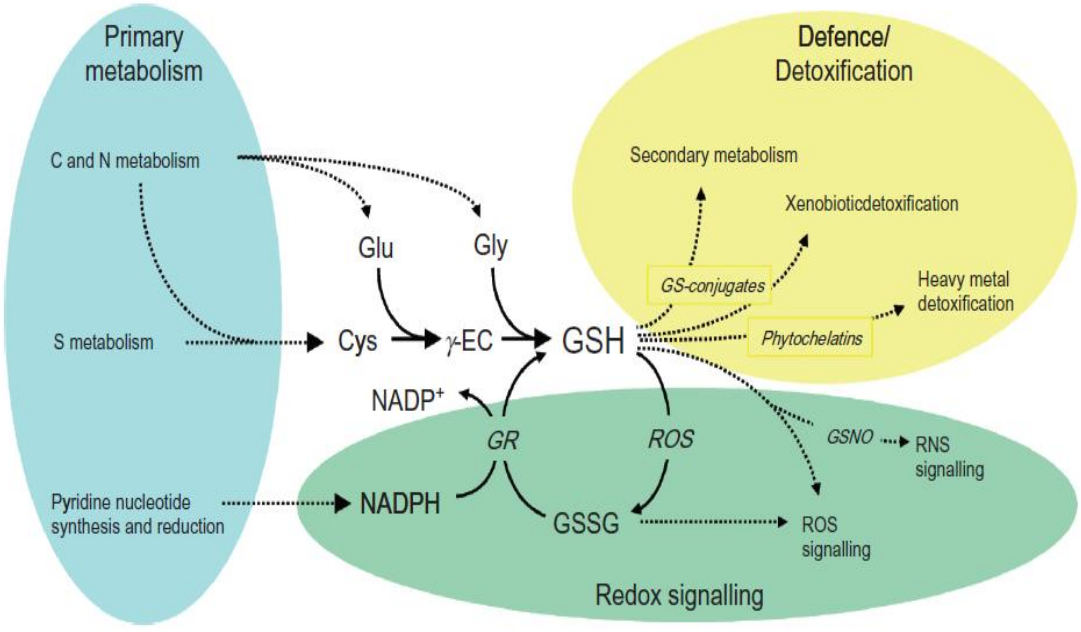


Fig. 3 General overview of some of the most important glutathione functions (Noctor *et al.*, 2012) (Synthesis, redox turnover, metabolism, signalling). Cys, cysteine; γ-EC, γ-glutamylcysteine; GS-conjugates, glutathione *S*-conjugates; GSNO, *S*-nitrosoglutathione; Glu, glutamate; Gly, glycine; RNS, reactive nitrogen species; ROS, reactive oxygen species.

1.4.3 Regulation of GSH biosynthesis

In plants, GSH1 forms a homodimer that are linked by two disulphide bonds (Hothorn *et al.*, 2006), one of which plays an important role in redox regulation (Hicks *et al.*, 2007;

Gromes *et al.,* 2008) and might be important in GSH biosynthesis during oxidative stress. GSH biosynthesis is affected by many factors like glycine and ATP (Buwalda *et al.,* 1990; Noctor *et al.,* 1997; Ogawa *et al.,* 2004) but the most important ones are GSH1 activity and availability of its precursor, cysteine (see section 1.5).

Increase in the level of GSH can be achieved by over expression of either GSH1 or enzymes in the cysteine biosynthestic pathway (Strohm *et al.,* 1995; Noctor *et al.,* 1996, 1998; Creissen *et al.,* 1999; Harms *et al.,* 2000; Noji and Saito, 2002; Wirtz and Hell, 2007). Under optimal conditions, over expression of the *Escherichia coli* GSH2 in popular showed little effect on GSH contents (Foyer *et al.,* 1995; Strohm *et al.,* 1995), while the expression of *E. coli* GSH1 caused a two- to four-fold increase in GSH in leaf (Noctor *et al.,* 1996, 1998; Arisi *et al.,* 1997). Creissen *et al.,* (1999) reported a substantial accumulation in GSH when GSH1 from *E. coli* was introduced into tobacco chloroplasts. GSH1-overexpressing transgenic Indian mustard and poplar also showed enhanced resistance to heavy metals and certain herbicides (Zhu *et al.,* 1999a; Gullner *et al.,* 2001; Ivanova *et al.,* 2011). Recently, it has been shown that overexpression of a bifunctional γ-ECS/GSH-S from *Streptococcus* also have positive effect on GSH accumulation (Liedschulte *et al.,* 2010).

Reduced glutathione (GSSG) formed by glutathione (GSH) oxidation is rapidly recycled by the glutathione reductase (GR) at the expanse of NADPH in key organelles and the cytosol (Halliwell and Foyer, 1978; Smith *et al.,* 1989; Edwards *et al.,* 1990; Jiménez *et al.,* 1997; Chew *et al.,* 2003b; Kataya and Reumann, 2010). Many stresses such as drought, cold, high light and ozone can induce GR activity, thereby maintaining the level of reduced glutathione at the cellular level (Creissen *et al.,* 1994; Gamble and Burke, 1984; Burke *et al.,* 1985). GSSG can also be reduced by NADPH-dependent thioredoxin reductase (NTR) in thioredoxins (TRX)-dependent manner (Marty *et al.,* 2009), but the efficiency of these enzymes is low compared to GR. GR, a homodimeric flavoprotein, is the last enzyme of the glutathione-ascorbate cycle that keeps the redox state of GSH pool in reduced form (Foyer *et al.,* 2002). GR1 is dual targeted to the cytosol and peroxisome while GR2 is present both in chloroplast and mitochondria (Creissen *et al.,* 1995; Chew *et al.,* 2003; Kataya and Reumann, 2010). In maize, GR activity is exclusively localized to mesophyll cells due to lack of reductant in bundle sheath cells (BSCs) while *GR* transcripts are present in both types of leaf cells (Doulis *et al.,* 1997; Pastori *et al.,* 2000b). In Arabidopsis, T-DNA mutants for the chloroplast/mitochondrial *GR2* are embryo-lethal (Tzafrir *et al.,* 2004), *gr1* knockout mutants show a reduction of 30-60% in

total extractable enzyme activity although no phenotypic effects was observed (Marty *et al.,* 2009; Mhamdi *et al.,* 2010a).

1.5 The role of sulfur assimilation pathway in the biosynthesis of GSH

Sulfur is one of the most important macronutrient in plants and is available in the environment primarily in oxidized state in the form of inorganic sulfate and further needs to be reduced for incorporation into organic compounds. Animals are unable to achieve this primary assimilation of sulfur and are dependent on plants, algae, bacteria and fungi for the supply of organic sulfur-containing compounds. Cysteine is the first organic compound containing reduced sulfur. It can be produced by plants, algae and most bacteria.

Cysteine is one of the important precursors in GSH biosynthesis and is considered as the last product from the sulfur assimilation and reduction pathway that can be used for the production of methionine, protein, GSH, and a variety of compounds containing reduced sulfur and is dependent on the assimilatory sulfate reduction pathway (Fig. 4).

1.5.1 Role of sulfate transporters in the uptake of sulfate

The uptake of sulfate from the soil into the root is carried out by different sulfate transporters. Sulfate is then distributed throughout the plants. Based on the protein sequence similarities, these putative sulfate transporters are classified into 5 groups, Sultr 1-5 (Hawkesford, 2003; Buchner *et al.,* 2004). In Arabidopsis, 14 members of this family have been identified so far. Sultr1;1 and Sultr1;2 are high affinity sulfate transporters that are localized to root epidermal, cortical plasma membrane and root apex and play an important role in initial uptake of sulfate from the soil (Takahashi *et al.,* 2000; Yoshimoto *et al.,* 2002). Sultr1;1 is a high affinity sulfate transporter (Vidmar *et al.,* 2000) but its expression level is lower compared to Sultr1;2 (Rouached *et al.,* 2008) and is strongly induced under sulfur deficiency (Takahashi *et al.,* 2000; Yoshimoto *et al.,* 2002). It has been reported that a mutation in the Sultr1;2 caused a significant decrease in the sulfate uptake capacity, thus making this transporter a major contributor in sulfate uptake (Shibagaki *et al.,* 2002; Yoshimoto, *et al.,* 2002; El Kassis *et al.,* 2007; Barberon *et al.,* 2008). The responses of these transporters towards biotic and abiotic stresses were found to be very different despite high homology (Barberon *et al.,* 2008; Rouached *et al.,* 2009). Abiotic stress such as drought also triggered the induction of *Sultr1;1* expression level in maize and grapevine (Cramer *et al.,* 2007; Ernst *et al.,* 2010). Phloem localized Sultr1;3 is another member of the high affinity sulfate transporter that is responsible for source to

sink sulfate transport (Yoshimoto *et al.,* 2003). Sultr2;1 and 2;2 are group 2 low affinity transporter which are highly expressed in the xylem parenchyma cells (Takahashi *et al.,* 1997, 2000) and are responsible for long distance transport. Members of the group 3 Sultr family are involved in the translocation of sulfate in the developing seeds due to their high expression at this developmental stage (Zuber *et al.,* 2010). It has been shown recently that Sultr3;1 is chloroplast-localized and is responsible for the sulfate transport into chloroplast (Cao *et al.,* 2012). In chick pea, a homologue of this group is involved in sulfate delivery to developing embryo (Tabe *et al.,* 2003). Members of the group 4 sulfate transporter are mainly involved in the efflux of sulfate from the vacuole to the cytosol. Both *Sultr4;1* and *Sultr4;2* are up-regulated upon sulfur deficiency, indicating an increase demand for sulfate release from the vacuole (Kataoka *et al.,* 2004). Less information is available about the group 5 sulfate transporters although Sultr5;2 functions as a molybdate transporter in Arabidopsis (Tomatsu *et al.,* 2007).

1.5.2 ATP sulfurlyase role in sulfate reduction

In order to assimilate inorganic sulfur from sulphate into cellular metabolism, sulfate first needs to be activated to adenosine 5'-phosphosulfate (APS) by ATP sulfurylase (ATPS EC: 2.7.7.4). In this reaction, sulfate is coupled with a phosphate residue produced by ATP cleavage. ATPS is considered as widespread enzyme present in sulfate assimilating plants, algae, fungi, and bacteria. Arabidopsis has 4 ATPS isoforms whereas spinach and potato contain two isoforms that are specifically present in plastids and cytosol (Lunn *et al.,* 1990; Renosto *et al.,* 1993; Klonus *et al.,* 1994). In maize, 75-100% total leaf ATPS activity was reported in bundle sheath cells (BSCs) by several groups (Gerwick and Black, 1979; Passera and Ghisi, 1982; Burnell, 1984; Schmutz and Brunold, 1984). Sulfate deficiency induced transcripts of sulfate transporter, *ATPS* and *APR* in leaves and roots of maize (Bolchi *et al.,* 1999; Hopkins *et al.,* 2004), while reduced cellular sulfur compounds caused down-regulation in the mRNA level of *ATPS* (Bolchi *et al.,* 1999). Exposure of maize seedling to cadmium or chilling stress caused an increase in ATPS activity due to a higher demand for reduced sulfur (Nussbaum *et al.,* 1988; Brunner *et al.,* 1995). The overexpression of ATPS in *Brassica juncea* showed elevated level of GSH and resistance to selenate (Pilon-Smith *et al.,* 1999). Since the reduction of APS exclusively takes place in plastids, the physiological role of cytosolic ATPS is still unclear, although there is some speculation that the production of APS in the cytosol might be further activated via PAPS for secondary metabolism (Rotte and Leustek, 2000).

In this reaction the phosphorylation of APS to PAPS is catalysed by APK. APK is essential for sulfate assimilation in some organism like yeast, fungi, and some c-proteobacteria where reduction of PAPS takes place instead of APS (Kopriva and Koprivova, 2004). In Arabidopsis, 4 APKS isoforms were identified, 3 of which are localized in plastids and one APK3 in the cytosol due to lack of transit peptide. *APK1* and *APK2* have higher expression level in leaves compared to *APK3* and *APK4* (Lunn *et al.,* 1990; Rotte and Leustek, 2000; Mugford *et al.,* 2009). The susceptibilty of APS kinase has been shown very recently to redox regulation (Ravilious *et al.,* 2012). A remarkable increase in cysteine and GSH but low glucosinolates level was observed in *apk1 apk2* mutants that showed semi dwarf phenotype (Mugford *et al.,* 2009). In these mutants, the flux through the primary reductive pathway was higher compared to wild type due to blockage of the PAPS branch of the sulfate assimilation (Mugford *et al.,* 2011). PAPS is also considered as a storage form of APS and is converted again back to APS upon oxidative stress in a reaction that is catalyzed by 3'(2'),5'-diphosphonucleoside 3'(2')-phosphohydrolase (DNPase; EC 3.1.3.7) (Peng and Verma, 1995). PAPS acts as a sulfate donor for various molecules such as glucosinolates, saccharides, proteins, flavonoids, and jasmonates in the sulfotransferases (SOT)-catalysed sulfation process, producing 3′-phosphoadenosine 5′-phosphate (PAP) as a by-product (Klein and Papenbrock, 2004). Accumulation of desulfo-glucosinolates and decrease in glucosinolates was recently shown in *fou8* alleles of *fry1* mutant encoding 2'(3'),5'-diphosphoadenosine (PAP) phosphatase that may be involved in glucosinolates biosynthetic pathway (Rodrigues *et al.,* 2010; Lee *et al.,* 2012). PAP plays an important role in RNA catabolism by inhibiting the *in vitro* activity of the two yeast (*Saccharomyces cerevisiae*) 5′ to 3′ exoribonucleases (XRNs) by 40-65% (Dichtl *et al.,* 1997). Recently, it has been shown in *fry1* mutant that the accumulation of PAP also functions in retrograde stress signals between chloroplasts and the nucleus that alter nuclear gene expression under high light or drought stress in Arabidopsis (Estavillo *et al.,* 2011).

1.5.3 APS reductase role in sulfate reduction

APS reductase (APR; EC 1.8.4.9) is one of the most important enzymes of the sulfate assimilation pathway that reduces APS further to sulfite in a GSH-dependent electron transfer (Suter *et al.,* 2000). APR is exclusively localized to the plastids and is encoded by small gene family. In Arabidopsis, genes encoding three isoforms of APR are present that are regulated in the same way but their response timing and strength are different (Kopriva and Koprivova, 2004). *APR2* response towards various hormone treatments was

found to be different from *APR1* and *APR3* (Koprivova *et al.,* 2008), indicating specific function of each isoform. APR2 is the major form that contributes 80% of the total APR activity in the cell (Loudet *et al.,* 2007). APR is highly regulated enzyme of the assimilatory sulfate reduction: the expression level of *APR2* is down regulated upon exposure to reduced sulfur compounds such as sulfide, cysteine, and GSH (Kopriva and Koprivova, 2004). Various stress treatments such as heavy metal, salinity, high light or cold caused up-regulation in the *APR* expression level (Lee and Leustek, 1999; Kopriva *et al.,* 2008; Queval *et al.,* 2009). An increase in APR activity was reported upon addition of sugars to the plant media (Hesse *et al.,* 2003). It was also found that APR shows a diurnal rhythm where higher activity was observed during day than in the night (Kopriva *et al.,* 1999). Under nitrogen starved condition, APR activity was decreased whereas the addition of amino acids or ammonium resulted in an increase in APR activity, highlighting the possible connection between sulfate and nitrogen assimilation (Brunold and Suter, 1984; Koprivova *et al.,* 2000).

1.5.4 Sulfite reductase role in sulfate reduction

The conversion of sulfite to sulfide is catalyzed by sulfite reductase (SiR) that utilizes reduced ferredoxin as an electron donor (Martin *et al.,* 2005) and is dependent on siroheme and FeS cluster as prosthetic groups (Nakayama *et al.,* 2000)**.** SiR is encoded by a single copy gene (Bork *et al.,* 1998; Nakayama *et al.,* 2000) and is located exclusively in plastids (Brunold and Suter, 1989). Its involvement in the regulation of sulfate assimilation and control over the sulfur flux is well described in Arabidopsis (Khan *et al.,* 2010). In pea and maize, interaction of SiR with plastidic DNA-protein complex, called nucleoid, plays an important role in compacting nucleoids in the plastids (Sekine *et al.,* 2007). SiR does not seem to be regulated at the mRNA level (Bork *et al.,* 1998). However, addition of OAS to nitrogen deficient Arabidopsis (Koprivova *et al.,* 2000) or methyl jasmonate application led to the up-regulation in *SiR* transcript level (Jost *et al.,* 2005).

1.5.5 Cysteine biosynthesis, final step of sulfate assimilation

Two enzymes, serine acetyltransferase (SAT; EC 2.3.1.30) and *O*-acetylserine (thiol)lyase (OAS-TL; EC 2.5.1.47) form a multi enzymes complex called the cysteine synthase complex (CSC) (Hell *et al.,* 2002) that is responsible for the synthesis of cysteine. SAT and OAS-TL are found in cytosol, mitochondrion and chloroplast of the plant cells (Saito, 2000) while other enzymes of the sulfate reduction pathway are

localized to the plastids. O-acetylserine (OAS) is formed by the activation of serine by SAT-catalyzed acetyl transfer from acetyl-coenzyme A, whereas OAS-TL incorporates sulfide produced by the assimilatory sulfur reduction pathway into OAS to form cysteine.

There are five members of the SAT protein family in Arabidopsis and each of the encoding genes is located on different chromosomes. SAT1, SAT3 and SAT5 are the major isoforms that are localized in plastids, mitochondria and cytosol, respectively. SAT2 and SAT4 are localized in cytosol and are less expressed compared to the other three isoforms (Kawashima *et al.,* 2005). SAT2 and SAT4 are strongly induced upon sulfur starvation, indicating their possible role under sulfur deficiency (Zimmermann *et al.,* 2004; Kawashima *et al.,* 2005).

In Arabidopsis there are 9 *OASTL* like isoforms, three of which, OAS-TLA, OAS-TLB and OAS-TL, are major ones based on their expression (Wirtz *et al.,* 2004; Zimmermann *et al.,* 2004). The major isoforms are localized in cytosol, plastids and mitochondria respectively. The contribution of these three OASTLs to the total enzyme activity is also different, i.e. OAS-TLA, OAS-TLB and OASTLC contribute approximately 50%, 45% and 5%, respectively to the total enzyme activity in Arabidopsis leaf (Heeg *et al.,* 2008; Watanabe *et al.,* 2008a).

The C-terminus of SAT plays an important role in interacting with OASTL in bacteria and Arabidopsis. No CSC formation was observed when the C-terminus of SAT is partially deleted (Mino *et al.,* 1999, 2000; Francois *et al.,* 2006; Wirtz and Hell, 2006). SAT is active when bound to OASTL in the CSC and this binding of C-terminus of SAT with active site of OASTL makes OASTL inactive (Ruffet *et al.,* 1994; Droux *et al.,* 1998; Huang *et al.,* 2005; Francois *et al.,* 2006). OAS accumulation dissociates CSC (Kredich *et al.,* 1969; Droux *et al.,* 1998) whereas sulfide stabilizes the complex (Wirtz and Hell, 2006; Wirtz and Hell, 2007).

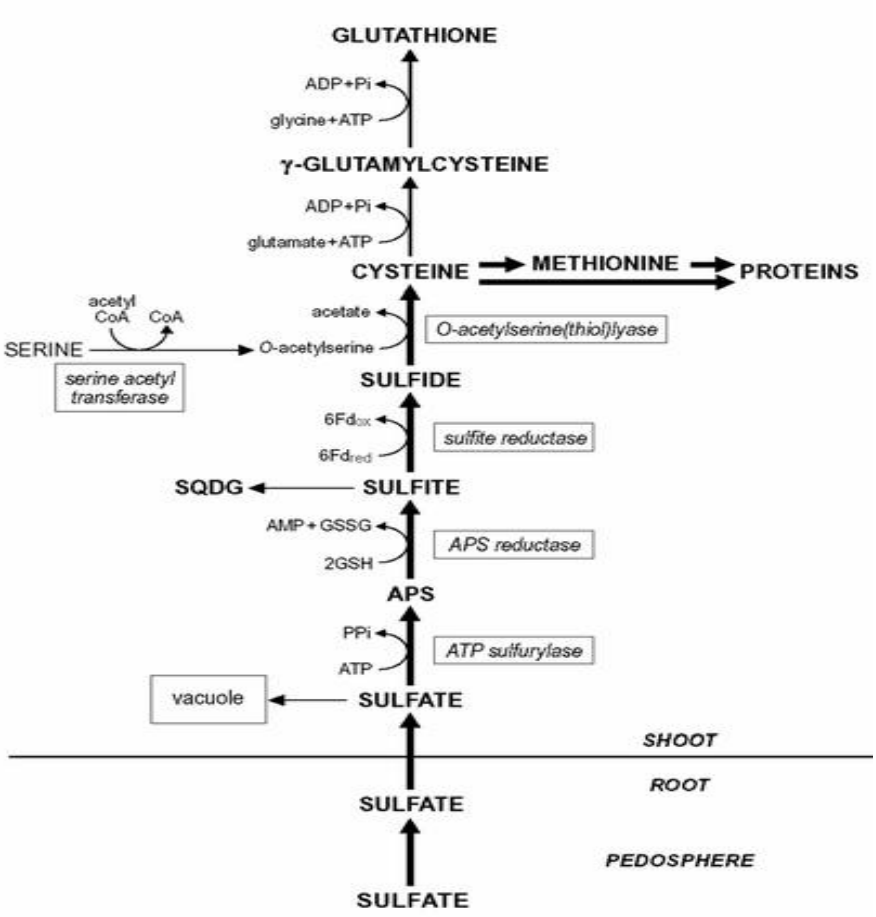


Fig. 4 Assimilatory sulfate reduction pathway in higher plants
Enzymes along with their products and substrates are shown. APS, adenosine 5′-phosphosulfate; Fdred, Fdox, reduced and oxidized ferredoxin; GSH, GSSG, reduced and oxidized glutathione; SQDG, sulfoquinovosyl diacylglycerol; OAS, *O*-acetylserine.

1.6 Aims of the project

Plants, being sessile in nature, encounter various environmental stresses that can ultimately lead to reduce growth and productivity. During these stresses including drought the normal homeostasis of the cell is disturbed, triggering the accumulation of reactive oxygen species that play a dual role in response to stress. They function as important signalling molecules in stress response pathways by activating stress defence/acclimation mechanisms. However, when reaching a certain level, ROS become deleterious, initiating damages of membranes and other cellular components. Plants have a well established antioxidant system that functions in an efficient way in order to keep the ROS level at a minimum level during stress. A lot of work was done on the role of antioxidant mechanism particularly on the glutathione-ascorbate cycle under different stresses that use reduced glutathione in the final step. GSH is an important and abundant non-protein thiol whose concentration in cells is regulated upon various stresses. However, high cysteine levels feedback inhibits the assimilatory sulfate reduction pathway in maize while in most other plant species glutathione concentrations are believed to control sulfate assimilation, the primary source of cysteine synthesis. While GSH is an important player in stress response and is dependent on the availability of cysteine and sulfur derived from sulfate reduction pathway, it is quite surprising that no attention has been given to the response of this pathway to different stresses such as drought.

This study aims to elucidate the mechanism in which assimilatory sulfate reduction is feedback inhibited by cysteine under drought in maize. Besides this, the consequences of drought stress on transcriptional and post-translational (metabolite) regulation of sulfur metabolism will be investigated. It is expected to provide useful information that may explain how sulfur assimilatory pathway operates in plants during drought stress. In addition to that, the regulation and coordination of sulfur assimilation pathway between roots and shoots will provide insight into how these organs cooperate at the metabolic and molecular level in response to water shortage.

2 Material and methods

2.1 Technical equipment, materials and IT

2.1.1 Technical equipment

6890N gas chromatograph	Agilent, Waldbronn
Autoclave Sanoklav	Sanoklav, Bad Überkingen-
Cooling / Heating block Thermostat KBT-2 133	HLC, Bovenden
Fraction collector LKB FRAC-100	Pharmacia, Freiburg
Growth chambers	Waiss, Gießen
Hausen Chromabond-SiOH-column, 500 mg	Macherey-Nagel, Düren
Heating block Thermostat HBT-2 132	HLC, Bovenden
Liquid Scintillation Counter, Tri-Carb 2810 TR	ParkinElmer, USA
Microscope Leica DM IRB	Leica, Bensheim
NanoDrop 2000 spectrophotometer	Peqlab, Erlangen
Photometer UvikonXL	Secoman, Kandsberg
PlateReader Fluostar Optima	BMG Labtechnologies, Offenburg
Rotor-Gene Q	Qiagen, Hilden
Spectral photometer LKB Ultraspec III	Pharmacia, Freiburg
Stereomicroscope Leica MZ FLIII	Leica, Bensheim
Sterile bench Lamin Air 2448 and HB 2472	Heraeus Instruments, Osterode
Uvikon® 900	Goebel Instrumentelle Analytik GmbH, Au i.d.H.

HPLC-Systems:

W600 controller	Waters, Milford (USA)
W600E pump multisolvent delivery system	Waters, Milford (USA)
Column Nova-Pak™C18 3,9 × 150 mm	Waters, Milford (USA)
Column Nova-Pak™C18 4,6 × 250 mm	Waters, Milford (USA)
W717plus autosampler	Waters, Milford (USA)
FP-920 fluorescence detector	Jasco, Groß-Umstadt

2. ICS 1000	Dionex, Idstein
AS 50 autosampler	Dionex, Idstein
Column Ion Pak® AS9-HC 2x250 mm	Dionex, Idstein
Column LiChroCART® 125-4 LiChrospher® 60 RP-select B (5 µm)	Merck, Darmstadt
Column Eurospher 100-C18 (5 µm, 250 × 4 mm)	Knauer, Berlin

Centrifuges:

Beckman J2-21 with JA-20 rotor	Beckman, Munich
or with SS-34 rotor	DuPont, Bad Homburg
Biofuge pico	Heraeus Instruments, Osterode
Megafuge 1.0 R with BS 4402/A Rotor	Heraeus Instruments, Osterode
Microcentrifuge 5415C and 5417R	Eppendorf, Hamburg
Sorvall RC5C with GSA Rotor	DuPont, Bad Homburg
SpeedVac Alpha RVC cmc-1 with Alpha 2-4 Loc-1m	Christ, Osterode

Further devices corresponded to the usual laboratory equipment.

2.1.2 Chemicals

AccQ-Tag™	Waters, Milford (USA)
Acid fuchsin	AppliChem, Karlsruhe
Agar Fluka	Biochemika, Fuchs
Agarose	Serva, Heidelberg
Albumin fraction V (BSA)	Roth, Karlsruhe
Ampholytes pH 3-10 for IEF	Amersham, Braunschweig
Ascorbic acid	AppliChem, Darmstadt
Boric acid	Merck, Darmstadt
Bovine serum albumine	Sigma-Aldrich, Steinheim
5-bromo-4-chloro-3-indolyl phosphate (BCIP)	Roche, Mannheim
Bromophenol blue	Feinchemie Kallies, Sebnitz
Chloral hydrate	Riedel-de Haën, Seelze
Coomassie Brilliant Blue G-250	Merck, Darmstadt or Bio-Rad,

	Hercules, CA (USA)
L-Cysteine	Duchefa, Haarlem (Netherlands)
Diethylpyrocarbonate (DEPC)	Roth, Karlsruhe
Dimethyl sulfoxide (DMSO)	Roth, Karlsruhe
Dithiothreitol (DTT_{red})	AppliChem, Karlsruhe
DNA loading buffer	Peqlab, Erlangen
Deoxynucleotide Solution Mix (dNTP)	New England Biolabs, Beverly (USA)
dNTPs (dATP, dGTP, dTTP, dCTP) for cDNA synthesis	Invitrogen, Karlsruhe
Ellman's reagent, DTNB (5, 5'-dithiobis-(2-nitrobenzoic acid))	Fluka Biochemika, Seelze
Ethylene diaminetetraacetic acid (EDTA)	Roth, Karlsruhe
Ethylene glycol tetraacetic acid (EGTA)	AppliChem, Karlsruhe
Ethanol	Merck, Darmstadt
Ethidium bromide	Sigma-Aldrich, Steinheim
Formaldehyde	Sigma-Aldrich, Steinheim
Formamid	Merck, Darmstadt
Glutathione (GSH)	Duchefa, Haarlem (Netherlands)
Glutathione disulfide (GSSG)	Sigma-Aldrich, Steinheim
Glutathione ethyl ether	Sigma-Aldrich, Steinheim
Glycerol	Roth, Karlsruhe
Iodoacetamine	Sigma-Aldrich, Steinheim
Isopropanol	Roth, Karlsruhe
Magnesium chloride	AppliChem, Karlsruhe
Magnesium sulfate	Merck, Darmstadt
β-mercaptoethanol	Merck, Darmstadt
MES	AppliChem, Karlsruhe
MOPS	AppliChem, Karlsruhe
Monobromobimane (MBB)	Invitrogen, Karlsruhe
Micro agar	Duchefa, Haarlem

	(Netherlands)
NADPH	Sigma-Aldrich, Steinheim
Nitroblue tetrazolium (NBT)	Roche, Mannheim
Nuclease free water	Ambion, Austin, TX (USA)
O-acetylserine	Bachem, Bubendorf (Switzerland)
Oil (mineral)	Sigma-Aldrich, Steinheim
Orange G	Sigma-Aldrich, Steinheim
Phenol	Fluka Biochemika, Seelze
O-phenylene dihydrochloride	Sigma-Aldrich, Steinheim
Phenylmethanesulphonylfluoride (PMSF)	Serva, Heidelberg
Phytagel	Sigma-Aldrich, Steinheim
Potassium dihydrogenphosphate	Fluka Biochemika, Seelze
Potassium hydrogenphosphate	Fluka Biochemika, Seelze
Protease inhibitor mix	Sigma-Aldrich, Steinheim
Protein Standard Mark12™	Invitrogen, Karlsruhe
Roti®-Quant Bradford reagent	Roth, Karlsruhe
Sodium azide	AppliChem, Darmstadt
Sodium chloride	AppliChem, Karlsruhe
Sodium dodecyl sulfate (SDS)	Fluka Biochemika, Seelze
Sodium dithionite	Merck, Darmstadt
Sodium pyrophosphate	Merck, Darmstadt
Sodium succinate	Sima-Adrich, Steinheim
Sodium sulfide	Sigma-Aldrich, Steinheim
Sodium thiosulfate	Sigma-Aldrich, Steinheim
Sucrose, D+	AppliChem, Darmstadt
TEMED	Roth, Karlsruhe
Trishydroxymethylaminomethane (Tris)	Roth, Karlsruhe
Thiourea	AppliChem, Darmstadt
Urea	Gerbu, Heidelberg

All not listed chemicals were obtained in *pro analysis* grade from providers listed above or from AppliChem, Biomol, Boehringer-Ingelheim, Riedel-de Haën or Sigma-Alrdich.

2.1.3 Consumables

384-well plate, white	Roche, Applied Science, Mannheim
96-well	Greiner, Frickenhausen
Microscope Slides	Marienfeld, Laude-Königshofen
Membrane Desalting Filters	Millipore, Eschborn
NAP5 TM columns	Amersham, Braunschweig
Rotilabo aseptic filters (0,45 µm and 0,22 µM)	Roth, Karlsruhe
SILGUR-25 thin layer chromatography plate	Macherey-Nagel, Düren

Further consumables corresponded to usual laboratory equipment.

2.1.4 Kits

2-D Quant Kit	GE Healthcare, Freiburg
EXPRESS SYBR® GreenER™ qPCR SuperMix Universal	Invitrogen, Karlsruhe
SensiMix™ SYBR No-ROX Kit	Bioline, Luckenwalde
RevertAid™ H Minus First Strand cDNA Synthesis Kit	Fermentas, St. Leon-Rot
RNeasy Plant Mini Kit®	Qiagen, Hilden
RNase free DNAse Mini Kit®	Qiagen, Hilden
SuperScript®III First-Strand Synthesis System for RT-PCR	Invitrogen, Karlsruhe

2.1.6 Primers

Primers for qRT-PCR

Primer No.	Description	Sequence
1625	GR_for	CATTTGGGCTGTTGGAGATG
1626	GR_rev	TGACGTAGCGCACACAAGAA
1742	APR_for	GTCCAGGTTGATCCTTCCTT
1743	APR_rev	GTCAATGTTGCCCTTGTGGA
2157	Sultr1;1_for	TCCTGGCATTCCTTCTGGTT
2158	Sultr1;1_rev	GAGGGCTGCAAATGTTCTTC
2159	Sultr1;2_for	GGCGTGGTTTTCAAGTCTGT
2160	Sultr1;2_rev	ATCTTCTTCGTCCGTCAACC
2405	Actin_for	CTCAACCCCAAGGCCAACAGAGAG
2406	Actin_rev	GGCTCACACCATCACCTGAATCCA
2409	GSH1_for	AGCTTAAGGAGCCATATCTGGACAG

2410	GSH1_rev	GCATAGTCCACATATTGCTCAAACCC
2540	β-tubulin_for	GATTTGCTCCACTGACCTCGCGG
2541	β-tubulin_rev	CGGAACATAGCAGATGCCGTGAG
2673	Sultr4;1_for	CTACATCAAAGACAGGTTGCGTGAG
2674	Sultr4;1_rev	GTA ACA GGG GAC ATC TCG AGG ATC AC
2677	ATPS_for	CAGATGATGTGCCTCTTAGTTGGAG
2678	ATPS_rev	GCATTGGAGAGGGAAAGATCGCAAC
2681	ATPS_for	TGATACAGTAGCAAAGGAGATGGCC
2682	ATPS_rev	GAAACCGTCTGGAGGATTCTCTCC
2683	ATPS_for	CCTGTTCTTCTGCTCCATCCACTG
2684	ATPS_rev	CAGTTGATTCTGGGTTGAGGACACC

2.1.7 Software

EndNote X2	Thomson Reuters, New York, NY (USA)
Fluostar Optima 1.30	BMG Labtechnologies, Offenburg
Millenium32 Waters	Waters, Milford MA, USA
Photoshop CS 8.0.1	Adobe Systems GmbH, Munich
Rotor-Gene® Q Series Software	Qiagen, Hilden
SigmaPlot 12.0	SPSS Inc., Munich
SigmaPlot Enzyme Kinetic Module	SPSS Inc., Munich

Web based software tools and websites:

Aramemnon	www.aramemnon.botanik.uni-koeln.de/
ClustalW2	www.ebi.ac.uk/Tools/msa/clustalw2/
Gene Investigator	www.genevestigator.com
MaizeGDB	www.maizesequence.org/index.html
Primer calc	www.basic.northwestern.edu/biotools/oligocalc.html
TAIR	www.arabidopsis.org

2.2 Plant methods

2.2.1 Plant material and growth conditions

Maize (*Zea mays* L) hybrid Severo (KWS Germany) was used for all experiments during this study. Seeds were sown individually in each pot containing 100% vermiculite media. Plants were grown in long day conditions with 16 h/8 h day/night cycle at a temperature between 20°C and 22°C. The humidity of growth chamber was set to 50% with a light intensity of 300 $\mu Em^{-2} s^{-1}$.

After one week of sowing, plants were started with application of ½ Hoagland solutions every 2 days.

Modified ½ Hoagland medium:

Macroelements:	Microelements (all without sulfate):
2.5 mM $Ca(NO_3)_2$	40 µM Fe-EDTA
2.5 mM KNO_3	25 µM H_3BO_3
0.5 mM $MgSO_4$ / $MgCl_2$	2.25 µM MnCl2
0.5 mM KH_2PO_4	1.9 µM $ZnCl_2$
	0.15 µM $CuCl_2$
	0.05 µM $(NH_4)_6Mo_7O_{24}$

The pH of all ½ Hoagland solutions was set to 5.8 with 5M KOH.

In addition to Hoagland solution, each pot was supplemented with solid fertilizer osmocote exact tablets (Scotts international, the Netherlands) in order to meet nutrients requirement of maize plants.

2.2.2 Drought stress treatment

Drought stress was imposed on 2 weeks old maize seedling by with-holding water for 7, 10 and 12 days whereas control plants were applied with water regularly.

2.2.3 Measurement of the relative water content (RWC)

Measurement of the relative water content (RWC) was performed on leaves taken from control and drought stressed plants. Individual leaves were removed from the stem using scissor and fresh weight (Fw) was recorded immediately. The leaves were then incubated in distilled water for at least 4 h at 4^0C in the dark, blotted dried and then turgid weight (Tw) was measured. Finally, dry weight (Dw) was determined after drying at 80^0C for 48h in the oven. The relative water content (RWC) was calculated with the following formula as described by Jones, 2007.

RWC (%) = [(FM - DW)/ (TW - DW)] * 100.

Where, RWC stands for relative water content. Fw, Tw and Dw are the fresh, turgid and dry weight respectively.

2.2.4 Measurement of soil water content

Soil water content of control and drought stressed plants pot was measured by weighing fresh weight of the pod with soil inside. Then soil dry weight was recorded by drying at 80^0C for 3-4 days in oven and the soil water content was determined using following formula,

SWC = [(FM - DM)/ (DM)]

SWC stands for soil water content, FW and DW represents fresh weight and dry weight, respectively.

2.2.5 Determination of phenotypic differences between control and drought stressed plants

The following morphological differences were recorded after 7, 10 and 12 days both in control and drought stressed plants. Fresh weight of leaves and roots was measured and root to shoot ratio was determined on fresh weight basis which is important indicator of drought stress.

2.2.6 Measurement of stomatal aperture

Quantification of stomata aperture was performed by doing a leaf imprint using a droplet of superglue on microscope slide. Truncated leaf discs from control and drought stressed plants were placed immediately on the slide with cuticle side up and the lower epidermis down on the glue droplet. The leaf discs were then gently pressed so that the lower part of the leaf is stick to the slide and afterwards with the help of forceps, leaf disc was removed forming image on the slide and stomata aperture was analyzed with microscope and image j.

2.3 Biochemical methods

2.3.1 Isolation of soluble proteins from plants

Approximately 100-150 mg grinded tissue of leaf and root of control and drought stressed maize plants were used for total protein extraction in 500 µl of extraction buffer containing 50 mM Hepes/KOH pH 7.4, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol supplemented with 0.5 mM PMSF and 30 mM DTT. After vortexing of the samples for 15 min on ice, the cell debris was removed by two steps of centrifugation at 25,000 x g for 10 min at 4°C. The supernatant was desalted by size-exclusion

chromatography using a NAP-5 column (Amersham, Braunschweig, Germany) and proteins were eluted in 500 µl of the resuspension buffer containing 50 mM HEPES-KOH pH 7.5, 1 mM EDTA, 2 mM DTT and 0.5 mM PMSF according to manufacturer's protocol.

2.3.2 Determination of the protein concentration by Bradford assay

The protein concentration was determined according to Bradford (1976) using bovine serum albumine (BSA) as standard. Each sample was diluted accordingly and 10 µl of the diluted sample or standard was added with 250 µl of Roti®-Quant Bradford reagent in 96-well plate. After incubation at room temperature for 5 min, the absorbance at 595 nm was measured in the Fluostar Optima plate reader and determination of the protein concentration of the samples was achieved on the basis of a standard calibration curve.

2.3.3 Enzymatic activity assays

2.3.3.1 Determination of GR activity

GR activity was measured of total protein extract as described in Smith et al., (1988). Approximately, 20 µg of proteins were used in a total volume of 250 µl reaction mixture containing 100 mM K_2HPO_4/KH_2PO_4 pH 7.4, 1 mM EDTA together with 750 µM Ellman's reagent (DTNB), 200 µM NADPH and 400 µM GSSG. DTNB and GSH react to generate 2-nitro-5-thiobenzoic acid and GSSG. Since 2-nitro-5-thiobenzoic acid is yellow, the GSH concentration in a sample solution can be determined by O.D. measurement at 412 nm absorbance in the plate reader. Respective blank samples without protein were used for subtraction of background absorption level at 412 nm. NADPH and GSSG were always prepared freshly.

2.3.3.2 Determination of OASTL activity

Determination of enzymatic OAS-TL activity was performed by quantification of the reaction product cysteine. The reaction was performed in an assay of 0.1 ml volume containing 50 mM Hepes; pH 7.5, 5 mM Na_2S, 5 mM DTT, 10 mM OAS and 1-2 µg crude protein. The reaction was started by the addition of master mix to the crude extract and incubation at 25^oC for 25 minutes. After stopping the reaction by addition of 50 µl 20% TCA, the samples were centrifuged at 25,000 x g for 10 min at 4^oC.The supernatant was transferred to new safe lock eppendorf tube quantitatively and then added 200 µl ninhydrin solution (250 mg ninhydrin in a mixture of 6 ml of 100% acetic acid and 4 ml of concentrated HCl) and 100 µl of 100% acetic acid. After incubation in water bath for

10 min at 99^{o}C, the samples were cooled down at room temperature, followed by the addition of 550 µl of 100% ethanol to each sample. The amount of cysteine was determined photometrically as described in Gaitonde (1967) with a cysteine standard calibration curve.

2.3.3.3 Determination of SAT activity

SAT activity was assayed by coupling to the OAS-TL reaction (Nakamura et al., 1987). 60 µl of the crude protein extracts from leaves and roots in a reaction volume of 100 µl were assayed to obtain a good signal. The reaction mixture consisted of 50 mM HEPES; pH 7.5, 10 mM Na_2S, 5 mM DTT, 10 mM serine, 1 mM Ac-CoA, The reaction was allowed to proceed for 60 min at 25°C. Afterwards 50 µl 20% of TCA was added in order to stop the reaction. The production of OAS by SAT was converted to cysteine by excess of OAS-TL activity. Subsequently, the cysteine obtained was determined as described in Gaitonde, (1967).

2.4 Metabolomics

2.4.1 Extraction of metabolites

Approximately 100 mg fresh weight grinded leaf and root material from control and drought stressed plants were used for extraction of metabolite in 500 µl 0.1 M HCl for 15 min by vortexing on ice. Cell debris was collected by two centrifugation steps with 20,000 x g at 4°C. The supernatant extract was transferred to another clean micorcentrifuge tube and used for further analysis.

2.4.2 Determination of thiol metabolites

Separation and detection of low molecular weight thiols was acheived after their full reduction and derivatization to a fluorescent conjugate. 270 µl of the reduction buffer containing 25 µl of HCl extract, 20 µl 1 M Tris pH 8.3, 190 µl water, 10 µl 10 mM DTT and 25 µl of 0.08 M NaOH was incubated for 1 h at room temperature in the dark for full reduction. 25 µl of 10 mM MBB (Synchem, Felsberg, Germany) was added to the derivatization assay which starts forming of thiol-bimane conjugate that is specific for reducing sulfhydryl groups containing metabolites followed by incubation for 15 min at room temperature in the dark. 705 µl of 5% acetic acid was added for stopping the reaction and stabilizing the thiol-bimane derivatives. Separation of thiol derivatized was performed by reverse phase HPLC (Waters 600E Controller and pump, Waters 717 plus autosampler and NovaPak C18 column 4.6x250 mm, 4 µm beads). Thiols were separated using an isocratic run of 91% buffer A (100 mM potassium acetate, pH 5.3, 0.02%

sodium azide) and 9% methanol and detection was achieved by using a Jasco FP-920 fluorescence detector at 480 nm after excitation with 380 nm and results were analyzed using the Millenium32 software.

2.4.3 Determination of reduced and oxidized glutathione

Approximately 25 mg fresh weight of leaf and root tissue was ground in liquid nitrogen for determination of reduced glutathione (GSH) and oxidized glutathione (GSSG). For GSH determination, extraction was performed with 0.5 ml PM buffer containing 0.1 M K_2HPO_4/KH_2PO_4 buffer, pH 7.1 and 50% methanol supplemented with 5 mM DTT. For GSSG, extraction was carried out in 0.5 ml PM buffer supplemented with 5mM N-ethylmaleimide (NEM). The extracts were then incubated at 60^{o}C under vigorous shaking for 10 min and subsequently centrifuged twice with 20,000 g at room temperature for 5 min. 10 µl of the supernatant was added to the reduction buffer containing 190 µl dH_2O, 40 µl 1M Tris-HCI pH 8.3 and 30 ul 5mM DTT in 1.5 ml tube and incubated for 1h in the dark for full reduction. Afterwards, 30 µl of 30 mM monobromobimane was added to the the reactions for 15 min to form conjugate with the free thiol groups. The reaction was then stopped by addition of 705 µl of 5% acetic acids. Detection and quantification was performed as described for thiols.

2.4.4 Determination of OAS and aminoacids

OAS and amino acids were quantified after derivatization with the fluorescent dye AccQ-TagTM (Waters). Derivatization was performed in 100 µl volume containing 70 µl 0.2 M borate buffer; pH 8.8, 20 µl AccQ-Tag in acetonitrile (3 mg/ml) and 10 µl of the metabolite extract (section 2.4.1) were used for the derivatization according to the manufacturer's specification. The derivatized amino acids were separated by reverse phase liquid chromatography (LC) using a Nova-PakTM C18, 3.9 x 150 mm column (Waters) as described in Hartmann et al. (2004). Separated AccQ-Tag derivatives were detected with a fluorescence detector Jasco FP-920 (Jasco, Groß-Umstadt, Germany) at 395 nm after excitation with 250 nm. Quantification was performed using the Waters LC control- and analysis software Millenium32 (Waters, USA). Standardization was carried out on the basis of standard calibration curve for each individual amino acid.

2.4.5 Determination of sulfide

Approximately 25 mg of plant tissue was grinded and homogenized in liquid nitrogen. The sample was directly derivatized in 155 µl alkaline extraction buffer (160 mM Hepes pH 8.0, 16 mM EDTA) and 5 µl 100 mM mBB. The reaction set was vigorously agitated

for 30 sec and incubated for 30 min at RT in dark and vertexed every 10 min. After incubation, the reactions were centrifuged twice for 15 min at 4°C with 20,000 g. Afterwards, 840 µl of 5% acetic acid was added for stopping the reaction and stabilizing the mBB-derivatized products, followed by sedimentation at 18°C for 45 min at 20,000 g. Sulfide was separated from other thiols on a LiChroCART® 125-4 LiChrospher® 60 RP-select B (5 µm) column (2.1.1) with a flow rate of 1.3 ml/min by increasing the hydrophobicity of the eluent A (0.25% acetic acid) by mixture with methanol (eluent B). Quantification was performed as described for thiols.

2.4.6 Determination of adenosines

For determination of adenosines, 150 µl of metabolite extract (2.4.1) or standards were mixed with 770 µl of CP buffer containing 620 mM citric acid pH 4, 760 mM disodium hydrogen phosphate. After addition of 80 µl of 45% CAA (chloroacetaldehyde) the samples were incubated for 10 min at 80°C and were allowed to cool down on ice to room temperature followed by sedimentation at 20°C for 45 min with 20,000 g. The supernatant was transferred to HPLC vials. Adenosine derivatives were separated by reversed phase HPLC with an XTerraTM MS C18, 5 µm, 3 mm × 159 mm column (2.1.1). 1× TBAS (5.7 mM TBAS, 30.5 mM potassium dihydrogen phosphate, pH 5.8) used as running buffer and acetonitrile:1× TBAS (2:1, v/v) as separation buffer. Detection of metabolites was performed with a Jasco FP-920 fluorescence detector (2.1.1) at 410 nm after excitation with 280 nm and results were quantified using the Millenium32 software.

2.4.7 Determination of the anions sulfate, phosphate, and nitrate

Prior to application of the sample, the metabolite extract (section 2.4.1) was diluted 10-times. The sample was separated by anion exchange chromatography using an IonPac AS9- HC 2 x 250 mm column (Dionex) that was connected to an ICS 1000 (Dionex). A carbonate buffer containing 8 mM $NaCO_3$, 1 mM $NaHCO_3$ was used as mobile phase with a flow rate of 0.3 ml/min. Detection of the separated ions was performed with a conductivity detector (Dionex) and Chromeleon 7.1 software provided by the manufacturer was used for the quantification of each ion on the basis of standard calibration curves.

2.4.8 Determination of total CNS contents

Determination of total carbon, nitrogen and sulfur contents of the leaves and roots of control and drought stressed maize plants was achieved using a Vario MAX CNS

elemental analyzer (Elementar, Hanau) in collaboration with Mr. Gerd Schukraft at the Geographical Institut at Heidelberg University.

The plant material was ground to fine powder after complete drying of leaves and roots in a 120°C incubator for 2-3 days. A sample of 30mg dry weight was incinerated in the elemental analyzer and separation of CO_2, SO_2 and N_2 was performed by adsorption of CO_2 and SO_2 to specific chromatography columns in the elemental analyzer. Desorption of a gas from the chromatography matrix was performed by heating of the respective column. Helium served as the mobile phase with a flow rate of 140 ml/min and for incineration of the sample, O_2 was added with a flow rate of 60 ml/min for 50 sec. The elements were detected in the forms of CO_2, N_2 and SO_2 by means of their thermal conductivity and quantified according to a standard calibration curve prepared with sulfadiazin.

2.4.9 Determination of leaf chlorophyll contents

Approximately 20 mg FW leaf material was grinded in liquid nitrogen and extraction was carried out in 750 µl of 80% acetone for 15 minutes on ice. After centrifugation at 18,000 x g at 4°C for 10 min, the supernatant was transferred to a new micro centrifuge tube. The same volume of 80% acetone was added to the pellet, mixed thoroughly and upon centrifugation, the resulting supernatant was combined with the first one and the absorbance of total extract was measured (against 80% acetone) at 645 nm and 663 nm. Chlorophyll contents were determined according to the following equation as described in Arnon (1949):

Equation

$C = Ca + Cb$ or $C = 20.2\ OD645 + 8.02\ OD663$

Whereas, C stands for total chlorophyll, Ca means chlorophyll a, and Cb represents chlorophyll b

2.4.10 Detection of H_2O_2

3,3'-diaminobenzidine (DAB) staining was used for visualization of H_2O_2 level as described by Thordal-Christensen (1997). DAB gives a brownish colour upon reaction with H_2O_2 in the presence of peroxidases. Leaf discs of control and drought stressed plants were cut and incubated in freshly prepared DAB solution (1.68 mg/ml in dH_2O; pH 3.8) in a 2 ml eppendorf tube. The leaf discs were completely immersed in the solution after vacuum infiltration and then allowed to stand for 24 h at room temperature. A discoloration of chlorophyll from the leaf tissue was performed with 100% ethanol for 24

h and the solution was replaced 3 times in order to make DAB staining visible and then stored in 40% glycerol (v/v) until further analysis.

2.5 Microscopic methods

2.5.1 Detection of DAB staining.

The leaf discs in 40% glycerol (v/v) were then put on slides, and analyzed microscopically by using the differential contrast. Images were taken with the color LCD 320 FX camera (Leica) with 2.5x magnification.

2.5.2 Measurment of stomatal aperture

The stomata on the slides were imaged with a Leica MZ FLII stereomicroscope equipped with a DFC 320 camera with 40x magnification.

2.6 Transcriptomics

2.6.1 mRNA Isolation

Approximately 100mg of leaf and root tissue was used for total RNA extraction using RNeasy Plant Mini Kit and RNase free DNAse Kit (2.1.4) according to manufacturer's protocols.

2.6.2 Determination of RNA concentration

RNA concentration was determined spectrophotometrically at 260 nm using NanoDrop 2000 spectrophotometer (2.1.1). The ratios A260 nm/A280 nm and A260 nm/A230 nm were compared to estimate protein and polysaccharide impurities. The values obtained were between 1.8 and 2.1 which is always used for good quality RNA.

2.6.3 Quantitative real time-PCR

2.6.3.1 cDNA synthesis

Synthesis of cDNA from total RNA extract was performed with RevertAid™ H Minus First Strand cDNA Synthesis Kit (2.1.4) according to manufacturer's protocols using 1 µg of total RNA extract.

2.6.3.2 qRT-PCR

The qRT-PCR reaction was performed with 1 µg cDNA and 2.5 pmol of each specific primer (2.1.5) and was mixed with 6.25 µl SYBR solution from SensiMix™ SYBR No-ROX Kit (2.1.4). The reaction took place in the Rotor-Gene Q (2.1.1) according to the manufacturer's protocol. Rotor-Gene® Q Series Software (2.1.7) was used for quantification. For leaf and root actin and β-tubulin were used for normalization as

reference genes, respectively. Each of at least 3-4 biological replicas from control and drought stressed plants was tested.

2.7 Physiological methods

2.7.1 Analysis of metabolic flux

2.7.1.1 Incorporation of ^{35}S into thiols and protein of maize leaves and roots

Incorporation rates of radioactively labeled sulfur into plant thiols and protein fraction of leaves and roots of control and drought stressed maize plants were determined by incubating on $^{35}SO_4^{2-}$ labeled solution. Approximately 30 mg of leaf discs of comparable sizes and 50 mg of roots were cut from the control and drought stressed plants and 1st rehydrated in dH_2O for 10 min followed by incubation on the ^{35}S labeling solution (½ Hoagland medium) for 30 and 60min for leaf and 30, 60, 180 and 360 min for roots with a total of 0.502 mM sulfate containing 125 nM $^{35}SO_4^{2-}$ on a horizontal shaker with 60 rpm in the light (17 µE). After incubation on $^{35}SO_4^{2-}$ labeled solution, the leaf and roots pieces were washed twice with the nonradioactive ½ Hoagland medium, dried on plastic- coated paper towel and immediately frozen in liquid nitrogen.

2.7.1.2 Extraction of radiolabeled metabolites

Metabolites were extracted in a volume of 0.3 ml 0.1M HCl as described in section 2.4.1. Homogenization of the radiolabeled leaf samples was performed using the Bio101 ThermoSavant Fast Prep system (Qbiogene) according to the manufacturer's instructions whereas root samples were grinded to a fine powder in liquid nitrogen.

2.7.1.3 Isolation and derivatization of radioactive labeled thiol

Isolation of the thiols (Cysteine and GSH) from the radiolabeled metabolite extract (section 2.7.2.2) was achieved by liquid chromatography as described in sections 2.4.1. 100 µl of the metabolite extract were added for derivatization to the reduction buffer (2.4.2) with addition of 100 µl of 0.08 M NaOH for neutralization of the hydrochloric metabolite extract. Derivatization was carried out as described in 2.4.2. 205 µl of 20% (v/v) acetic acid were added for stabilization of thiol derivates resulting in a total volume of 500 µl. 50 µl was injected on the column of the derivatized sample for separation by HPLC.

Cysteine and GSH containing fractions were collected after elution from the column using a fraction collector connected with the HPLC. The collected metabolite fractions were subjected to liquid scintillation counting (section 2.7.1.5).

2.7.1.4 Isolation of the radioactive labeled protein fraction

Protein fractions from the radioactively fed leaves and roots pieces were isolated from the pellets of the 0.1 M HCl extract(section 2.7.1.2) and washed the pellets with 1ml of the 0.1M HCI. 500 µl 8 M urea was added to the pellet containing 100 µl of residual HCl extract mixed thoroughly and incubated at 37°C over night. Samples were centrifuged at 16,000 x g for 5 min at room temperature after mixing. 50 µl of the resulting supernatant was used for precipitation of proteins using the 2-D-Quant Kit according to the manufacturer's instructions. After precipitation, 100 µl copper solutions (supplied with the Kit) and 400 µl dH_2O was used to dissolve the pellet. The entire protein fraction was used for liquid scintillation counting (section 2.7.1.2).

2.7.1.5 Determination of incorporated radioactivity by liquid scintillation counting

Liquid samples of the fractions containing the individual metabolites or proteins (sections 2.7.1.3 and 2.7.1.4) were mixed each with 10 ml of Ultima Gold liquid scintillation amplifier in a 20 ml scintillation vial. The incorporated radioactivity of the samples was determined for 5 min using standard settings for ^{35}S with the liquid scintillation counter (2.1.1). After calibration with a solution containing a defined activity of ^{35}S using the specifications of the supplier regarding the activity correspondence to molarity, the cpm or dpm values of the samples were converted to the corresponding molar content of the respective isotope.

2.7.2 Determination of transport rate of $^{35}SO_4^{2-}$ through stem of maize

The transport rate of $^{35}SO_4^{2-}$ from root to shoot was determined in control and drought stressed plants by injecting ^{35}S labeled solution into the stem. 1ul of the $^{35}SO_4^{2-}$ labeled solution containing 750 nM $^{35}SO_4^{2-}$ was injected into the stem of the intact plant, 5 cm above the soil level with 1µl Hamilton syringe penetrating directly to the middle of the stem. After 1 min of injection, the stem of the plant was cut into 4 pieces each at 3 cm distance. Afterwards each piece was again cut into smaller pieces with the help of blade in order to expose each portion of the stem to scintillation chemical for reaction and the amount of radioactivity was then measured in each segment with scintillation counter (2.1.1).

2.8 Statistical Analyses

Means of different data sets were analyzed for statistical significance using unpaired t-test or ANOVA test. Constant variance and normal distribution of data were checked with

SigmaStat 12.0 (2.1.7) prior to statistical analysis. The Mann-Whitney rank sum test was used to analyze samples that did not follow normal Gaussian distribution. Asterisks in all figures indicate the significance: *, $0.05 \geq p > 0.01$; **, $0.01 \geq p > 0.001$; ***, $p \leq 0.001$.

3 Results

3.1 Impact of drought on water contents and growth of maize

Maize hybrid Severo was obtained from KWS Germany for drought stress experiments and grown under long day condition (16 h day/8 h dark) in vermiculite growth medium because it was easy to analyzed roots as well compared to soil grown plants. Plants were grown for 2 weeks in ½ Hoagland solution and water was either withheld for 7, 10 and 12 days (drought stress) or supplied regularly (mock treatment) (Fig. 5A, B, C & D).

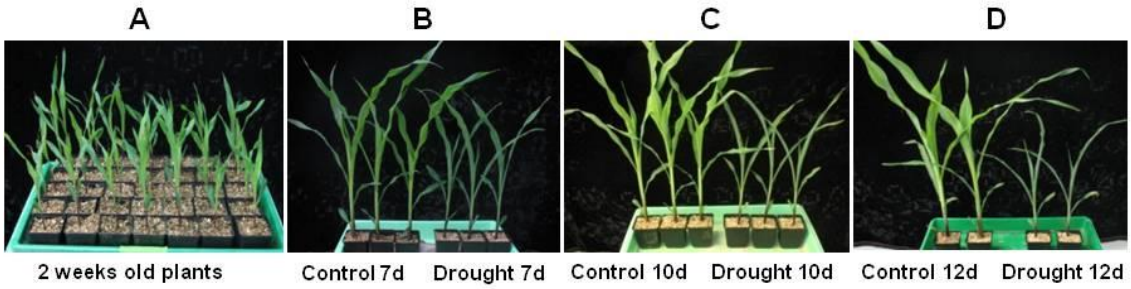


Fig. 5 Phenotype of control and drought stressed maize plants
Phenotype of maize plants grown on vermiculite media under long day condition for (A) 2 weeks and then subjected to drought stress for (B) 7 days (C), 10 days and (D) 12 days

After 7, 10 and 12 days, the relative water content (RWC) in leaf and soil, which are the important indicators of drought, was determined. RWC of leaf from control plants grown under optimum water condition remained at 96%, whereas RWC was significantly decreased to approximately 93, 78 and 66 % after 7, 10 and 12 days, respectively, in drought stressed plants compared to the control (Fig. 6A).

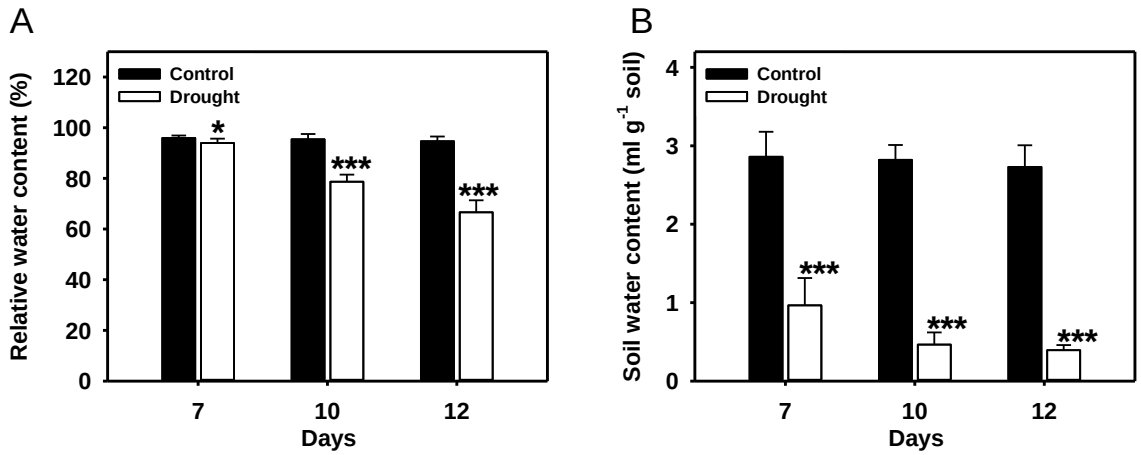


Fig. 6 Impact of drought stress on relative water content of leaf and soil
(A) Relative water content of leaf and (B) Soil water content was determined in leaves and soil of control and drought stressed plants grown on vermiculite under long day condition (n=8). Mean ± standard deviations are shown.

In addition to the decrease in RWC of leaf, the soil water content (SWC) was also significantly decreased compared to the control. This decrease in SWC was approximately 66, 83 and 85% after 7, 10 and 12 days of drought stress, respectively, compared to the control (Fig. 6B). However, this magnitude of difference in soil water content after 7 days of drought was much lower than observed for relative water content of leaf.

In order to determine if the observed differences in RWC and SWC also affect plant growth, fresh weight of leaves and roots of both control and drought stressed plants was measured. An average reduction of about 42, 74 and 80 % was observed in total leaves biomass after 7, 10 and 12 days of drought stress respectively, compared to control plants (Fig. 7A). This reduction was pronounced when longer period of drought stress was applied.

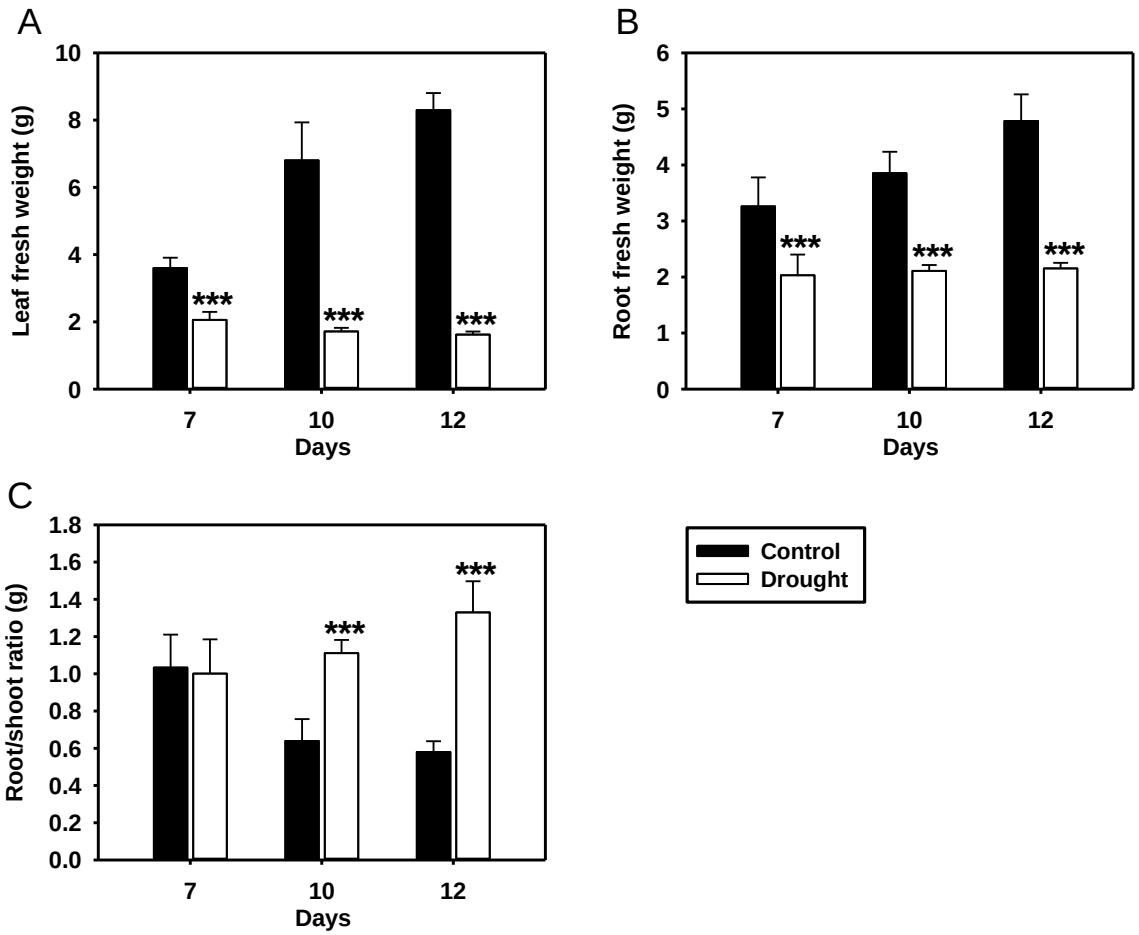


Fig. 7 Impact of drought on growth of maize

(A) Total leaf fresh weight (B) Total root fresh weight and (C) root to shoot ratio. Total fresh weight of leaves, roots and then root to shoot was determined in control and drought stressed plants after 7, 10 and 12 days grown on vermiculite media under long day condition (n=8). Mean ± standard deviations are shown.

Beside decrease in leaf growth, a significant reduction in root fresh weight was also observed in drought-treated plants. An average decrease of approximately 37, 45 and 54%

was observed in root biomass after 7, 10 and 12 days of drought, respectively (Fig. 7B). But this decrease was not as severe as observed for leaves.

Although the biomass of shoots and roots was significantly decreased, root to shoot ratio was significantly increased by 1.73 and 2.29-fold on a fresh weight basis after 10 and 12 days. No changes were observed in plants subjected to drought, which might indicate that roots continued to grow in order to cope with prolonged water shortage (Fig. 7C).

3.2 Analysis of stomata aperture and chlorophyll content

One of the early responses of plant to drought stress is stomata closure in order to reduce water loss due to transpiration. The decrease in soil water contents and subsequent curling of leaves led us to determine the stomata aperture of leaves from the control and drought stressed plants grown on vermiculite. A leaf disc from the control and drought plants was taken and slides were prepared for analyzing stomata with microscope and afterwards with image j software (section 2.2.6). 35 stomata were analysed from 3 different plants at each time point. Analysis of stomata revealed a significant decrease in stomata aperture after 7, 10 and 12 days in drought-treated plants compared to the control. This decrease continued even after 7 days of drought, indicating that the plant roots could sense water shortage due to decrease in soil water content and altered root to shoot transport of water and metabolites might play an important role in stomata closure (Fig. 8).

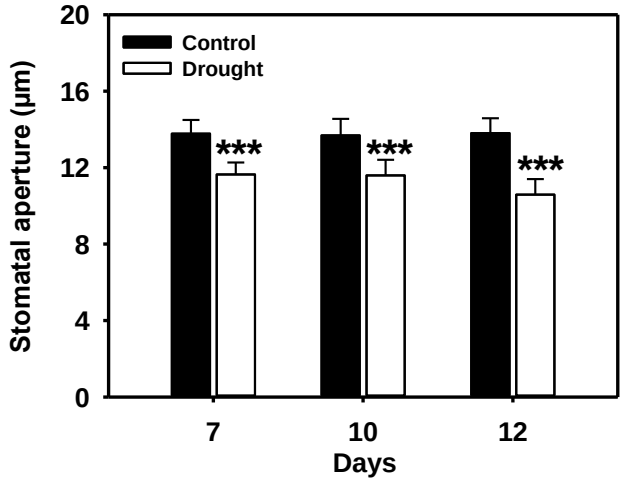


Fig. 8 Impact of drought on stomata aperture in leaves
Quantification of stomata aperture was performed by doing a leaf imprint using a droplet of superglue on microscope slide. Leaf disc was then removed forming image on the slide and stomata aperture was analysed with microscope and image j in leaves of control and drought stressed plants at each time point grown on vermiculite media under long day condition (n=35). Mean ± standard deviations are shown.

The observed decrease in stomata aperture might have an effect on photosynthesis. Therefore, chlorophyll contents were determined in leaves of control and drought stressed

plants grown on vermiculite media (section 2.4.9). A significant decrease in total chlorophyll contents was observed under drought stress at each time point. An average decrease of approx. 20, 26 and 24% was observed in total chlorophyll when plants were subjected to 7, 10 and 12 days of drought, respectively compared to the control (Fig. 9A). The reduction in total chlorophyll contents was attributed to the significant decrease in both chlorophyll a and b amounts (Fig. 9B & C).

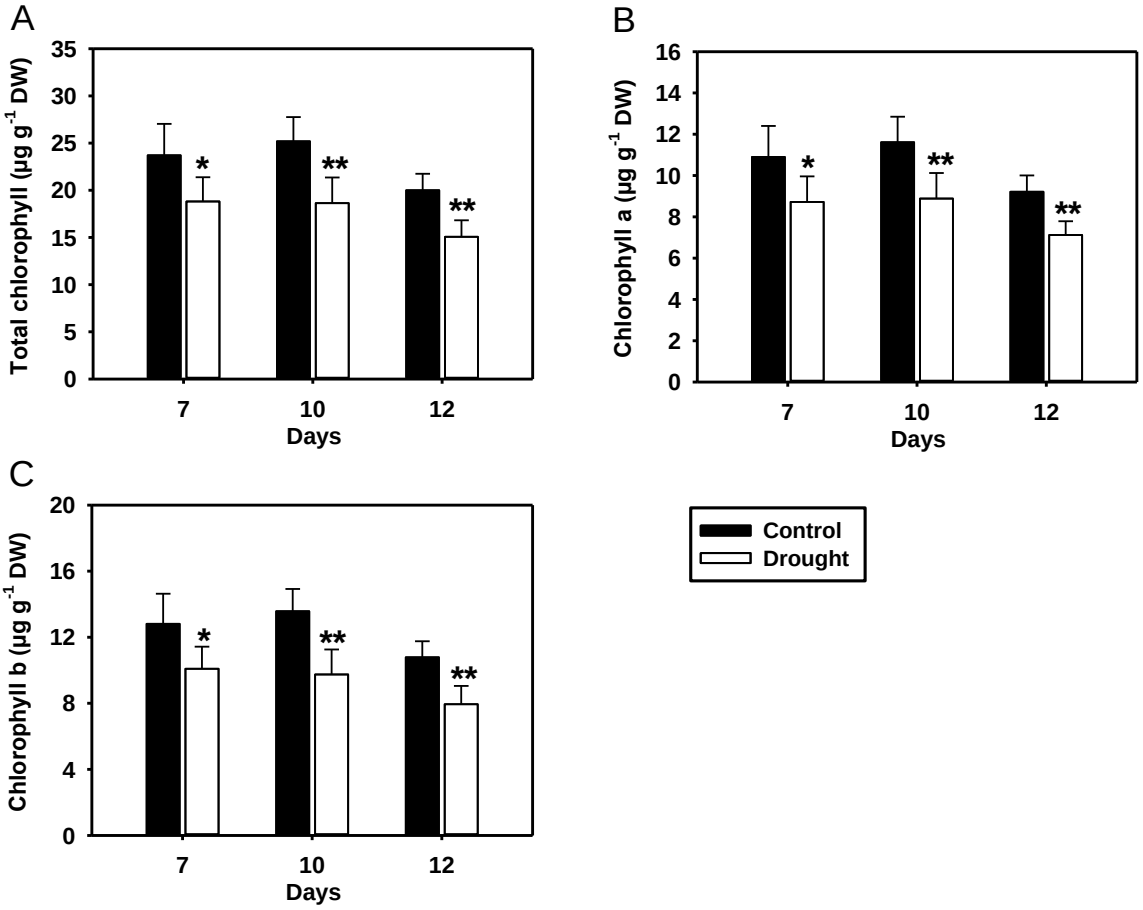


Fig. 9 Impact of drought on chlorophyll content in leaves of maize
Comparison of (A) Total (B) Chlorophyll a and (C) Chlorophyll b contents in leaves of 2 weeks old plants subjected to drought stress for 7, 10 and 12 days grown on vermiculite media under long day condition. (n=8). Mean ± standard deviations are shown.

3.3 Impact of drought on stress markers in leaves and roots

3.3.1 Analysis of proline contents in leaves and roots

To evaluate the extent in which drought stress affected maize growth, the content of proline, a known drought stress marker in higher plants (Hanson, 1980; Lutts *et al.*, 1999; Secenji *et al.*, 2010), was quantified in leaves of control and drought stressed plant. A significant 2, 4 and 7-fold increase was observed in proline contents in leaves after 7, 10 and 12 days of drought stress, respectively, compared to the control (Fig. 10).

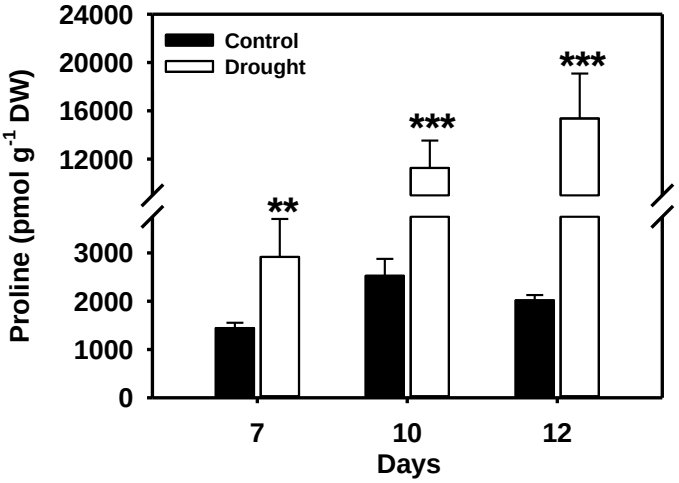


Fig. 10 Impact of drought on stress marker proline in leaves of maize
A marker for stress, proline content was quantified by HPLC in leaves of control and drought stressed plants subjected to drought for 7, 10 and 12 day. (n=5). Mean ± standard deviations are shown.

Proline contents were also determined in roots where drought was first encountered. As observed in leaves, a huge increase in proline content was observed under drought stress in roots. Proline contents were approximately increased by 7, 8 and 21-fold in roots after 7, 10 and 12 days of drought stress, respectively compared to the control (Fig. 11). This huge accumulation of proline indicates that roots are the first tissue in plants that sense drought.

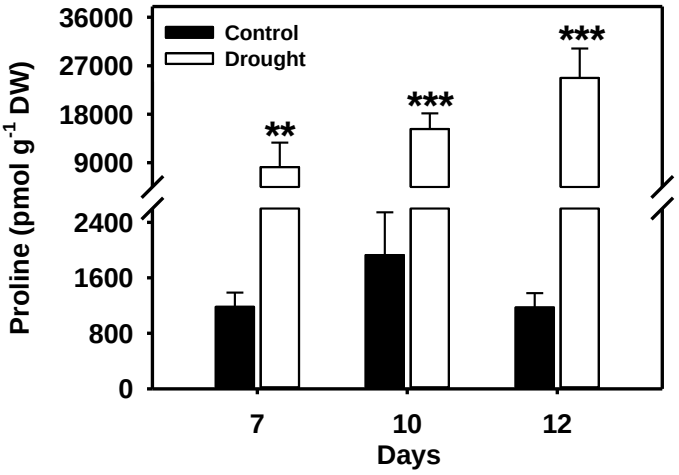


Fig. 11 Impact of drought on stress marker proline in roots of maize
A marker for stress, proline content was quantified by HPLC in roots of control and drought stressed plants subjected to drought for 7, 10 and 12 day. (n=5). Mean ± standard deviations are shown.

3.3.2 Detection of H_2O_2 in leaves of maize

To further investigate whether drought caused an oxidative stress in maize, H_2O_2 level was measured in leaves of control and drought stressed plants grown on vermiculite

media under long day condition. Leaf discs of control and drought stressed plants were incubated in 3,3'-diaminobenzidine (DAB) solution. DAB reacts with H_2O_2 to give a brownish colour. H_2O_2 level as indicated by DAB staining intensity was then visualized with microscope (section 2.4.10). The staining was more pronounced in 10 and 12 days drought-treated leaf disc compared to the control, indicating higher level of H_2O_2 production under drought (Fig. 12). However, no difference in DAB staining was observed for 7 days drought stressed leaf disc.

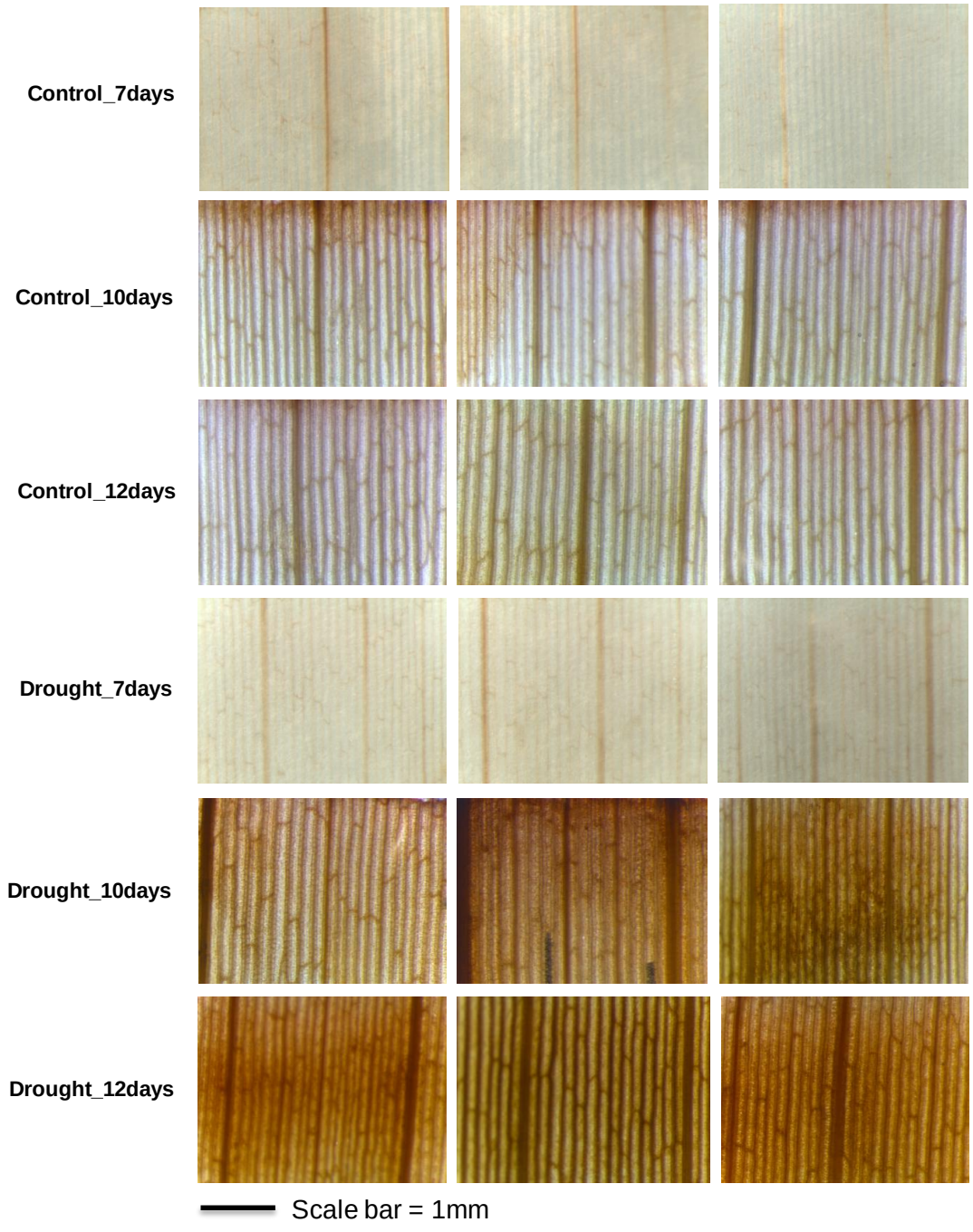


Fig. 12 Detection of H_2O_2 in leaves of non- and drought stressed maize by DAB staining
Leaf discs of control and drought stressed plants were treated with DAB solution overnight, washed with ethanol and afterwards was analyzed with microscope using 2.5x magnification (n=3)

3.4 Impact of drought on thiol and other sulfur containing compounds

3.4.1 Alteration of GSH steady-state level in drought-treated leaves and roots

In order to determine if the redox state of GSH pool is altered as a result of a higher level of H_2O_2, the ratio of oxidized to reduced GSH was determined in leaves of control and drought stressed plants by HPLC (section 2.4.3). Consistent with the observed DAB staining patterns, GSSG:GSH ratio was significantly increased by approximately 2.5 and 2.6-fold after 10 and 12 days of drought, respectively, whereas 7 days of drought had no significant effect on GSSG to GSH ratio in leaves of maize plant (Fig. 13).

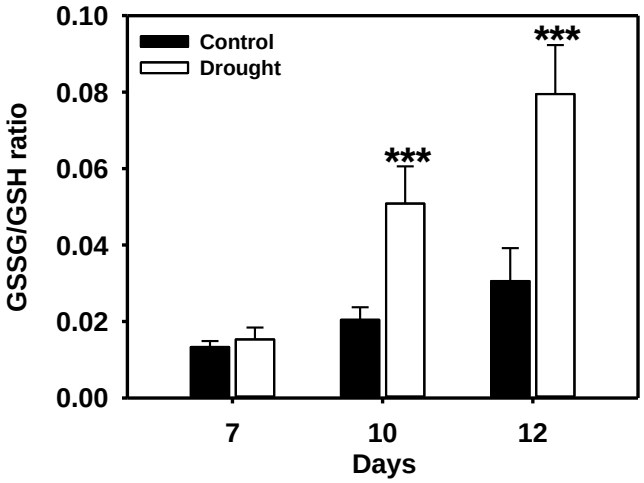


Fig. 13 Impact of drought on GSSG/GSH ratio in leaves
Total and oxidized glutathione were quantified by HPLC and the GSSG/GSH ratio was then determined in leaves of control and drought stressed plants at each time point grown on vermiculite media under long day condition (n=5). Mean ± standard deviations are shown.

In leaves the ratio was not changed after 7 days of drought stress, whereas in roots 3.5-fold change in GSSG to GSH ratio was observed. Roots of drought stressed plants also showed an increase in GSSG to GSH ratio by 2-to 3.5-fold relative to the control (Fig 14). This change in ratio to a more oxidized glutathione state is one of the indicators of oxidative stress in roots as observed for leaves.

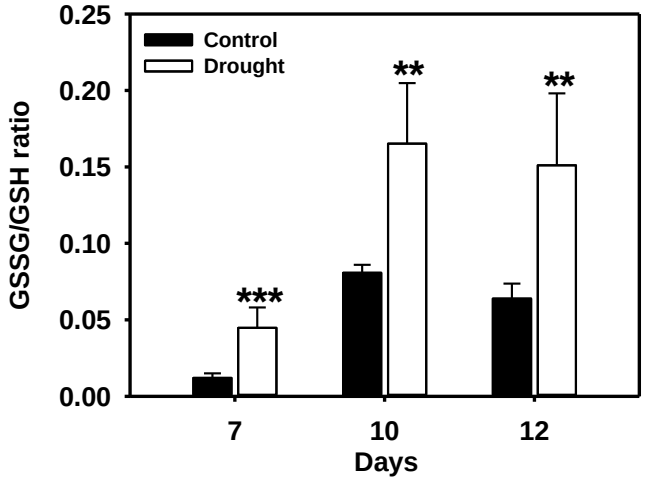


Fig. 14 Impact of drought on GSSG/GSH ratio in roots
Total and oxidized glutathione were quantified by HPLC and the GSSG/GSH ratio was then analyzed in roots of control and drought stressed plants at each time point. (n=5). Mean ± standard deviations are shown.

3.4.2 Analysis of *GR* transcript and GR activity in leaves and roots

Glutathione reductase regenerates GSH on the expanse of NADPH during ROS detoxification via the ascorbate-glutathione cycle (Noctor *et al.,* 2002). In Arabidopsis, two GRs are present that encode for the cytosolic GR1 (At3g24710), and the plastidic and mitochondrial localized GR2 (At2g54660) (Creissen et al., 1995; Chew et al., 2003a). To identify GR isoforms in maize, the protein sequence of Arabidopsis GR1 and GR2 were blasted against the maize database (maizegenome.org). Only one GR in maize was found based on sequence identity with Arabidopsis GRs. When these protein sequences were aligned using CLUSTALW software, maize GR showed sequence identity of approximately 53% with GR1 whereas 75% with GR2 from Arabidopsis (Fig. 15). Furthermore, bioinformatic tool such as Aramemnon-plant membrane protein database (release 7.0) was used for the prediction of subcellular location of maize GR. This database strongly predicted maize GR as chloroplast localized.

```
MaizeGR           MAAHATLPFSCSS---TLQTLTRTL--SSRGAHQLRGGFLRLP----SLAAL-PRLAHPC 50
ArabidopsisGR2    MASTPKLTSTISSSSPSLQFLCKKLPIAIHLPSSSSSSFLSLPKTLTSLYSLRPRIALLS 60
ArabidopsisGR1    MARKMLVDGEIDK------------------------------------------------ 13
                  **    :    ..

MaizeGR           RRHVSAS-----AAAAPNGASVEGEYDYDLFTIGAGSGGVRASRFASALYGSRVAICEMP 105
ArabidopsisGR2    NHRYYHSRRFSVCASTDNGAESDRHYDFDLFTIGAGSGGVRASRFATS-FGASAAVCELP 119
ArabidopsisGR1    ------------VAADEANAT---HYDFDLFVIGAGSGGVRAARFSAN-HGAKVGICELP 57
                              *:   .*    .**:***.*********:**::  .*: ..:**:*

MaizeGR           FATIASDELGGLGGTCVLRGCVPKKLLVYASKYSHEFEESRGFGWTYETDPKHDWSTLIA 165
ArabidopsisGR2    FSTISSDTAGGVGGTCVLRGCVPKKLLVYASKYSHEFEDSHGFGWKYETEPSHDWTTLIA 179
ArabidopsisGR1    FHPISSEEIGGVGGTCVIRGCVPKKILVYGATYGGELEDAKNYGWEINEKVDFTWKKLLQ 117
                  * .*:*:  **:*****:******:***.:.*. *:*:::.:**  : . .. *..*:

MaizeGR           NKNTELQRLVGIYRNILNNAGVTLIEGRGKIVDPHTVSV---NG--KLYTAKHILVSVGG 220
ArabidopsisGR2    NKNAELQRLTGIYKNILSKANVKLIEGRGKVIDPHTVDV---DG--KIYTTRNILIAVGG 234
ArabidopsisGR1    KKTDEILRLNNIYKRLLANAAVKLYEGEGRVVGPNEVEVRQIDGTKISYTAKHILIATGS 177
                  :*. *: ** .**:.:* :* *.* **.*:::.*: *.*   :*    **:::**::.*.

MaizeGR           RPSMPDIPGIEHVIDSDAALDLPSKPEKIAIVGGGYIALEFAGIFNGLKSEVHVFIRQKK 280
ArabidopsisGR2    RPFIPDIPGKEFAIDSDAALDLPSKPKKIAIVGGGYIALEFAGIFNGLNCEVHVFIRQKK 294
ArabidopsisGR1    RAQKPNIPGHELAITSDEALSLEEFPKRAIVLGGGYIAVEFASIWRGMGATVDLFFRKEL 237
                  *.  *:*** * .* ** **.* . *::  ::******:***.*:.*: . *.:*:*::

MaizeGR           VLRGFDEEVRDFVAEQMSLRGITFHTEQSPQAITKSNDGLLSLKTNKENFGGFSHVMFAT 340
ArabidopsisGR2    VLRGFDEDVRDFVGEQMSLRGIEFHTEESPEAIIKAGDGSFSLKTSKGTVEGFSHVMFAT 354
ArabidopsisGR1    PLRGFDDEMRALVARNLEGRGVNLHPQTSLTQLTKTDQG-IKVISSHGEEFVADVVLFAT 296
                   *****:::* :*..::. **: :*.: *   : *:.:* :.: :.:      . *:***

MaizeGR           GRRPNSKNLGLEAVGVEMDKNGAIVVDEYSRTSVDSIWAVGDVTNRVNLTPVALMEGGAF 400
ArabidopsisGR2    GRKPNTKNLGLENVGVKMAKNGAIEVDEYSQTSVPSIWAVGDVIDRINLTPVALMEGGAL 414
ArabidopsisGR1    GRSPNTKRLNLEAVGVELDQAGAVKVDEYSRTNIPSIWAVGDATNRINLTPVALMEATCF 356
                  ** **:*.*.** ***:: : **: *****:*.: *******.*:*:*********. .:

MaizeGR           AKTVFGNEPTKPDYRAIPSAVFSQPPIGQVGLTEEQAIEEY-GDVDVFVANFRPLKATLS 459
ArabidopsisGR2    AKTLFQNEPTKPDYRAVPCAVFSQPPIGTVGLTEEQAIEQY-GDVDVYTSNFRPLKATLS 473
ArabidopsisGR1    ANTAFGGKPTKAEYSNVACAVFCIPPLAVVGLSEEEAVEQATGDILVFTSGFNPMKNTIS 416
                  *:* * .:***.:*  :..***. **:. ***:**:*:*:  **: *:.:.*.*:* *:*

MaizeGR           GLPDRVLMKILVCATSNKVVGVHMCGDDAPEIIQGIAIAVKAGLTKQDFDATIGIHPTSA 519
ArabidopsisGR2    GLPDRVFMKLIVCANTNKVLGVHMCGEDSPEIIQGFGVAVKAGLTKADFDATVGVHPTAA 533
ArabidopsisGR1    GRQEKTLMKLIVDEKSDKVIGASMCGPDAAEIMQGIAIALKCGATKAQFDSTVGIHPSSA 476
                  *  ::.:**::*  .::**:*. *** *:.**:**:.:*:*.* ** :**:*:*:**::*

MaizeGR           EEFVTMRSPTRKIRKSSTDQVESKDEVVSKQ- 550
ArabidopsisGR2    EEFVTMRAPTRKFRKDSSEGKASPEAKTAAGV 565
ArabidopsisGR1    EEFVTMRSVTRRIAHKPKPKTNL--------- 499
                  *******: **:: :...
```

Fig. 15 Alignment of GR protein sequences
Sequence of maize GR and Arabidopsis GR1 and GR2 were aligned using CLUSTALW software

Since the redox state of GSH pool changed towards a more oxidized state by an elevated level of ROS during drought, the levels of *GR* transcript was analyzed by qRT-PCR (section 2.6.3.2) in leaves and roots to ascertain its role in GSH turnover. The *GR* transcript was up-regulated 1.7 and 2.2-fold in leaves after 10 and 12 days of drought stress, respectively compared to the control (Fig. 16A). GR activity was also determined using protein extracted from the same plants. Interestingly, an increase of approximately 25-30% was also observed in GR activity in leaves of drought stressed plants in

comparison to the control (Fig. 16B). This up-regulation in *GR* transcript level and activity shows the importance of the glutathione-ascorbate pathway in ROS detoxification under drought stress.

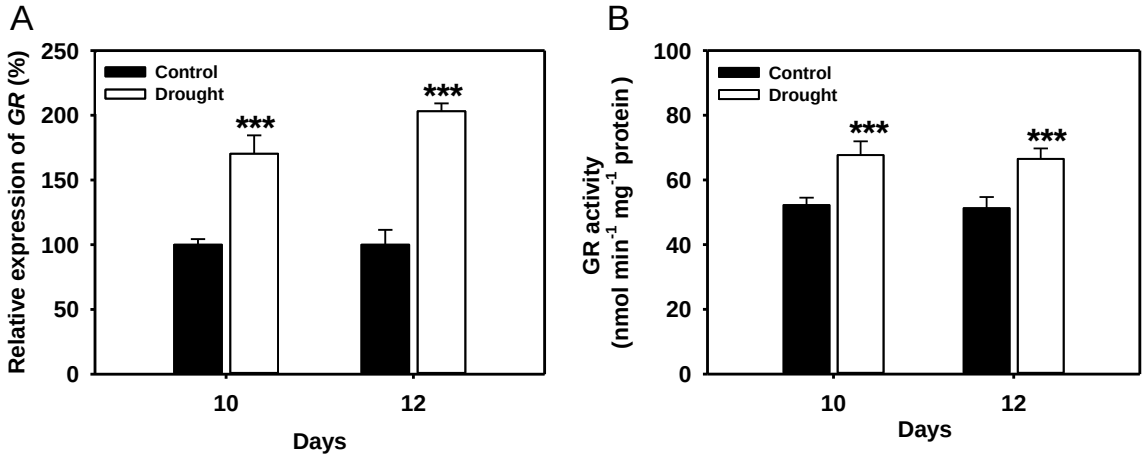


Fig. 16 Impact of drought on *GR* transcripts and activity in leaves of maize
Plants were grown in vermiculite media under long day condition for drought stress (A) After RNA isolation and cDNA synthesis, qRT-PCR was performed for quantification of *GR* transcripts level (n=3) (B) GR activity was measured of protein extracted from leaves of control and drought stressed plants (n=4)**.** Mean ± standard deviations are shown.

In roots, qRT-PCR analysis showed up-regulation of approximately 1.7- and 2-fold in *GR* transcript abundance after 10 and 12 days of drought respectively compared to the control (Fig. 17A). In contrast to leaves where 25-30% increase was observed, GR activity was not significantly changed in roots, indicating it is regulated differently at the post-transcriptional level in roots (Fig.17B).

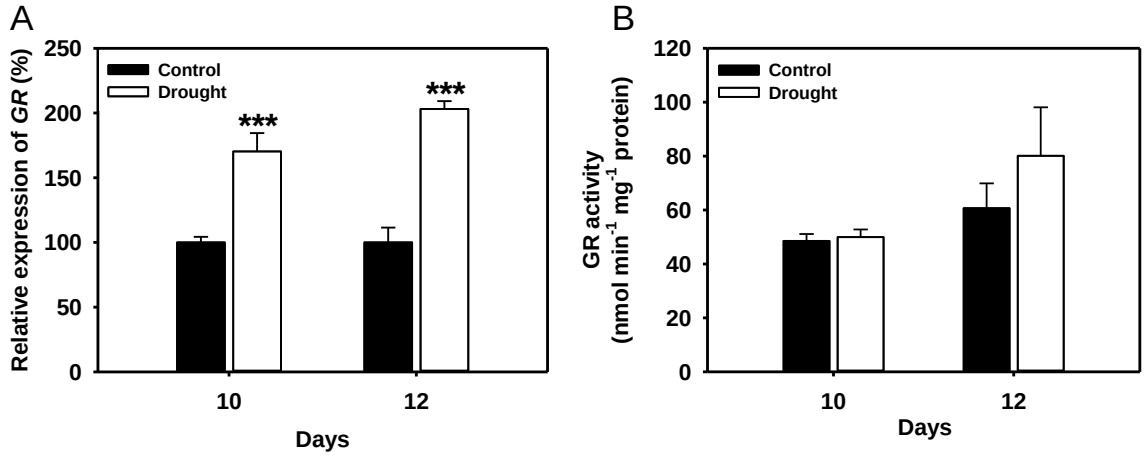


Fig. 17 Impact of drought on *GR* transcript and activity in roots of maize
Plants were grown in vermiculite media under long day condition for drought stress (A) After RNA isolation and cDNA synthesis, qRT-PCR was performed for quantification of *GR* transcript level (n=3) (B) GR activity was measured of protein extracted from roots of control and drought stressed plants (n=4)**.** Mean ± standard deviations are shown.

3.4.3 Analysis of total GSH, γ-EC in leaves and roots

The increase in GSH pool towards more oxidized state in leaves of drought stressed maize seedlings prompted us to test GSH contents in leaves and roots of control and drought stressed plants. The steady state level of GSH in leaves was decrease by approximately 50% after 10 and 12 days of drought stress (Fig. 18A). To further evaluate whether the observed decrease in GSH was due to the down-regulation of its precursor γ-EC, the steady state level of γ-EC was quantified both in control and drought stressed leaves.

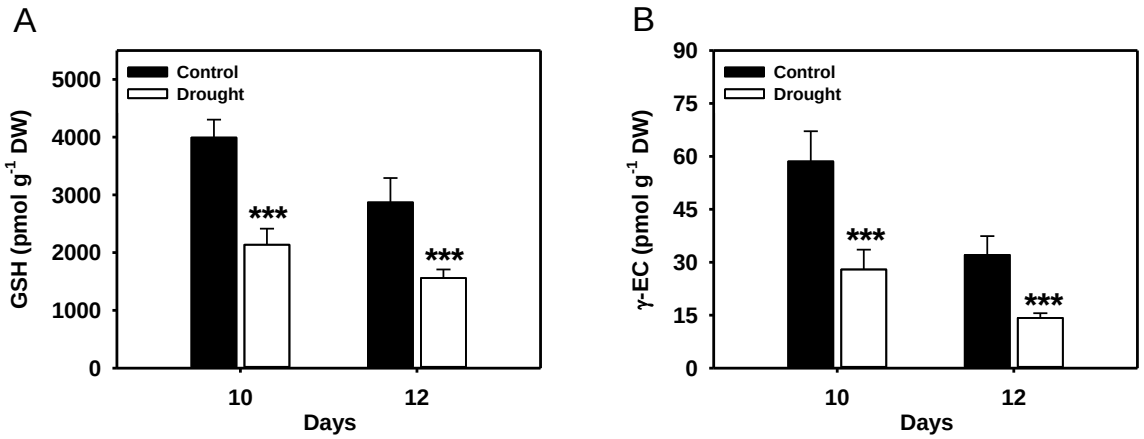


Fig. 18 GSH and γ-EC contents in leaves of maize under drought
Metabolites were extracted with 0.1 M HCl from leaves of control and drought stressed plants grown under long day condition in vermiculite media. Thiols were derivatized with monobromobimane and quantified using HPLC (n=5). Mean ± standard deviations are shown.

As expected, in leaves the steady-state level of γ-EC was decreased by 50% at each time point under drought in comparison to the control, suggesting that a decrease in GSH contents was indeed due to a decrease in precursor γ-EC (Fig. 18B).

In contrast to leaves, roots showed elevated level of GSH of approximately 1.8 and 2.3-fold after 10 and 12 days respectively in drought-treated roots in comparison to the control (Fig. 19A). γ-EC contents in roots also showed an increase by 2.7 and 4-fold after 10 and 12 days in drought stressed plants, respectively (Fig. 19B).

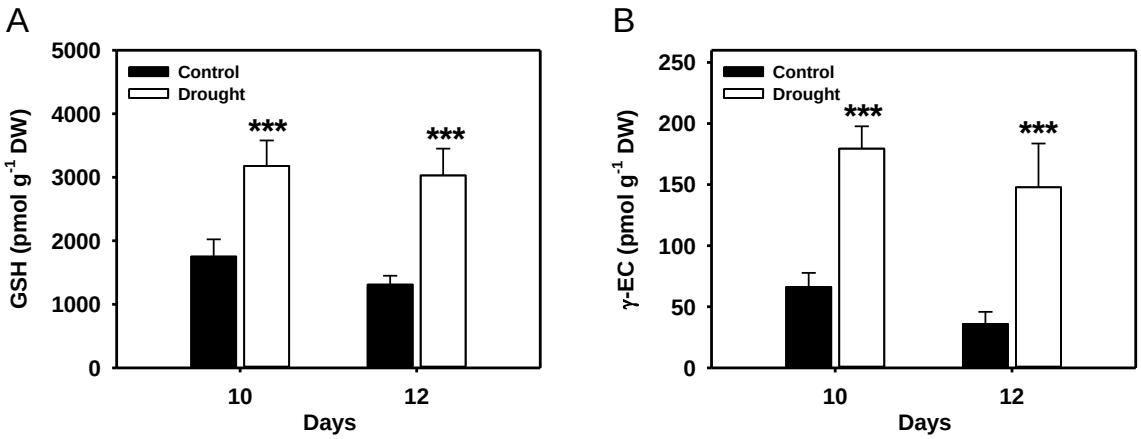


Fig. 19 GSH and γ-EC contents in roots of maize under drought
Metabolites were extracted with 0.1 M HCl from roots of control and drought stressed plants grown under long day condition in vermiculite media. Thiols were derivatized with monobromobimane and quantified using HPLC (n=5). Mean ± standard deviations are shown.

3.4.4 Quantification of *GSH1* transcript in leaves and roots

Since the steady state level of GSH and γ-EC was decreased in drought stressed leaves and *GSH1* is rate limiting in GSH biosynthesis (Jez *et al.*, 2004), it was important to check the transcript abundance of *GSH1*. The transcript level of *GSH1* as determined by qRT-PCR was up-regulated by approximately 2-fold in leaves at each time point under drought compared to the control (Fig. 20) This further indicates that *GSH1* is also rate limiting for GSH biosynthesis in leaves of maize plants.

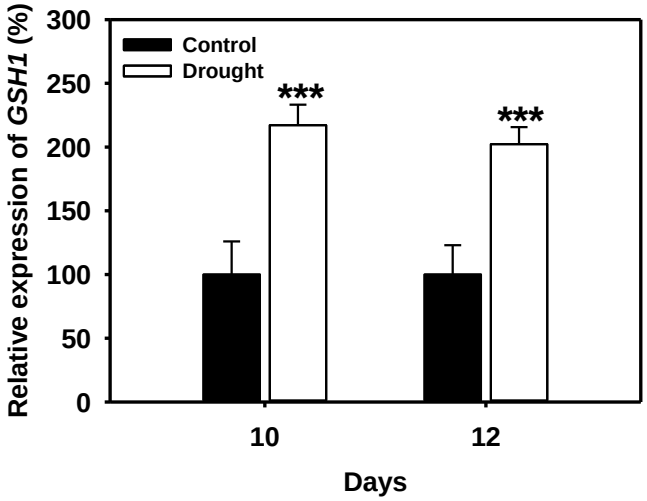


Fig. 20 Impact of drought on the expression level of *GSH1* in leaves of maize
RNA was extracted from leaves of control and drought stressed plants and cDNA synthesized and quantification was performed by qRT PCR (n=3). Mean ± standard deviations are shown.

In roots, the transcript abundance of *GSH1* was also significantly up-regulated by approximately 1.5 and 1.62-fold after 10 and 12 days of drought, respectively relative to control (Fig. 21).

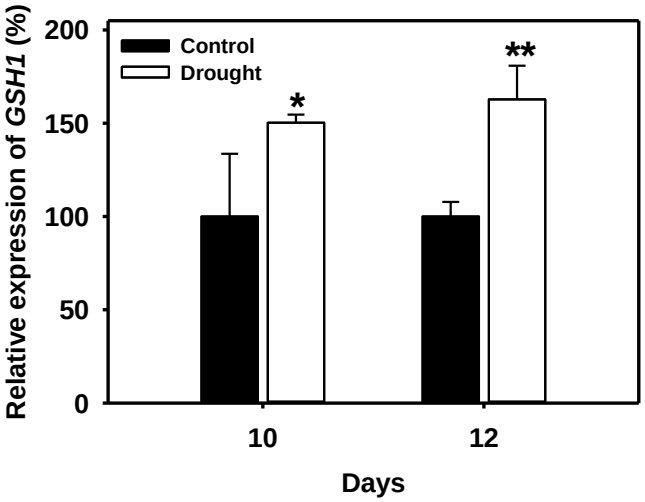


Fig. 21 Impact of drought on the expression level of *GSH1* in roots of maize
RNA was extracted from roots of control and drought stressed plants and cDNA synthesized and quantification was performed by qRT PCR (n=3). Mean ± standard deviations are shown.

3.5 Impact of drought on the capacity for cysteine synthesis

3.5.1 Analysis of cysteine contents in leaves and roots

One of the important precursors for glutathione formation is cysteine and a lower steady state level of GSH and γ-EC should result from a decrease in cysteine biosynthesis. To address this, cysteine contents were analyzed in leaves of control and drought stressed plants.

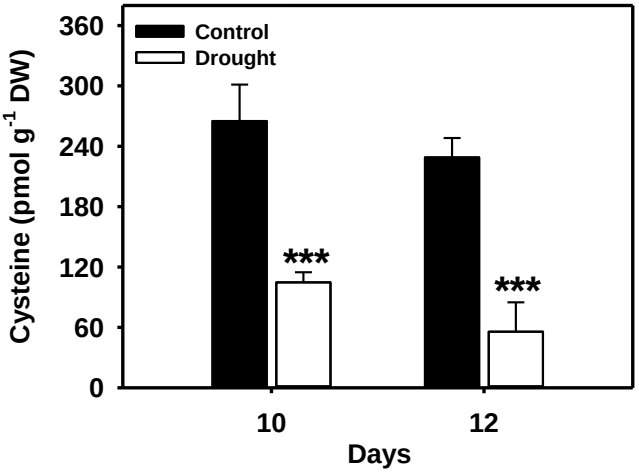


Fig. 22 Impact of drought on cysteine contents in leaves of maize
Cysteine contents were quantified by HPLC in leaves of control and drought stressed plants after 10 and 12 days grown on vermiculite under long day condition (n=5). Mean ± standard deviations are shown.

Analysis of leaf cysteine steady state level revealed a significant reduction of 60 and 75 % after 10 and 12 days of drought stress respectively relative to control (Fig. 22).

In contrast to leaves, roots showed elevated steady state level of cysteine where a significant increase by 1.75 and 1.82-fold was observed after 10 and 12 days of drought stress, respectively relative to control (Fig.23).

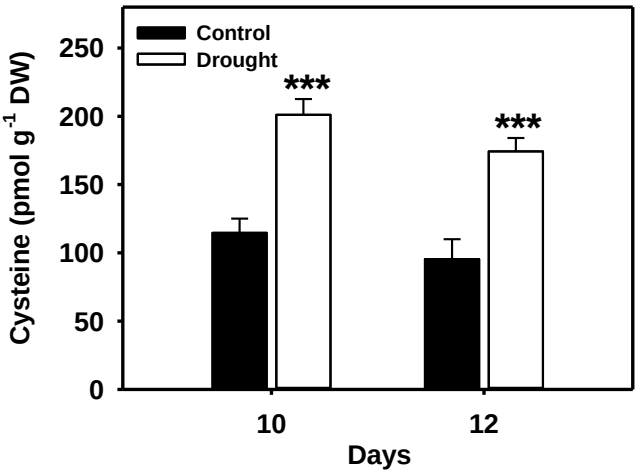


Fig. 23 Impact of drought on cysteine contents in roots of maize
Cysteine contents were quantified by HPLC in roots of control and drought stressed plants after 10 and 12 days grown on vermiculite under long day condition (n=5). Mean ± standard deviations are shown.

3.5.2 Enzymatic activity of SAT and OASTL in leaves and roots

Cysteine synthesis is catalyzed by two important enzymes SAT and OASTL that form cysteine synthase complex. SAT synthesizes OAS from serine and acetyl coenzyme A and is considered rate limiting for cysteine biosynthesis, whereas OASTL substitutes the acetyl group of OAS with sulfide to produce cysteine. Since the steady state level of cysteine decreased, we determined if lowered cysteine amount is due to drought-induced change in enzymatic activities of SAT and OASTL. Therefore *in vitro* activities of SAT and OASTL was measured in leaves of control and drought stressed plant after 10 and 12 days of drought stress. Total SAT activity was significantly decreased by 50% in drought stressed plant compared to the control (Fig. 24A). On the other hand, OASTL activity was not significantly altered in leaves of drought stressed plants (Fig. 24B).

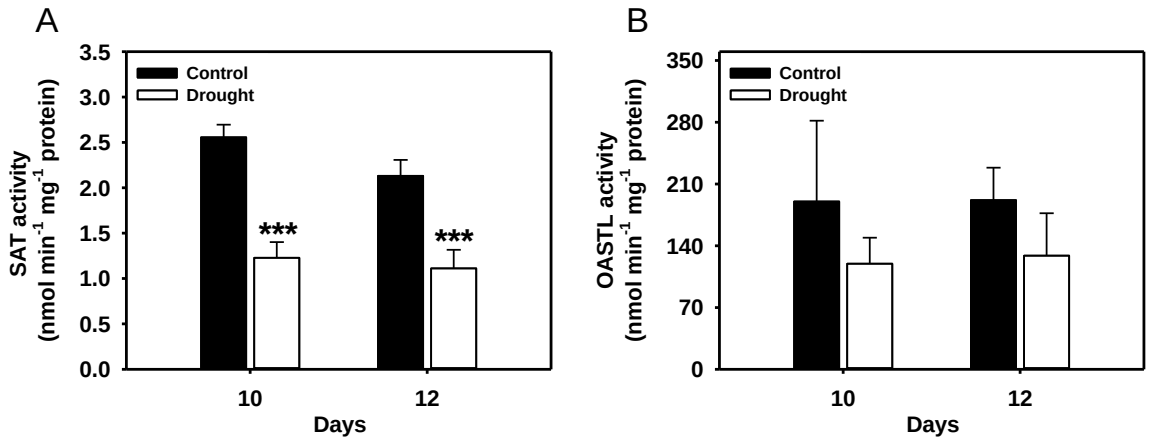


Fig. 24 Enzymatic activities of SAT and OASTL under drought in leaves of maize
Specific activity of SAT (A) and OASTL (B) was measured in the soluble protein extracts from leaves of control and drought stressed plants grown on vermiculite media (n=5). Mean ± standard deviations are shown.

In roots, the total SAT activity did not show any noticeable difference between control and drought stressed plant (Fig. 25A) whereas OASTL activity was significantly decreased by 41 and 53% after 10 and 12 days, respectively in roots of drought stressed plants compared to control (Fig. 25B).

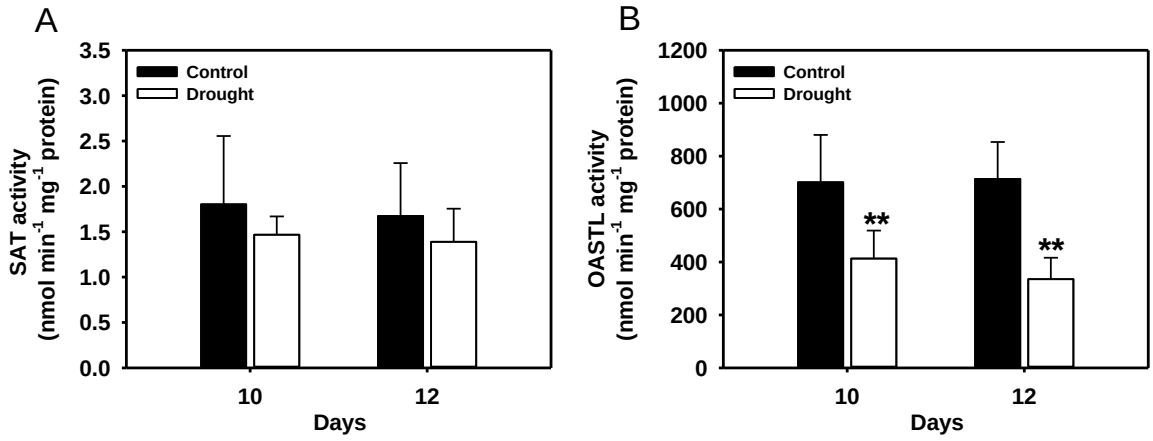


Fig. 25 Enzymatic activities of SAT and OASTL under drought in roots of maize
Specific activity of SAT (A) and OASTL (B) was measured in the soluble protein extracts from roots of control and drought stressed plants grown on vermiculite media (n=5). Mean ± standard deviations are shown.

3.5.3 Analysis of OAS and serine in leaves and roots

SAT catalyzes the synthesis of OAS from serine and acetyl coenzyme A. The lowered SAT activity should lead to a decrease in the steady state level of OAS and an accumulation of serine. To confirm this, OAS and serine contents were analysed in leaves of both control and drought stressed plants. As expected, OAS contents were significantly decreased by 25 and 30% after 10 and 12 days in drought stressed plants, respectively

compared to control (Fig. 26A). Serine accumulated in drought-stressed plants at a higher level by approximately 2.4 to 3-fold in leaves after 10 and 12 days relative to the control (Fig. 26B).

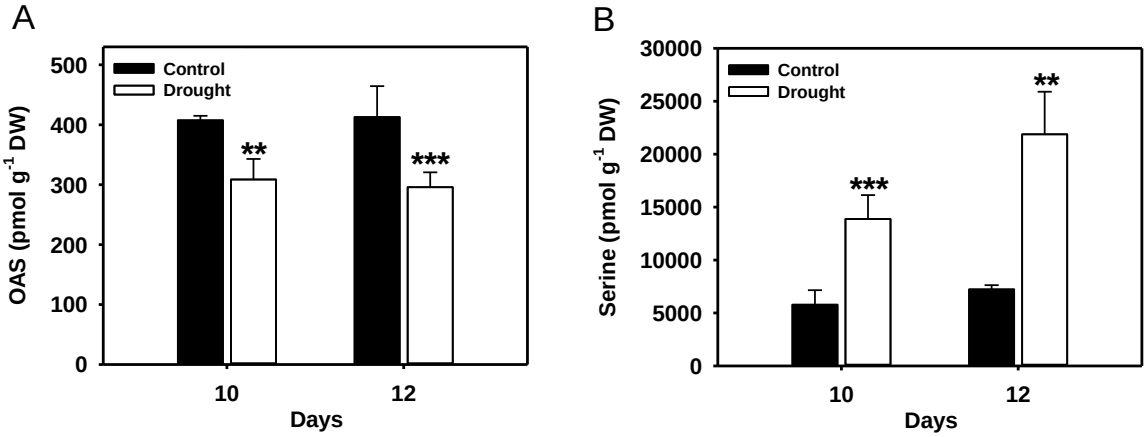


Fig. 26 Impact of drought on OAS and serine contents in leaves of maize
Metabolites were extracted with 0.1 M HCl from leaves of control and drought stressed plants grown in vermiculite under long day condition. OAS (A) and serine (B) were derivatized with AccQTag and quantified by HPLC analysis (n=5). Mean ± standard deviations are shown.

In roots, the steady state level of OAS revealed an average decrease of approximately 24 and 21% after 10 and 12 days in drought stressed plants, respectively in comparison to the control (Fig. 27A).

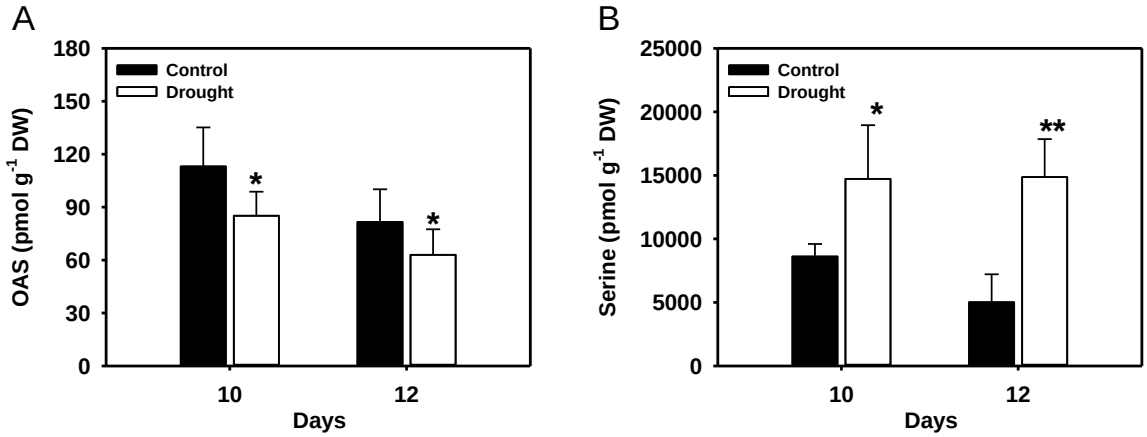


Fig. 27 Impact of drought on OAS and serine contents in roots of maize
Metabolites were extracted with 0.1 M HCl from roots of control and drought stressed plants grown in vermiculite under long day condition. OAS (A) and serine (B) were derivatized with AccQTag and quantified by HPLC analysis (n=5). Mean ± standard deviations are shown.

Interestingly in roots serine contents also showed an accumulation under drought as observed in leaves. An average increase of approximately 1.7 and 2.9-fold was observed in serine contents in roots after 10 and 12 days, respectively in drought stressed plants relative to control (Fig. 27B).

3.5.4 Analysis of sulfide contents in leaves and roots

Next, sulfide content was measured to examine whether the lower steady state level of cysteine and consequently increase in GSSG/GSH ratio might also be caused by altered flux in the sulfur assimilation pathway. After 10 and 12 days of water stress, sulfide steady state level in leaves were significantly decreased by 70 and 80%, respectively, relative to the control (Fig. 28). Therefore, it is very likely that sulfur assimilation pathway, which produces sulfide as the final product, could be affected by drought.

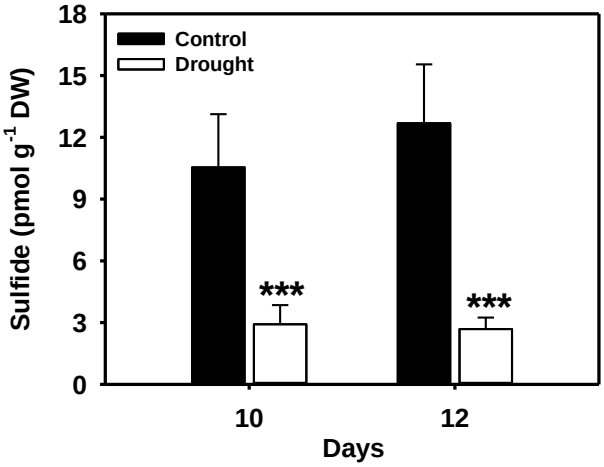


Fig. 28 Impact of drought on sulfide contents in leaves of maize
Sulfide contents were quantified by HPLC in leaves of control and drought stressed plants after 10 and 12 days grown on vermiculite under long day condition (n=7). Mean ± standard deviations are shown.

In contrast to leaves, sulfide contents were significantly increased by 2.57 and 2.63-fold, in roots after 10 and 12 days of drought stressed plants, respectively compared to control (Fig. 29).

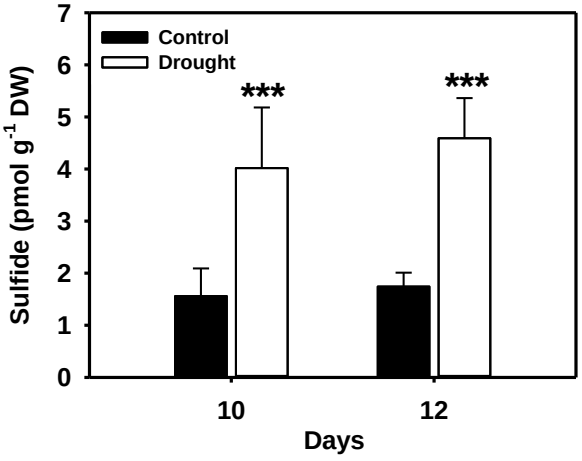


Fig. 29 Impact of drought on sulfide contents in roots of maize
Sulfide levels were quantified by HPLC in roots of control and drought stressed plants after 10 and 12 days grown on vermiculite under long day condition (n=7). Mean ± standard deviations are shown.

3.5.5 Analysis of amino acids in leaves and roots

Amino acids were also analyzed in leaves and roots of control and drought stressed plants in order to assess the response of different amino acids, which are required for protein synthesis, to drought stress. These amino acids were grouped into acidic and their amides, basic, aliphatic and aromatic based on their properties.

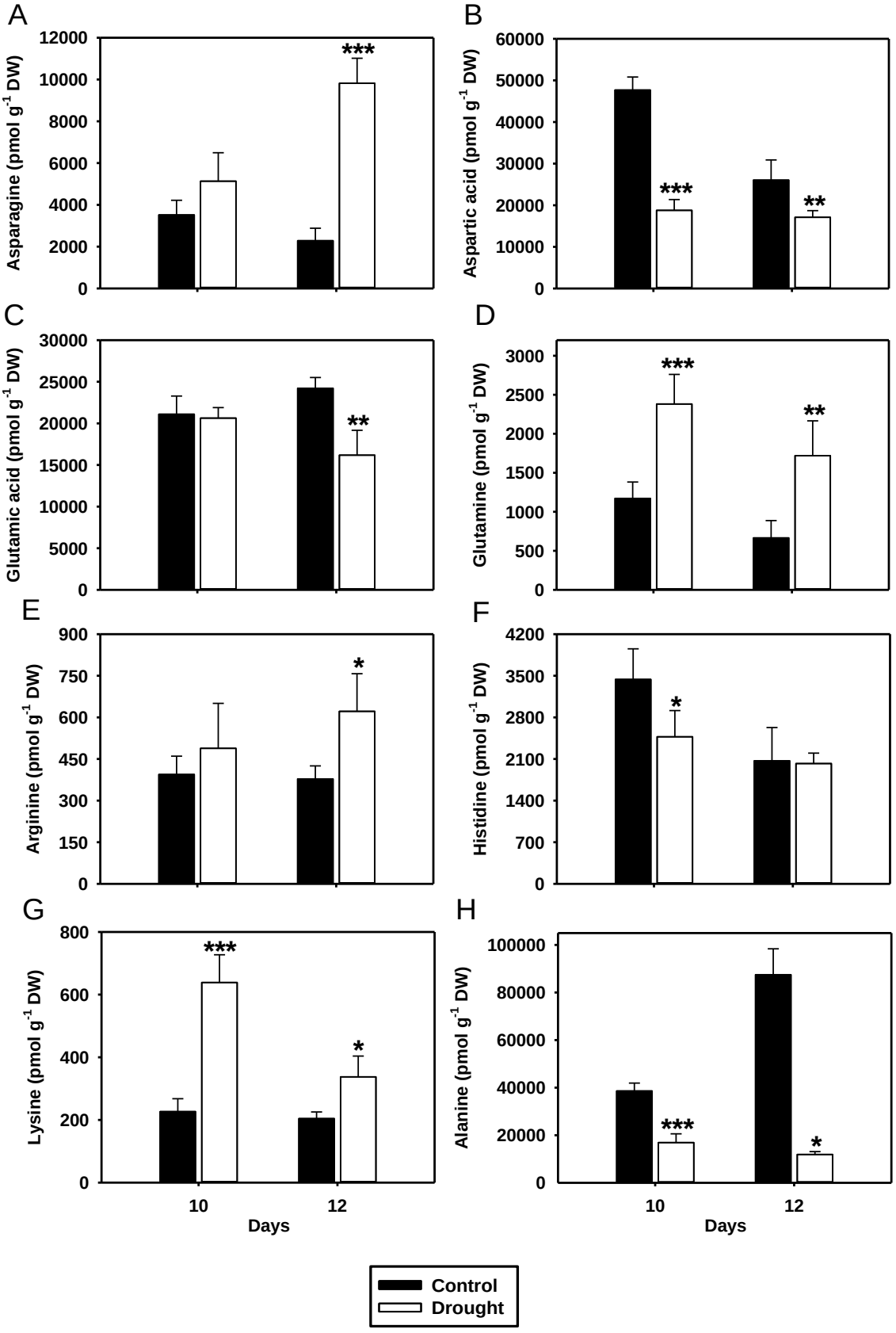
Acidic and their amides include amino acids asparagine, aspartic acid, glutamic acid and glutamine. Quantitative analysis of amino acids in leaves revealed that asparagine showed an increase only after 12 days whereas glutamine was significantly accumulated after 10 and 12 days of drought stress compared to control (Fig. 30A & D). Among others, glutamic acid was decreased only after 12 days of drought while aspartic acids showed reduction at both time points under drought relative to the control (Fig. 30B & C).

Arginine, histidine and lysine are basic amino acids. Arginine showed an accumulation following 12 days of drought while lysine showed significant increase at both time points of drought (Fig. 30E & G). On the other hand, a significant decrease was observed after 10 days of drought in histidine contents (Fig. 30F).

Alanine, glycine, isoleucine, leucine, and valine are aromatic amino acids that showed different response to drought. Alanine was the only aromatic amino acid that was significantly decreased after 10 and 12 days and glycine only after 10 days of drought relative to control (Fig. 30H & I). The accumulation of isoleucine and leucine was recorded after 10 and 12 days whereas valine was increased after 12 days of drought stress relative to control (Fig. 30J, K & L).

Aromatic amino acids contain phenylalanine and tyrosine, were significantly increased under drought in comparison to control after 10 and 12 days (Fig. 30M & N).

Methionine was the only amino acids that was not changed under drought at each time points (Fig. 30 O).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Asparagine (pmol g-1 DW)
12000
10000
8000
6000
4000
2000
0
***
B
Aspartic acid (pmol g-1 DW)
60000
50000
40000
30000
20000
10000
0
***
**
C
Glutamic acid (pmol g-1 DW)
30000
25000
20000
15000
10000
5000
0
**
D
Glutamine (pmol g-1 DW)
3000
2500
2000
1500
1000
500
0
***
**
E
Arginine (pmol g-1 DW)
900
750
600
450
300
150
0
*
F
Histidine (pmol g-1 DW)
4200
3500
2800
2100
1400
700
0
*
G
Lysine (pmol g-1 DW)
800
600
400
200
0
***
*
H
Alanine (pmol g-1 DW)
100000
80000
60000
40000
20000
0
***
*
10
12
Days
Control
Drought

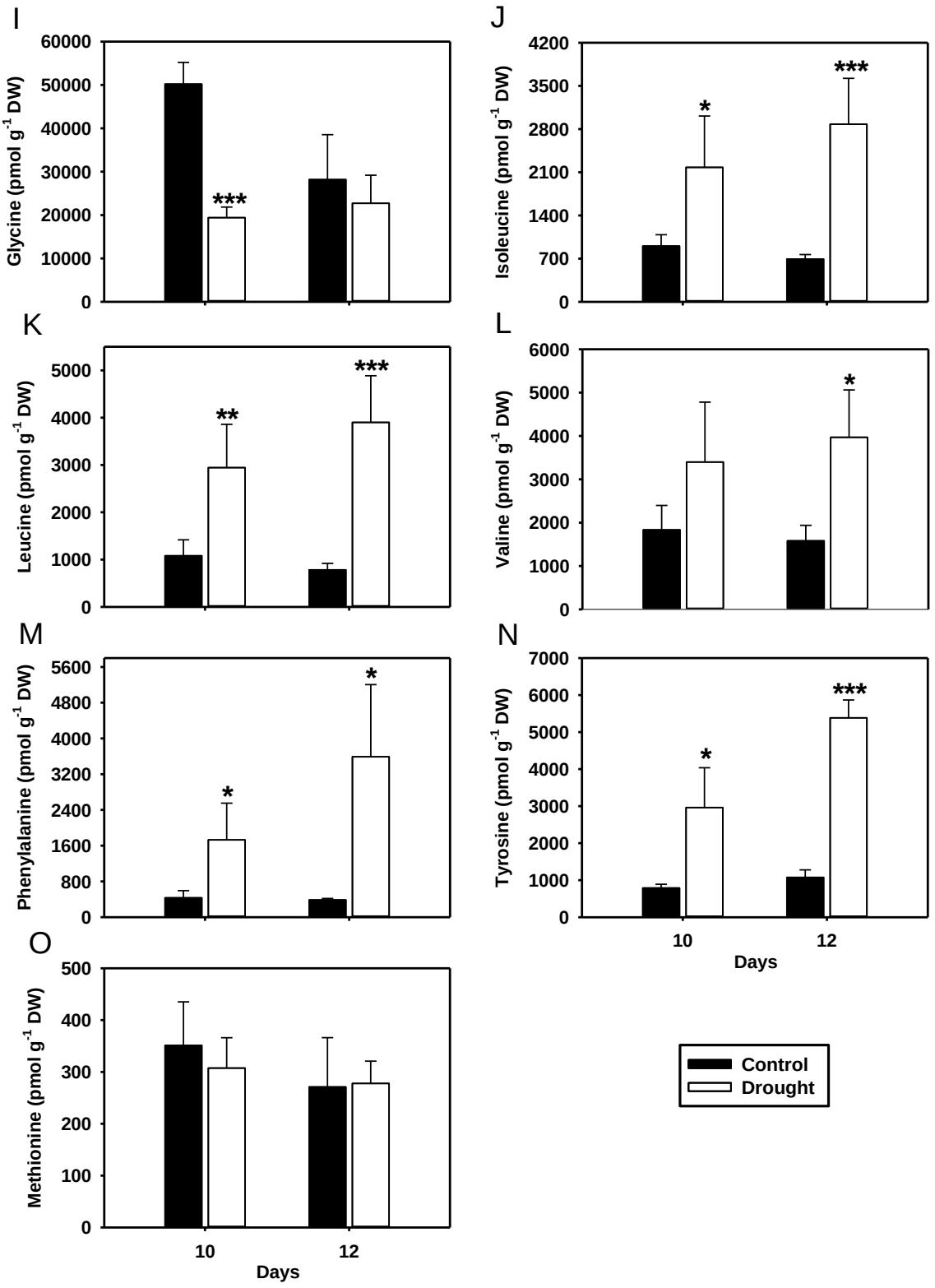


Fig. 30 Impact of drought on amino acids contents in leaves of maize
Metabolites were extracted with 0.1 M HCl from leaves of control and drought stressed plants grown in vermiculite under long day condition. Amino acids were derivatized with AccQTag and quantified by HPLC analysis. (n=4). Mean ± standard deviations are shown.

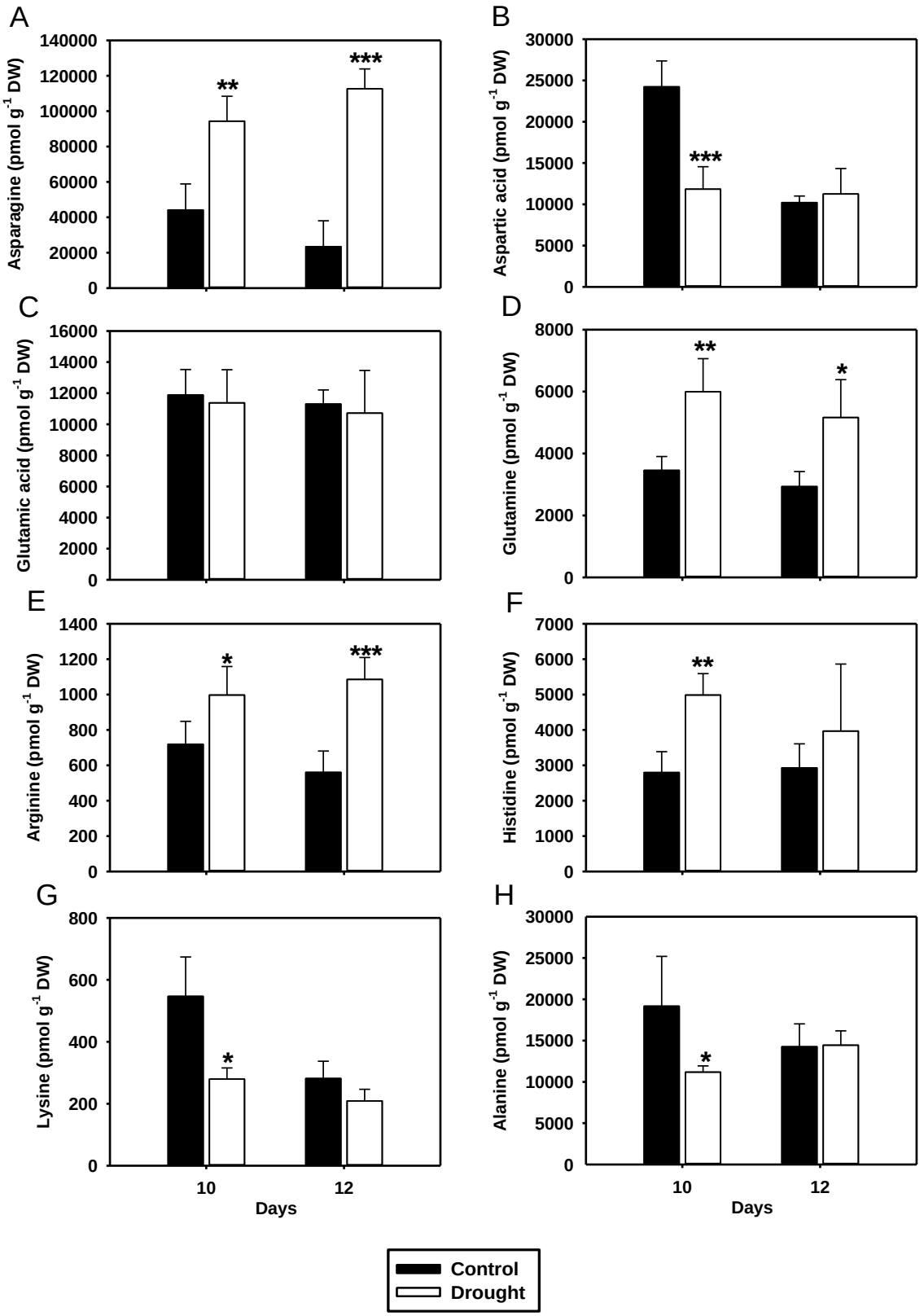
To test the response of different amino acids to drought stress in roots, the same analysis was performed as for leaves.

As observed for leaves, analysis of acidic amino acids in roots showed that asparagine and glutamine were also significantly increased following 10 and 12 days of drought stress compared to control (Fig. 31A & D). In contrast, glutamic acid was not affected by drought whereas aspartic acid was significantly decreased only after 10 days of drought (Fig. 31B & C).

Analysis of basic amino acids revealed that arginine showed an accumulation upon drought at each time point whereas histidine was significantly increased following 10 days of drought stress (31E & F). Moreover, lysine showed a reduction after 10 days of water stress (Fig. 31G).

Among aliphatic amino acids, alanine showed a decrease whereas glycine was not changed during drought (Fig. 31H & I). However, isoleucine and valine were significantly increased following 10 and 12 days of drought compared to control (Fig. 31J & L). Leucine was also accumulated but this was observed only after 12 days (Fig. 31K).

In contrast to leaves, phenylalanine did not show significant difference between control and drought stressed roots while tyrosine showed an accumulation following 10 days of drought in comparison to control (Fig. 31M & N).


A
Asparagine (pmol g-1 DW)
140000
120000
100000
80000
60000
40000
20000
0
**
***
B
Aspartic acid (pmol g-1 DW)
30000
25000
20000
15000
10000
5000
0
***
C
Glutamic acid (pmol g-1 DW)
16000
14000
12000
10000
8000
6000
4000
2000
0
D
Glutamine (pmol g-1 DW)
8000
6000
4000
2000
0
**
*
E
Arginine (pmol g-1 DW)
1400
1200
1000
800
600
400
200
0
*
***
F
Histidine (pmol g-1 DW)
7000
6000
5000
4000
3000
2000
1000
0
**
G
Lysine (pmol g-1 DW)
800
600
400
200
0
*
H
Alanine (pmol g-1 DW)
30000
25000
20000
15000
10000
5000
0
*
10
12
Days
Control
Drought

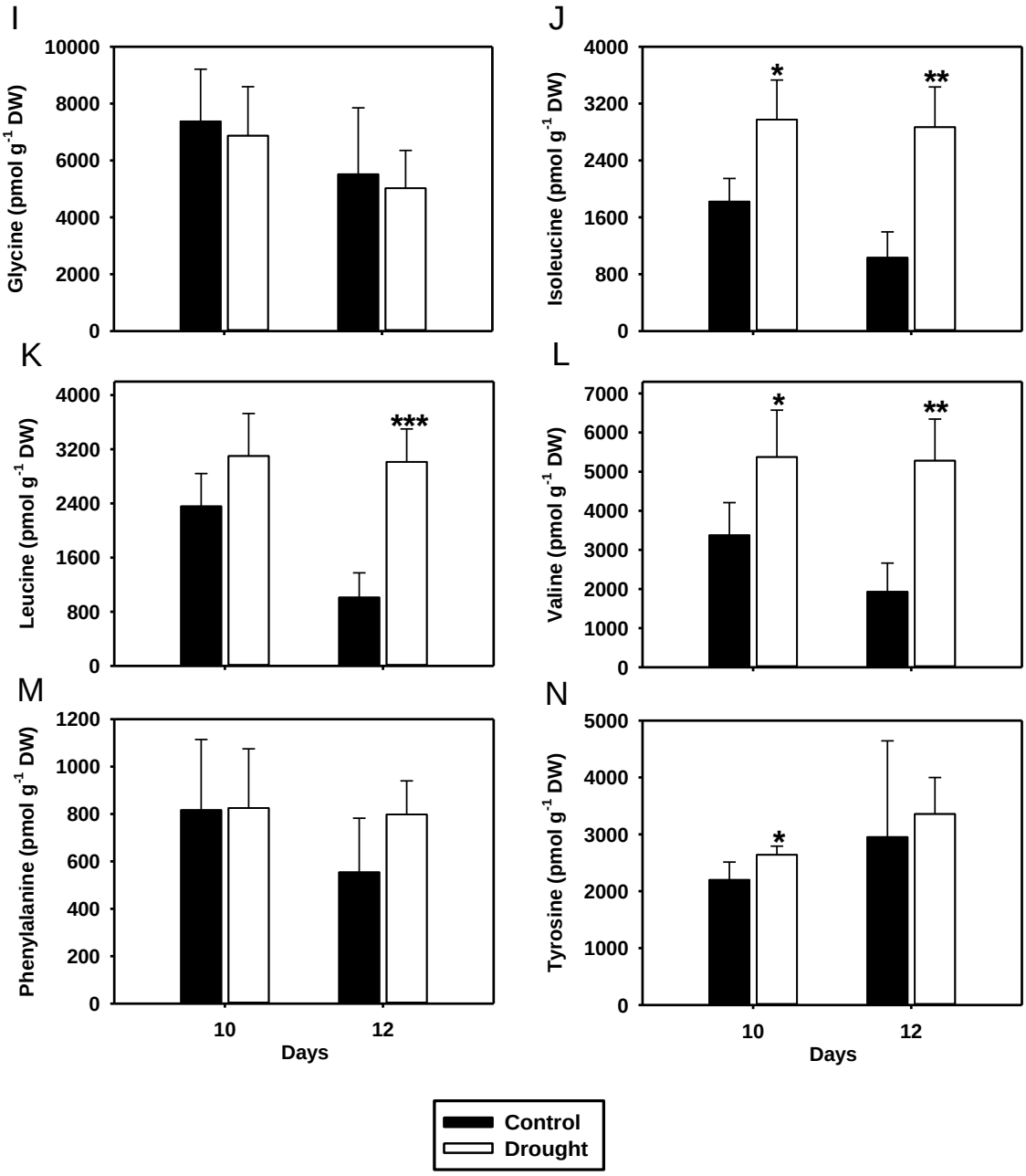


Fig. 31 Impact of drought on amino acids contents in roots of maize
Metabolites were extracted with 0.1 M HCl from roots of control and drought stressed plants grown in vermiculite under long day condition. Amino acids were derivatized with AccQTag and quantified by HPLC analysis (A-N). (n=4). Mean ± standard deviations are shown.

3.6 Impact of drought on alteration of adenosine related metabolites

3.6.1 Analysis of drought-induced changes in adenosine in leaves and roots

To investigate further effect of drought on adenosine related metabolites, quantification of sulfation, methylation and energy related metabolites was performed by HPLC (section 2.4.4) in leaves and roots of both control and drought stressed plants grown on vermiculite media. Analysis of adenosine-5′-phosphosulfate (APS) contents in leaves showed an increase of approximately 1.6- and 2.6-fold after 10 and 12 days, respectively in leaves of drought stressed plants compared to the control (Fig 32A). 3′-phosphoadenosine 5′-phosphate (PAP) and S-adenosylmethionine (SAM) contents were not affected by drought (Fig. 32B & H) whereas ADP and ATP steady state levels were significantly decreased in leaves of drought stressed plants. An average decrease of approximately 60 and 70% was observed for ADP whereas for ATP this decrease was 65 and 74% in leaves after 10 and 12 days in drought stressed plants, respectively compared to the control (Fig. 32D & E). A significant reduction in S- adenosylhomocysteine (SHC) and 3′-phosphoadenosine 5′-phosphosulfate (PAPS) amount was seen after 10 and 12 days, respectively. Methylthioadenosine (MTA) significantly accumulated only after 10 days of drought (Fig. 32C & F).

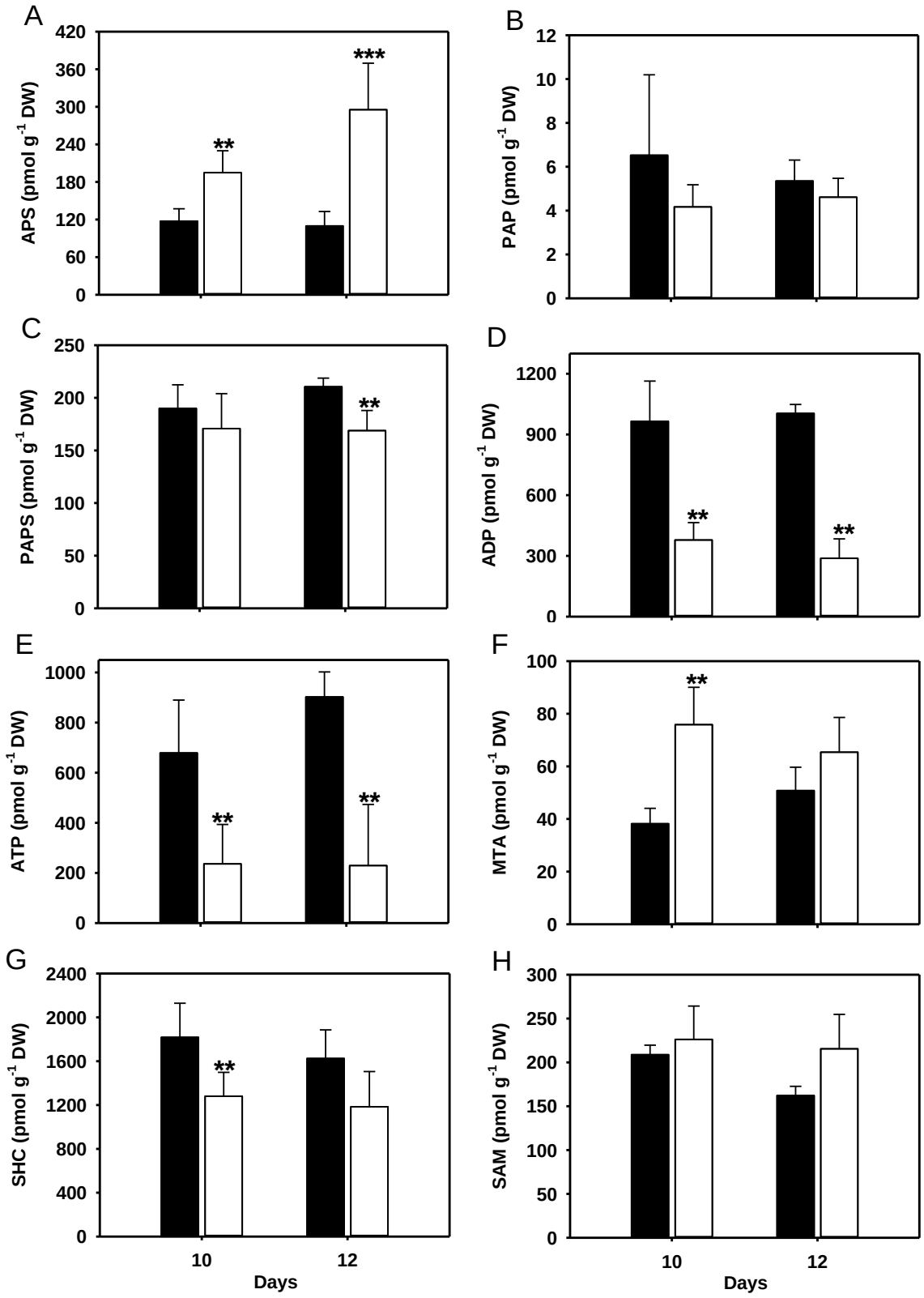


Fig. 32 Impact of drought on adenosine contents in leaves of maize
Leaves of control and drought stressed plants were used for extraction and quantification of adenosines (A) APS, adenosine-5′-phosphosulfate (n=5) (B) PAP, phosphoadenosine phosphate. (C) PAPS, phosphoadenosine phosphosulfate. (D) ADP, adenosine diphosphate. (E) ATP, adenosine triphosphate. (F) MTA, methylthioadenosine. (G) SHC, S-adenosylhomocysteine. (H) SAM,S-adenosyl methionine; (n=4). Mean ± standard deviations are shown.

As observed in leaves, the steady state level of APS was also increased by approximately 1.5 and 2.1-fold in roots after 10 and 12 days of drought, respectively relative to the control (Fig 33A). The steady state level of ADP was decreased by approximately 70 and 66% whereas even more reduction of 80 and 86% was recorded in ATP contents after 10 and 12 days in drought stressed plants, respectively relative to control (Fig. 33D & E). Among other adenosines, SHC and SAM contents did not show significant differences between control and drought (Fig. 33G & H) whereas PAPS was significantly increased in roots of drought stressed plants (Fig. 33C). Moreover, PAP analysis revealed a significant reduction only after 10 days and MTA following 12 days of drought stress in comparison to control (Fig. 33B & F).

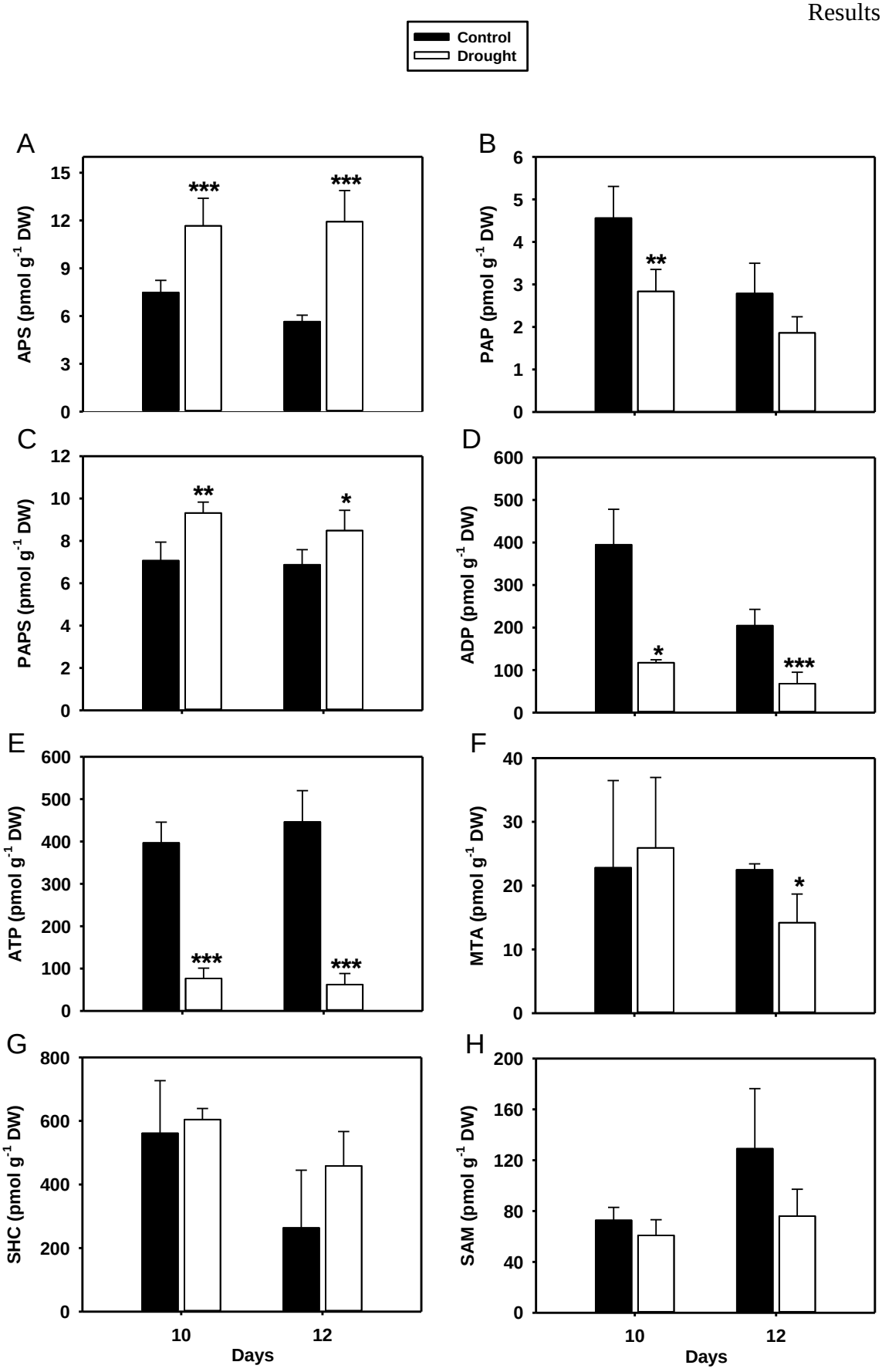


Fig. 33 Impact of drought on adenosine contents in roots of maize.
Roots of control and drought stressed plants were used for extraction and quantification of adenosines (A) APS, adenosine-5′-phosphosulfate. (B) PAP, phosphoadenosine phosphate. (C) PAPS, phosphoadenosine phosphosulfate. (D) ADP, adenosine diphosphate. (E) ATP, adenosine triphosphate. (F) MTA, methylthioadenosine. (G) SHC, S-adenosylhomocysteine. (H) SAM,S-adenosyl methionine; (n=4). Mean ± standard deviations are shown.

3.6.2 Quantification of *APR* transcript in leaves and roots

To further investigate the role of APR during drought in maize, the transcript level of *APR* in leaves was determined. Surprisingly, the expression data revealed no change in *APR* transcript abundance between control and drought stressed leaves (Fig 34).

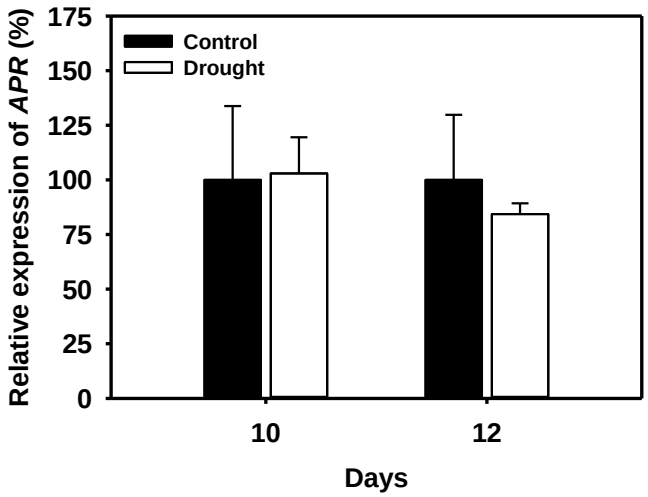


Fig. 34 Expression level of *APR* under drought in leaves of maize
RNA was extracted from leaves of control and drought stressed plants and cDNA synthesized and quantification was performed by qRT PCR (n=3). Mean ± standard deviations are shown.

In contrast to leaves where no change in *APR* expression level was observed, roots showed up-regulation in *APR* transcript abundance by 1.57 and 1.7-fold after 10 and 12 days of drought, respectively compared to control (Fig. 35).

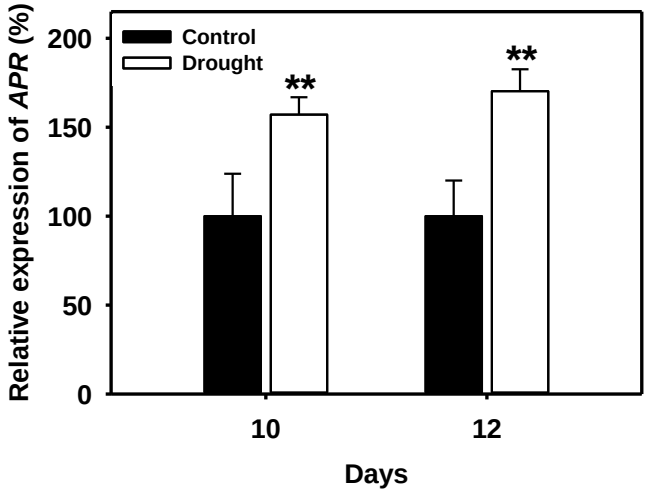


Fig. 35 Expression level of *APR* under drought in roots of maize
RNA was extracted from roots of control and drought stressed plants and cDNA synthesized and quantification was performed by qRT PCR (n=3). Mean ± standard deviations are shown.

3.6.3 Quantification of *ATPS* transcript in leaves and roots

The only entry step for assimilation and metabolism of activated sulfate is catalyzed by ATP sulfurylase. In maize, only one ATPS isoform was found so far in the NCBI database, which is in contrast to Arabidopsis that has 4 ATPS isoforms. To search for other potential isoforms in maize, the protein sequence of Arabidopsis ATPS isoforms were blasted against the maize database. Three isoforms were found that showed 70-80% protein sequence identity with Arabidopsis ATPS isoforms and showed more than 80% identity among each other (Fig. 36 & 37).

```
GRMZM2G149952      MAMQAAFLAGFSQLAAQPSRDCAVAVA------PTRVAVAAVGSAKVGARVGLKAGIAAP 54
GRMZM2G051270      MATQAAFLAGFSQLAAQPGRDRAVAVAVAPAPGPARVAVAAVGSAKLG----VKAGTSRT 56
GRMZM2G158147      MATTHLLTP---PRVHHPSPSASSSVAR------VRATASLAHPLHLCRLRLAAPRSRSP 51
                   **    : .     . :*. . : :**       .*.:.: . . ::       .    .

GRMZM2G149952      APAAG-RGVRCRASLIEPDGGQLVDLVAPEEGGRRAALRREAAELPHRLRLGRVDKEWVH 113
GRMZM2G051270      AAVAR-LGVRCRASLIEPDGGRLVDLVAPEEGGRRAALRREAAELPHRLRLGRVDKEWVH 115
GRMZM2G158147      SPRHGRRAMSVRSSLIDPDGGALVELVAPPD--RLPALRAEAEALP-RVRLAPVDLQWAH 108
                   :.     .:  *:***:**** **:**** :  * .*** **  ** *:**. ** :*.*

GRMZM2G149952      VLSEGWASPLQGFMREHEFLQTLHFNAIRGQDGRMVNMSVPIVLSVGDAQRRAIQADGAT 173
GRMZM2G051270      VLSEGWASPLQGFMREHEFLQALHFNAIRGQDGRMVNMSVPIVLSVGDAQRRAIQADGAT 175
GRMZM2G158147      VLAEGWASPLRGFMREHEYLQSLHFNCVRLPDGGLVNMSLPIVLAIGDADK--EQIGGKP 166
                   **:*******:*******:**:****.:*  ** :****:****::***::   * .* .

GRMZM2G149952      RVALVDERDRPIAVLSDIEIYKHNKEERIARTWGTTAPGLPYVEEAITNAGDWLIGGDLE 233
GRMZM2G051270      RVALVDERDRPIAVLSDIEIYKHNKEERVARTWGTTAPGLPYVEEAITNAGDWLVGGDLE 235
GRMZM2G158147      DVALQGPDGGVVAILRRVEIYPHNKEERIARTWGTTAPGLPYVDEAIASAGNWLIGGDLE 226
                    *** .  .  :*:*  :*** ******:***************:***:.**:**:*****

GRMZM2G149952      IIEPIKYNDGLDQYRLSPAQLREEFARRNADAVFAFQLRNPVHNGHALLMTDTRKRLLEM 293
GRMZM2G051270      VIEPIKYNDGLDQYRLSPAQLREEFARRNADAVFAFQLRNPVHNGHALLMTDTRKRLLEM 295
GRMZM2G158147      VLEPIKYNDGLDHYRLSPRQLRKEFDKRGADAVFAFQLRNPVHNGHALLMNDTRRRLLEM 286
                   ::**********:***** ***:** :*.********************.***:*****

GRMZM2G149952      GYKNPVLLLHPLGGFTKADDVPLSWRMKQHEKVLEEGVLNPESTVVAIFPSPMHYAGPTE 353
GRMZM2G051270      GYKNPVLLLHPLGGFTKADDVPLSWRMKQHEKVLEEGVLNPESTVVAIFPSPMHYAGPTE 355
GRMZM2G158147      GYKNPILLLHPLGGYTKADDVPLPVRMEQHSKVLEDGVLDPETTIVSIFPSPMHYAGPTE 346
                   *****:********:********. **:**.****:***:**:*:*:************

GRMZM2G149952      VQWHAKARINAGANFYIVGRDPAGMSHPTEKRDLYDADHGKKVLSMAPGLERLNILPFKV 413
GRMZM2G051270      VQWHAKARINAGANFYIVGRDPAGMSHPTEKRDLYDADHGKKVLSMAPGLERLNILPFKV 415
GRMZM2G158147      VQWHAKARINAGANFYIVGRDPAGMGHPTEKRDLYNPDHGKKVLSMAPGLEKLNILPFKV 406
                   ************************.*********:.**************:********

GRMZM2G149952      AAYDTKQKKMDFFDPSRKDDFLFISGTKMRTLAKNRESPPDGFMCPGGWKVLVEYYDSLV 473
GRMZM2G051270      AAYDTKQKKMDFFDPSRKDDFLFISGTKMRTLAKNRESPPDGFMCPGGWKVLVEYYDSLV 475
GRMZM2G158147      AAYDTVAKEMAFFDPSRSQDFLFISGTKMRTYAKTGENPPDGFMCPGGWKVLVDYYNSLQ 466
                   *****  *:* ******.:************ **. *.***************:**:**

GRMZM2G149952      PSEGSSKLREPIAA 487
GRMZM2G051270      PSEGSSKLREPVAA 489
GRMZM2G158147      AEEAT-----PVPV 475
                   ..*.:     *:..
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Fig. 36 Alignment of protein sequences of ATPS isoforms in maize
Sequence of maize ATPS isoforms were aligned using CLUSTALW software

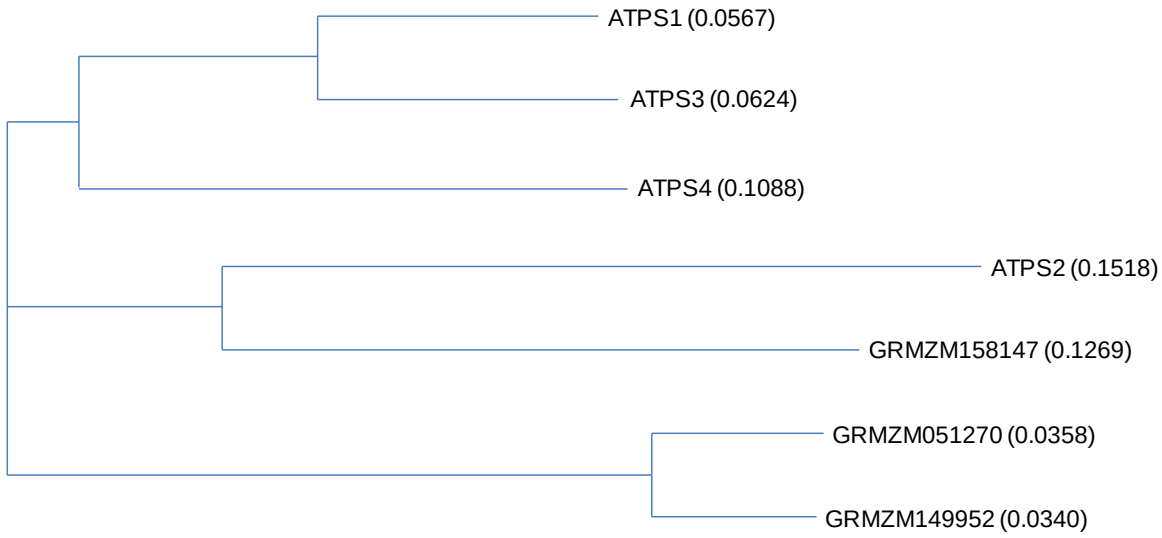

Fig. 37 Phylogenetic tree for protein sequences of ATPS isoforms of maize and Arabidopsis
Sequences of ATPS isoforms of maize and Arabidopsis were aligned and tree was drawn using Vector NTI 9 software. Arabidopsis isoforms accession numbers: ATPS1 (AT3G22890), ATPS2 (AT1G19920), ATPS3 (AT4G14680) and ATPS4 (AT5G43780).

In order to analyze if increase in the steady state level of APS is a consequence of up-regulation of *ATPS*, quantification of these *ATPS* isoforms in leaves of control and drought stressed was performed with qRT-PCR. Expression analysis revealed that all isoforms were up-regulated by more than 2-fold after 10 and 12 days of drought stress in comparison to the control (Fig. 38A, B & C).

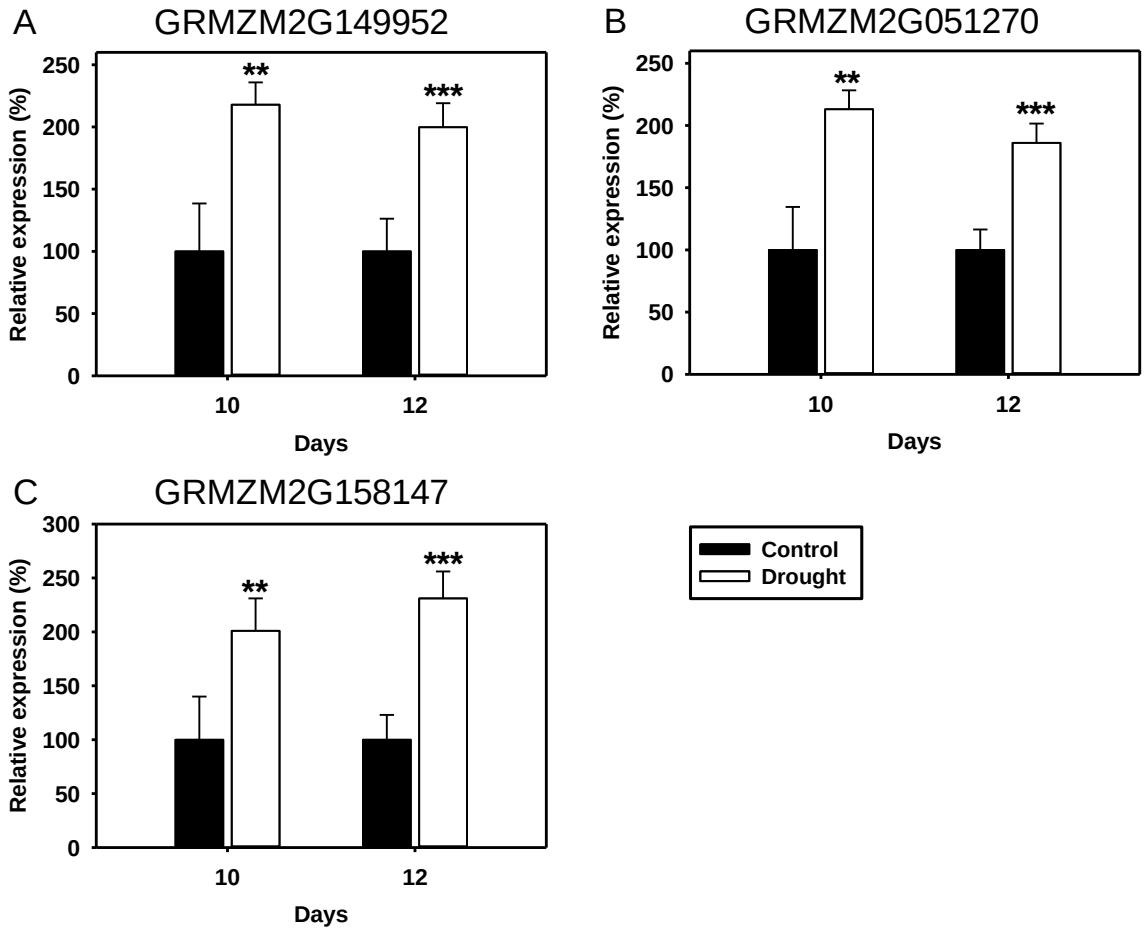

Fig. 38 Impact of drought on expression of *ATPS* isoforms in leaves of maize
Expression levels of *ATPS* isoforms (A) Accession # GRMZM2G149952 (B) Accession # GRMZM2G051270 and (C) Accession # GRMZM2G158147 were quantified with qRT PCR. RNA was extracted from leaves of control and drought stressed plants and cDNA synthesized for the quantification of all isoforms (n=3). Mean ± standard deviations are shown.

In contrast to leaves, roots showed down-regulation in the transcripts abundance of all *ATPS* isoforms where more than 50-70% decrease was observed following 10 and 12 days of drought relative to control (Fig. 39A, B & C).

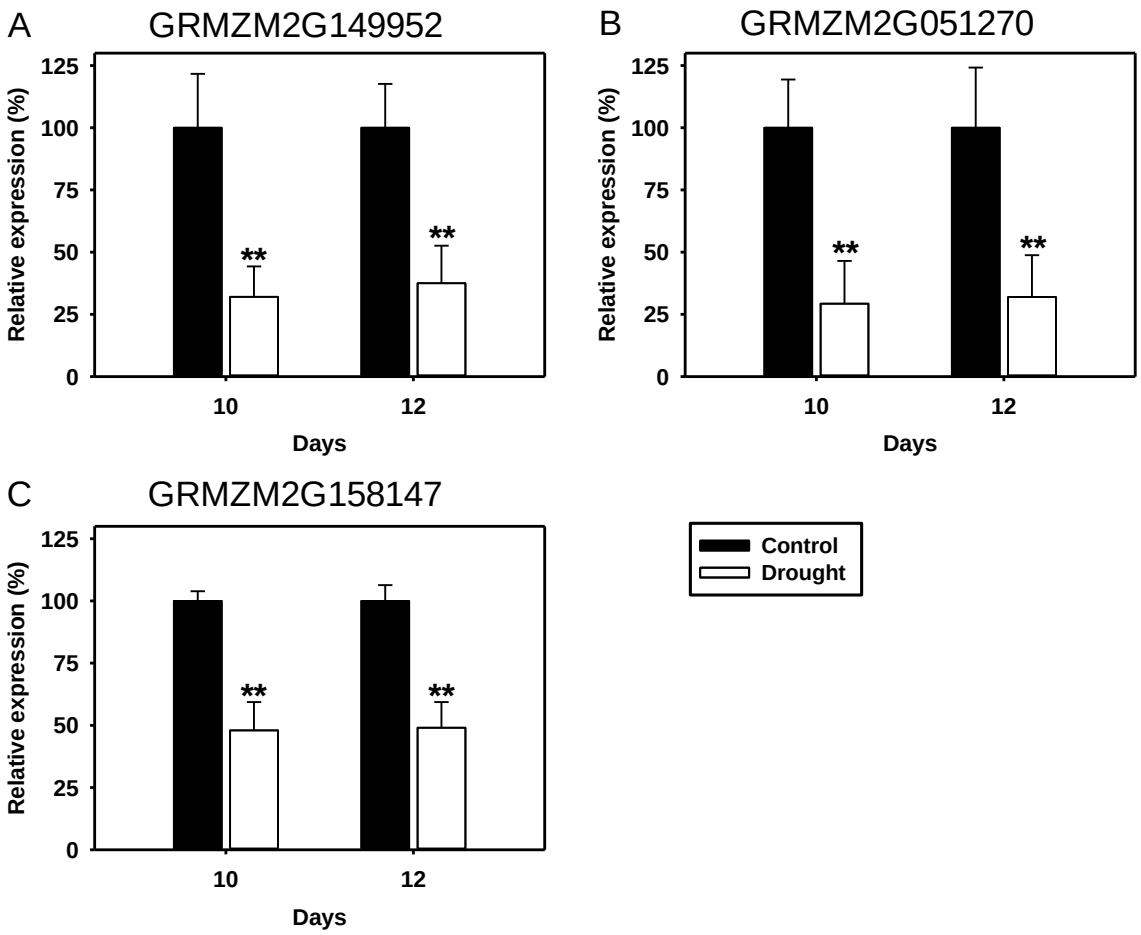

Fig. 39 Impact of drought on expression of *ATPS* isoforms in roots of maize
Expression levels of *ATPS* isoforms (A) Accession # GRMZM2G149952 (B) Accession # GRMZM2G051270 and (C) Accession # GRMZM2G158147 were quantified with qRT PCR. RNA was extracted from roots of control and drought stressed plants and cDNA synthesized for the quantification of all isoforms (n=3). Mean ± standard deviations are shown.

3.7 Impact of drought on total CNS, inorganic anions and sulfate transporters

3.7.1 Analysis of total carbon, nitrogen and sulfur contents in leaves and roots

Next, we measured the total carbon, nitrogen and sulfur (CNS) contents to determine the most possible assimilation pathways which were affected by drought in maize. Analysis of total CNS showed that carbon was not significantly affected by drought after 10 and 12 days (Fig. 40A). A significant decrease in nitrogen was observed only for 10 days of drought and after 12 days of drought, nitrogen content was not significantly affected (Fig. 40B). In contrast, total sulfur was found to be significantly lower in leaves of maize subjected to drought for 10 and 12 days in comparison to control (Fig. 40C).

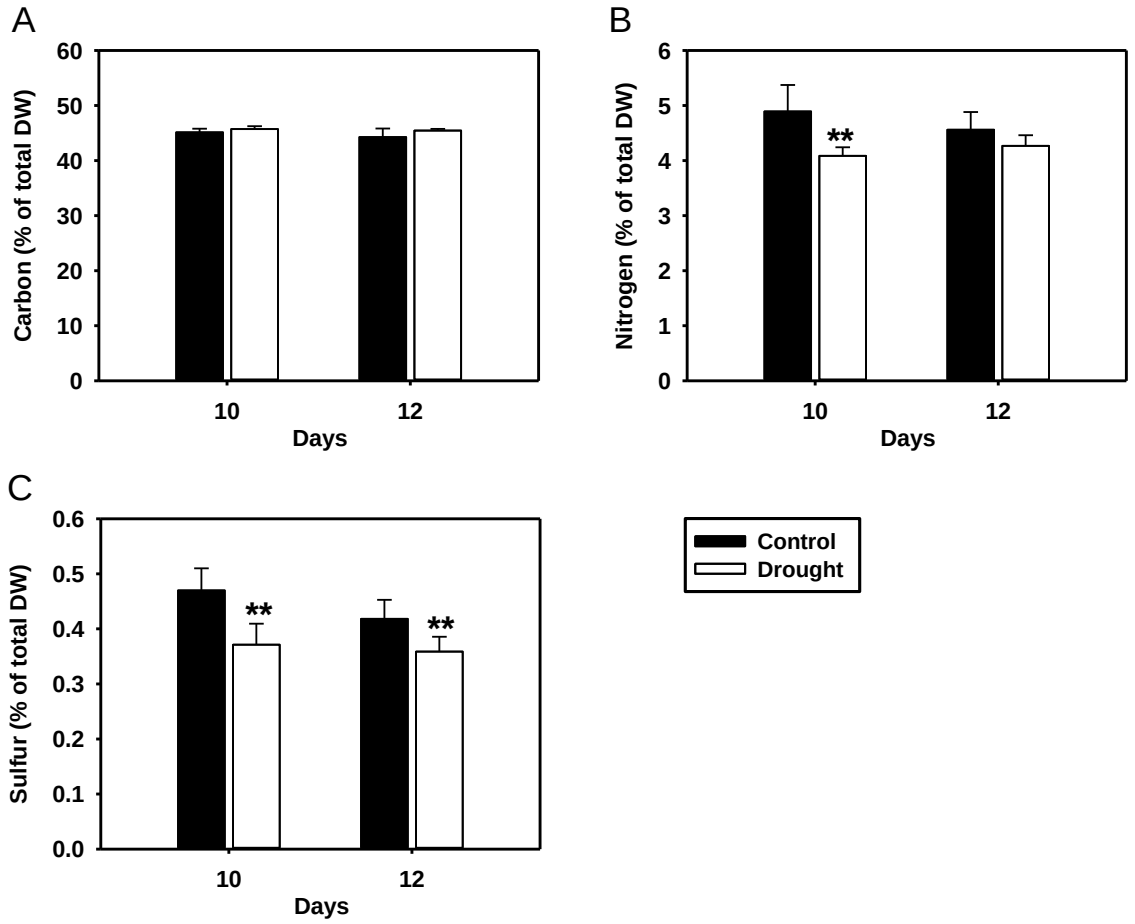

Fig. 40 Impact of drought on total CNS in leaves of maize
Total contents of carbon (A), nitrogen (B) and sulfur (C) in leaves of control and drought stressed plants as percent of the dry weight (n=7). The elements were quantified in a Vario MAX CNS elemental analyzer in collaboration with Mr. Gerd Schukraft (Geographical Institute, Heidelberg University). Mean ± standard deviations are shown.

Major differences in leaf CNS during drought led us to further investigate if similar patterns of changes during drought might also be observed in roots. Analysis of total CNS revealed significant decrease in carbon between control and drought stressed roots at each time point (Fig. 41A). However no significant difference for nitrogen was observed under drought (Fig. 41B). In contrast to foliar sulfur contents, root sulfur was not significantly decreased after 10 days of drought although after 12 days a significant reduction was observed compared to the control (Fig. 41C).

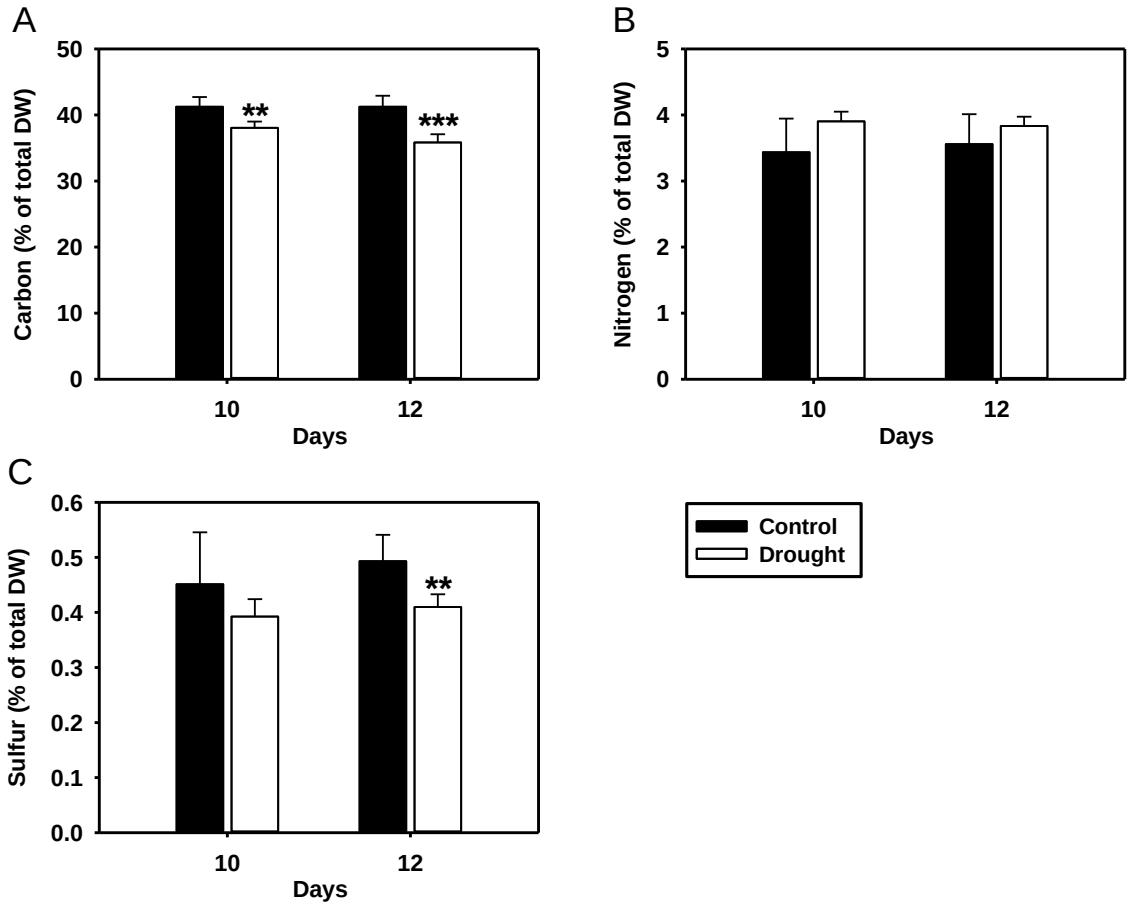

Fig. 41 Impact of drought on total CNS in roots of maize
Total contents of carbon (A), nitrogen (B) and sulfur (C) in roots of control and drought stressed plants as percent of the dry weight (n=5). The elements were quantified in a Vario MAX CNS elemental analyzer in collaboration with Mr. Gerd Schukraft (Geographical Institute, Heidelberg University). Mean ± standard deviations are shown.

3.7.2 Alteration of inorganic anions in drought stressed leaves and roots

It appeared that the sulfur metabolism was sensitive to drought, at least in maize. To further confirm this, the amounts of anions which are the first substrate for phosphorous (phosphate), nitrogen (nitrate) and sulfur (sulfate) assimilatory pathway were measured by anion HPLC (section 2.4.7). Analysis of sulfate contents in leaves showed a strong decrease by approximately 62 and 68% following 10 and 12 days drought treatment, respectively (Fig. 42A). In contrast to sulfate, nitrate contents were increased by 3.6 and 5.2-fold under drought after 10 and 12 days respectively (Fig. 42B) while phosphate contents were not significantly affected by drought (Fig. 42C).

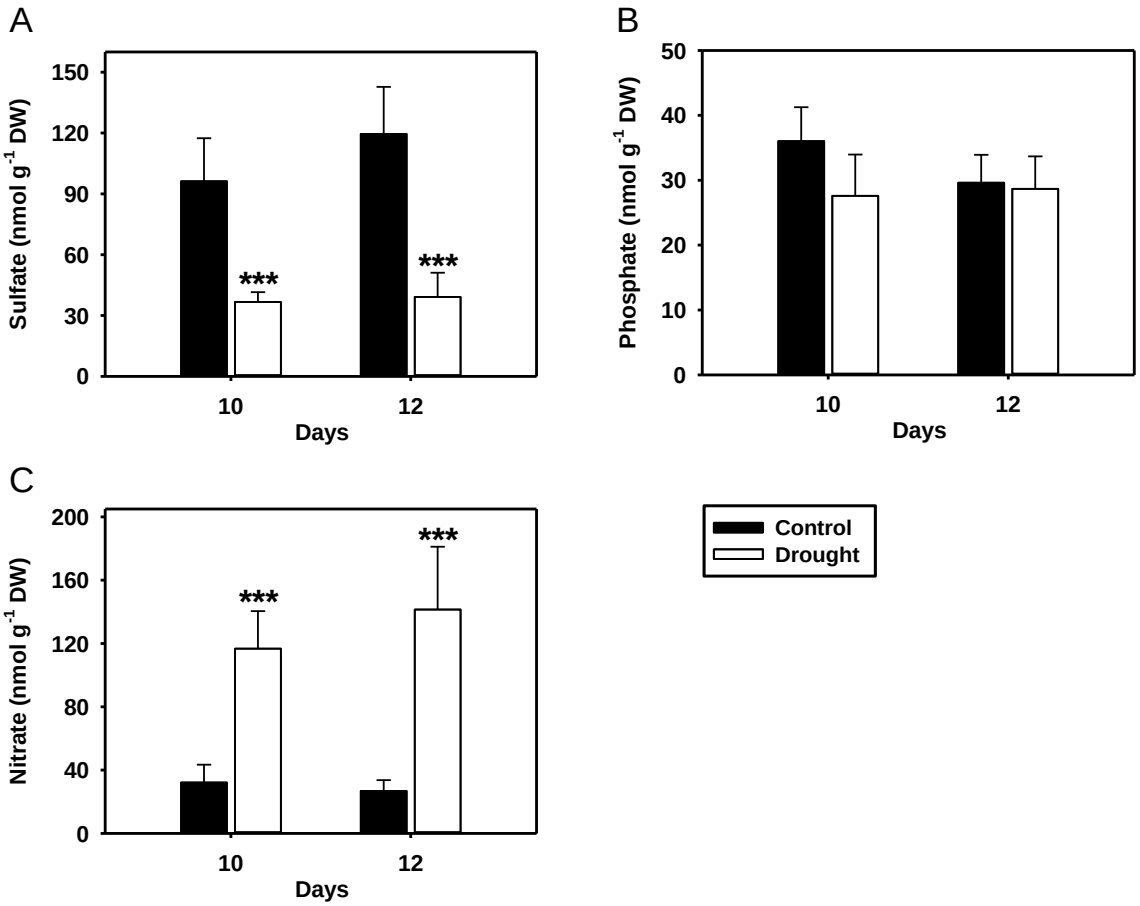

Fig. 42 Impact of drought on anions contents in leaves of maize
Sulfate (A), phosphate (B) and sulfate (C) were quantified by anion HPLC from 0.1M HCl extract in leaves of control and drought stressed plants grown on vermiculite media under long day condition (n=5). Mean ± standard deviations are shown.

The abundance of anions was also measured in roots to analyze the potential impact of drought on the function of major assimilatory pathways in maize. Analysis of anions revealed elevated steady-state level of sulfate by approximately 3 and 3.64-fold in drought stressed roots compared to control (Fig. 43A). Under drought, phosphate contents were increased by 2-to 3-fold (Fig 43B). The increase in nitrate levels in roots was even higher compared to sulfate and phosphate where 3.92 and 5.32-fold was recorded after 10 and 12 days of drought, respectively compared to the control (Fig. 43C).

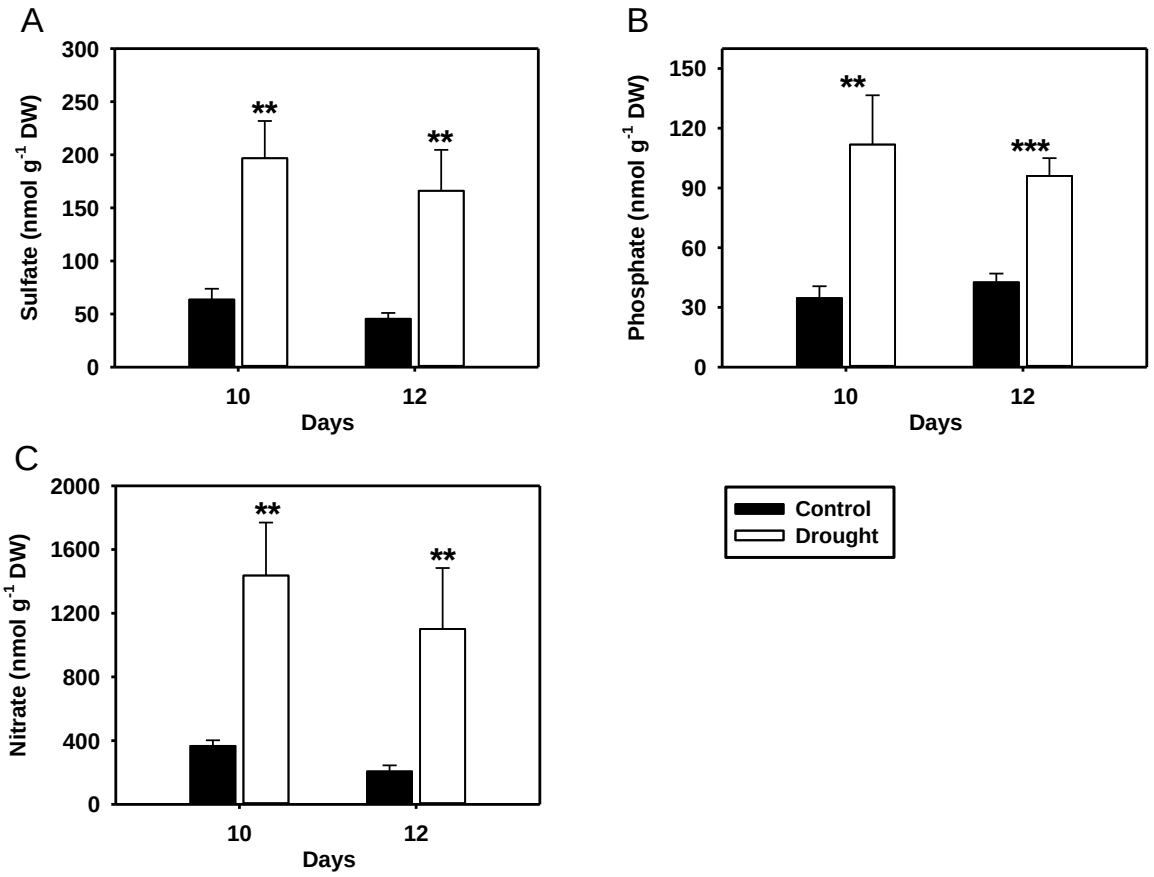

Fig. 43 Impact of drought on anions contents in roots of maize
Sulfate (A), phosphate (B) and nitrate(C) in roots of control and drought stressed plants were quantified by anion HPLC from 0.1M HCl extract (n=5). Mean ± standard deviations are shown.

3.7.3 Quantification of sulfate transporters in leaves and roots

A drastically lower steady state level of sulfate and also a significant decrease in total elemental sulfur (section 3.7.1) under drought stress mimics sulfur-starved condition. We therefore investigated the expression of sulfate transporter *Sultr1;1* and *Sultr1;2* in leaves that are responsible for sulfate uptake from the soil. In addition to those, *Sultr4;1* was also analyzed which is mainly responsible for sulfate efflux from the vacuole. The expression of *Sultr1;1* was up-regulated by 2.5-fold while *Sultr1;2* was down-regulated by 90% in leaves under drought stress (Fig. 44A & B).

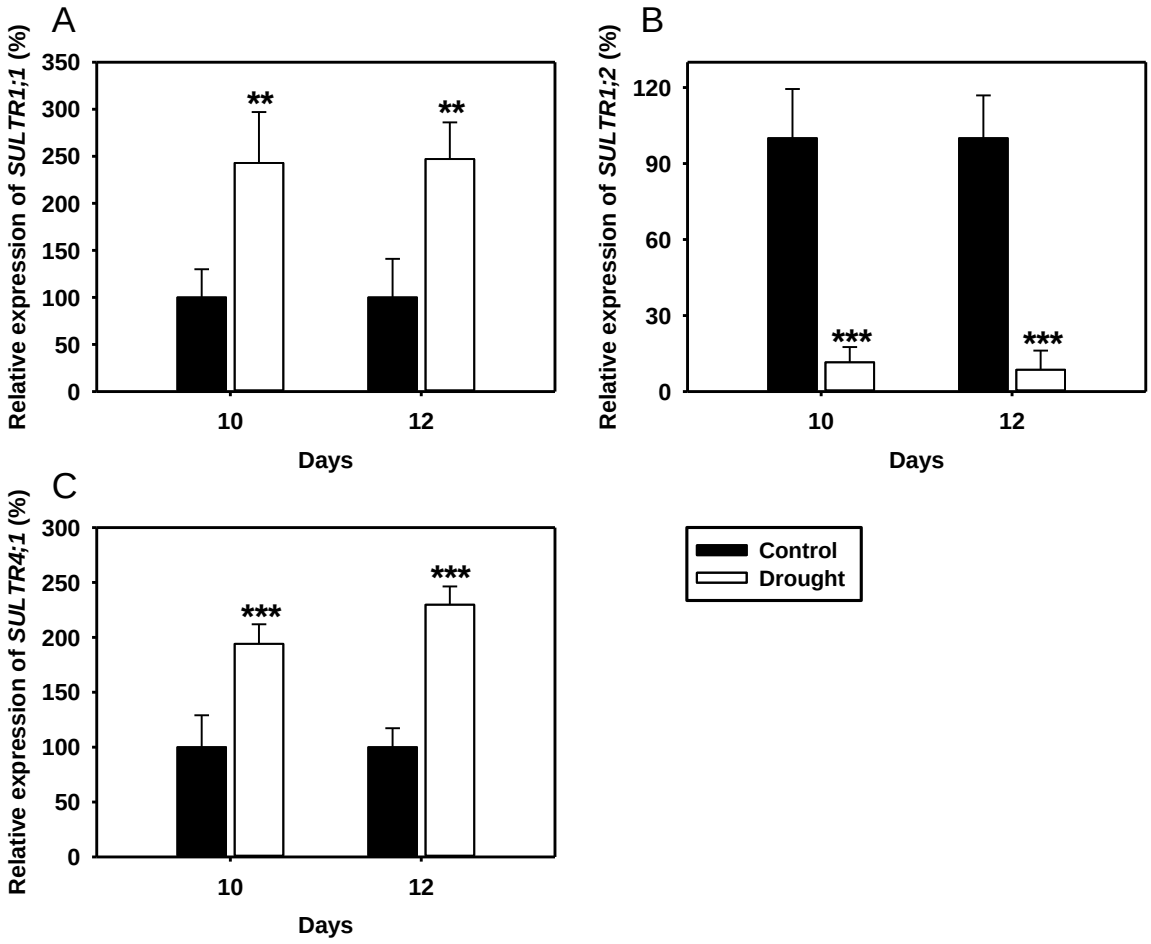

Fig. 44 Impact of drought on expression of sulfate transporters in leaves of maize
Expression levels of sulfate transporters (A) *Sultr1;1* (B) *Sultr1;2* and (C) *Sultr4;1* were quantified with qRT PCR. RNA was extracted from leaves of control and drought stressed plants and cDNA synthesized for the quantification of these transporters (n=3). Mean ± standard deviations are shown.

On the other hand, the *Sultr4;1* was up-regulated by approximately 2 and 2.3-fold after 10 and 12 days in drought stressed leaves, respectively, in comparison to the control (Fig. 44C). This expression pattern of *Sultr4;1* indicates that the stored sulfate in vacuole is exported and used due to less sulfate availability to leaves from roots.

To account for the opposing observations in leaves vs roots, the transcript abundance of the key sulfate transporters *Sultr1;1*, *Sultr1;2* and *Sultr4;1* in roots were also quantified by qRT PCR. *Sultr1;1* was up-regulated by approximately 2-fold under drought stress in roots, whereas no change in expression of *Sultr1;2* was observed (Fig 45A & B). In contrast to leaves, *Sultr4;1* was down-regulated by approximately 40% in the drought stressed roots compared to the control (Fig. 45C) indicating sulfate storage in the vacuole.

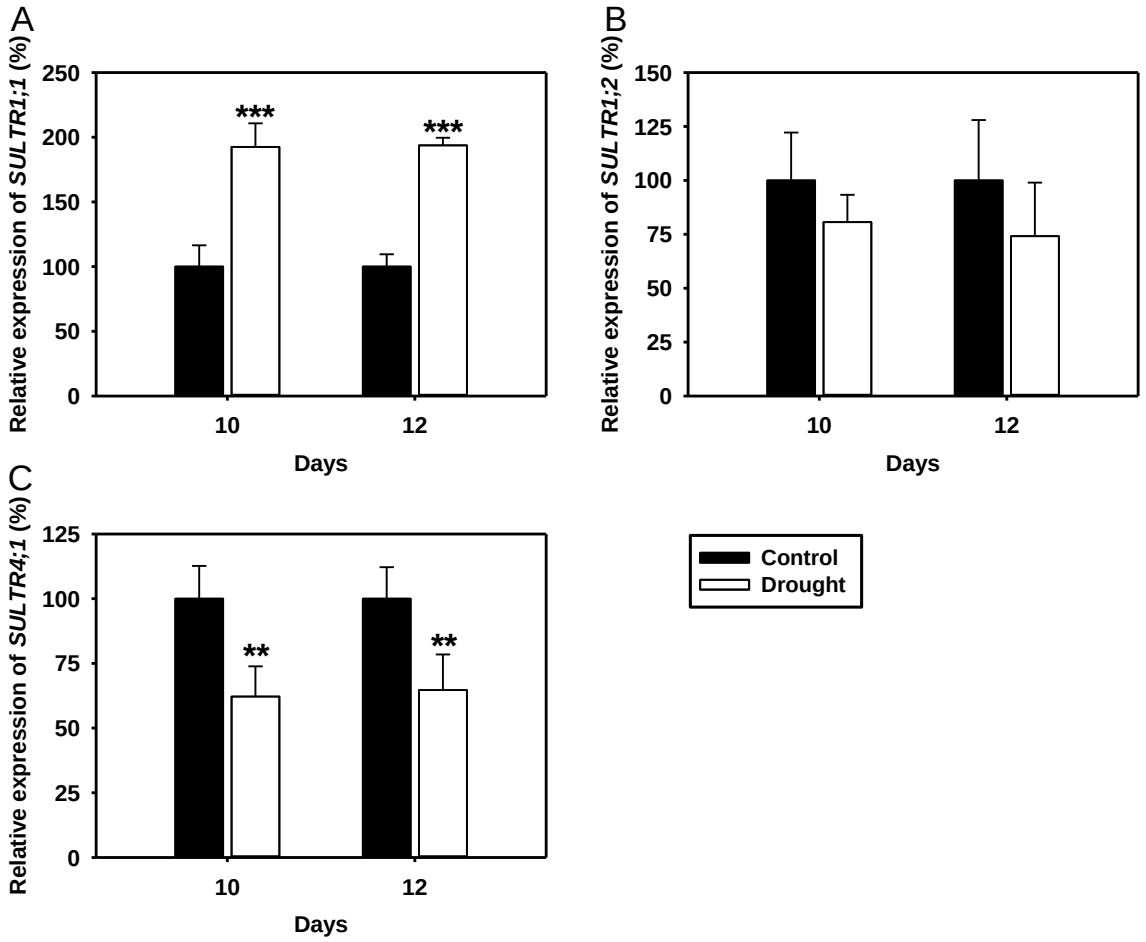

Fig. 45 Impact of drought on expression of sulfate transporters in roots of maize
Expression levels of sulfate transporters (A) *Sultr1;1* (B) *Sultr1;2* and (C) *Sultr4;1* were quantified with qRT PCR. RNA was extracted from roots of control and drought stressed plants and cDNA synthesized for the quantification of these transporters (n=3). Mean ± standard deviations are shown.

3.8 Impact of drought on ^{35}S incorporation rate *in vivo* in leaves and roots

3.8.1 Analysis of ^{35}S incorporation rate into thiols in leaves

Due to the decrease in steady state levels of sulfate, cysteine and GSH, we decided to monitor the flux of sulfur into the sulfate reduction pathway *in vivo*. To achieve this, feeding experiment was performed for determination of the incorporation of ^{35}S radioactively labeled sulfate into these metabolites. Leaf pieces of comparable sizes from both control and drought stressed plants were first rehydrated in dH_2O for 10 min, followed by an incubation in ½ Hoagland medium supplied with radioactive labelled sulfate ($^{35}SO_4^{2-}$) for 30 and 60 min to monitor the incorporation rate into cysteine and GSH. Extraction and derivatization were performed in order to determine the amount of ^{35}S incorporated into HPLC-separated cysteine and GSH fractions (section 2.7.2.3).

The degree of $^{35}SO_4^{2-}$ uptake by drought-treated leaves was significantly increased by approximately 2-to 3-fold in ^{35}S after 30 and 60 min incubation relative to the control (Fig. 46A). As expected, a reduction in the incorporation rate of ^{35}S into both cysteine and GSH was observed after 30 and 60 min incubation in drought stressed leaves compared to control. This decrease in the incorporation of ^{35}S into cysteine in leaves was approximately 70 and 80% following 10 and 12 days drought treatment, respectively in comparison to the control (Fig. 46B). Moreover, the incorporation of ^{35}S into GSH was decreased by approximately 65 and 70 % in leaves after 10 and 12 days in drought stressed plants, respectively (Fig. 46C).

Incorporation of ^{35}S label into the protein fraction revealed a reduction in drought stressed leaves in comparison to non-stressed. Taking together, an average decrease of approximately 65 and 73% was observed after 10 and 12 days in drought stressed leaves, respectively, in the incorporation of ^{35}S label into protein fractions relative to control (Fig. 46D).

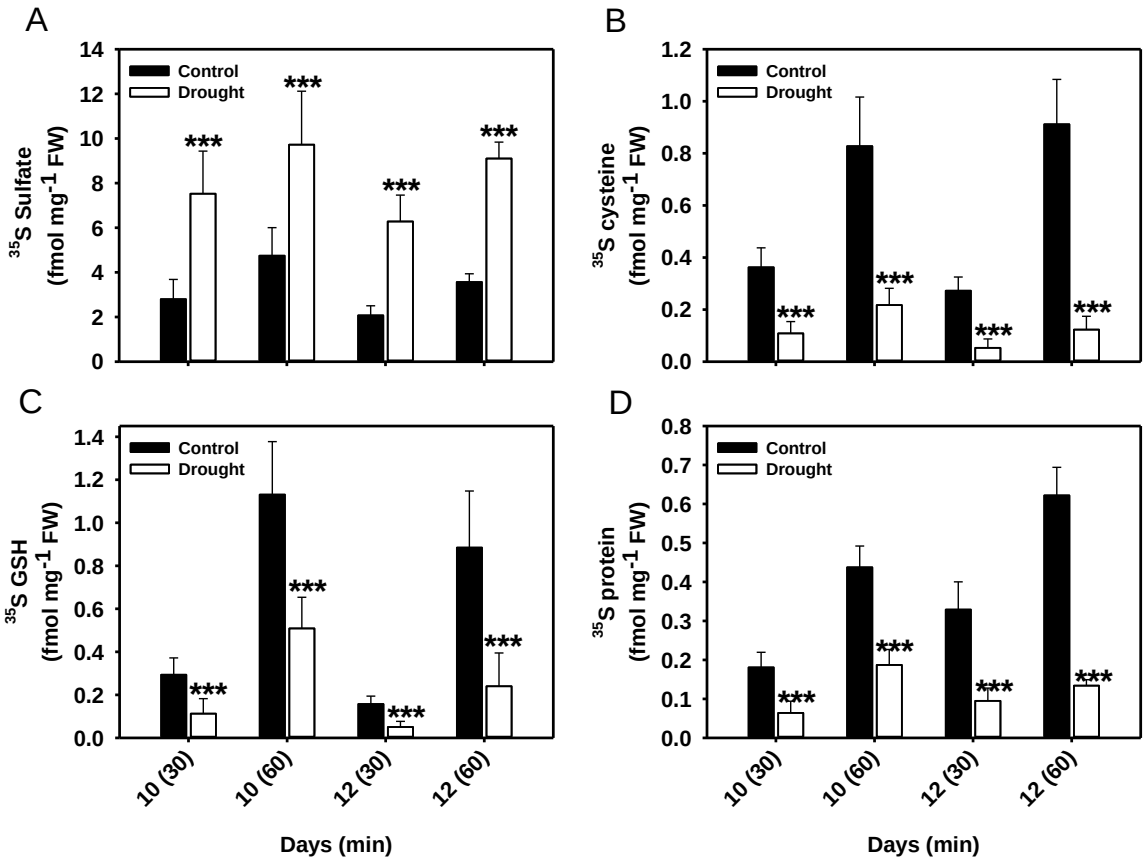

Fig. 46 Impact of drought on the incorporation of $^{35}SO_4^{2-}$ into sulfur-containing compounds *in vivo* in leaves of maize

Leaf pieces of 30 mg fresh weight (FW) of control and drought stressed plants were 1st rehydrated in dH_2O and then incubated in $^{35}SO_4^{2-}$ labeled solution for 30 and 60 min. Samples were taken at the indicated time points. Sulfate was precipitated with barium chloride and thiols were separated by HPLC while proteins were quantified by specific precipitation. The ^{35}S that was incorporated into the fractions was quantified by scintillation counting (sulfate n=6; thiol and protein n=8). Mean ± standard deviations are shown.

3.8.2 Analysis of ^{35}S incorporation rate into thiols in roots

The flux of sulfur in the assimilatory sulfate reduction and its incorporation into sulfate, cysteine and GSH were analyzed *in vivo* due to increase in steady state levels of these metabolites in drought-stressed roots. To do this, the same feeding procedure was used as described for leaves. Briefly, root pieces of comparable sizes from both control and drought stressed plants were first rehydrated in dH_2O for 10 min and afterwards incubated for 30, 60, 180 and 360 min on ½ Hoagland medium supplied with radioactive labeled sulfate ($^{35}SO_4^{2-}$) to monitor its incorporation rate into cysteine and GSH. After extraction and derivatization, the amount of ^{35}S label was determined into HPLC-fractionated cysteine and GSH.

Interestingly, the degree of sulfate uptake was significantly increased by more than 2-fold in drought stressed root pieces compared to control. Moreover, no significant difference

was observed between control and drought stressed roots in the incorporation of ^{35}S into sulfate after 180 and 360 minutes (Fig. 47).

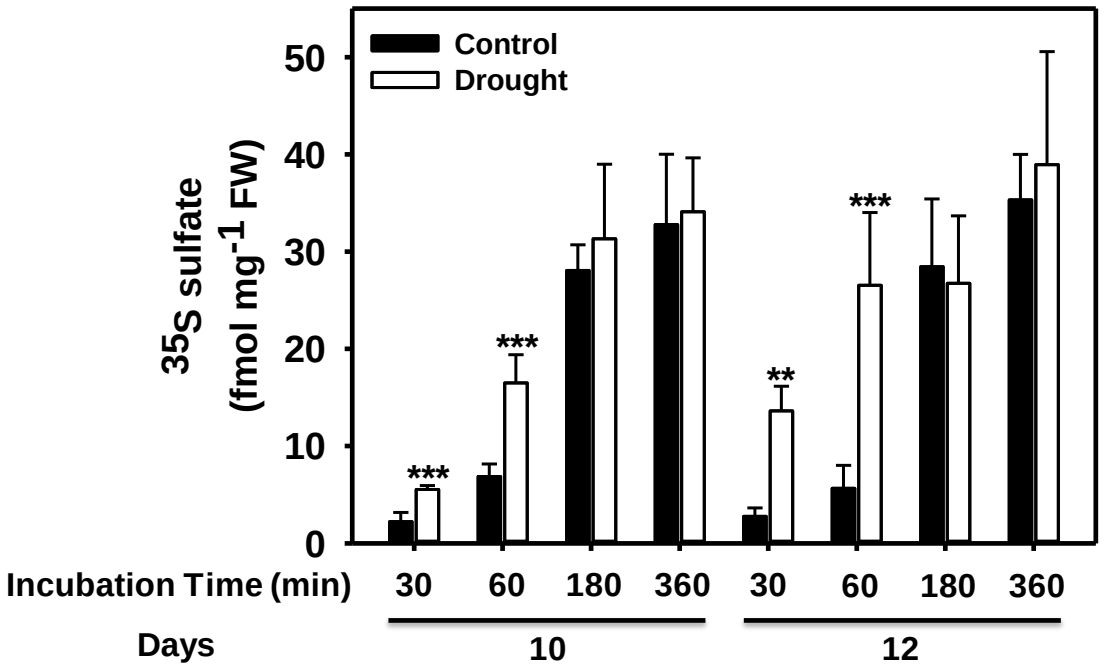

Fig. 47 Impact of drought on the incorporation of ^{35}S into sulfate *in vivo* in roots of maize
Roots pieces were incubated in $^{35}SO_4^{2-}$ labeled solution for 30, 60, 180 and 360 min. Samples were taken at the indicated time points. Sulfate was precipitated with barium chloride. The ^{35}S that was incorporated into the fractions was quantified by scintillation counting (n=6). Mean ± standard deviations are shown.

In roots, after 30 and 60 min incubation in the labeled solution, the incorporation of ^{35}S into cysteine and GSH was significantly slower than leaves and below detection level. Therefore, the incubation time was increased to 180 and 360 min. Analysis of the incorporation rate of ^{35}S into cysteine revealed at least 50-62% reduction after 180 and 360 min incubation in drought stressed root pieces compared to the control (Fig 48A).

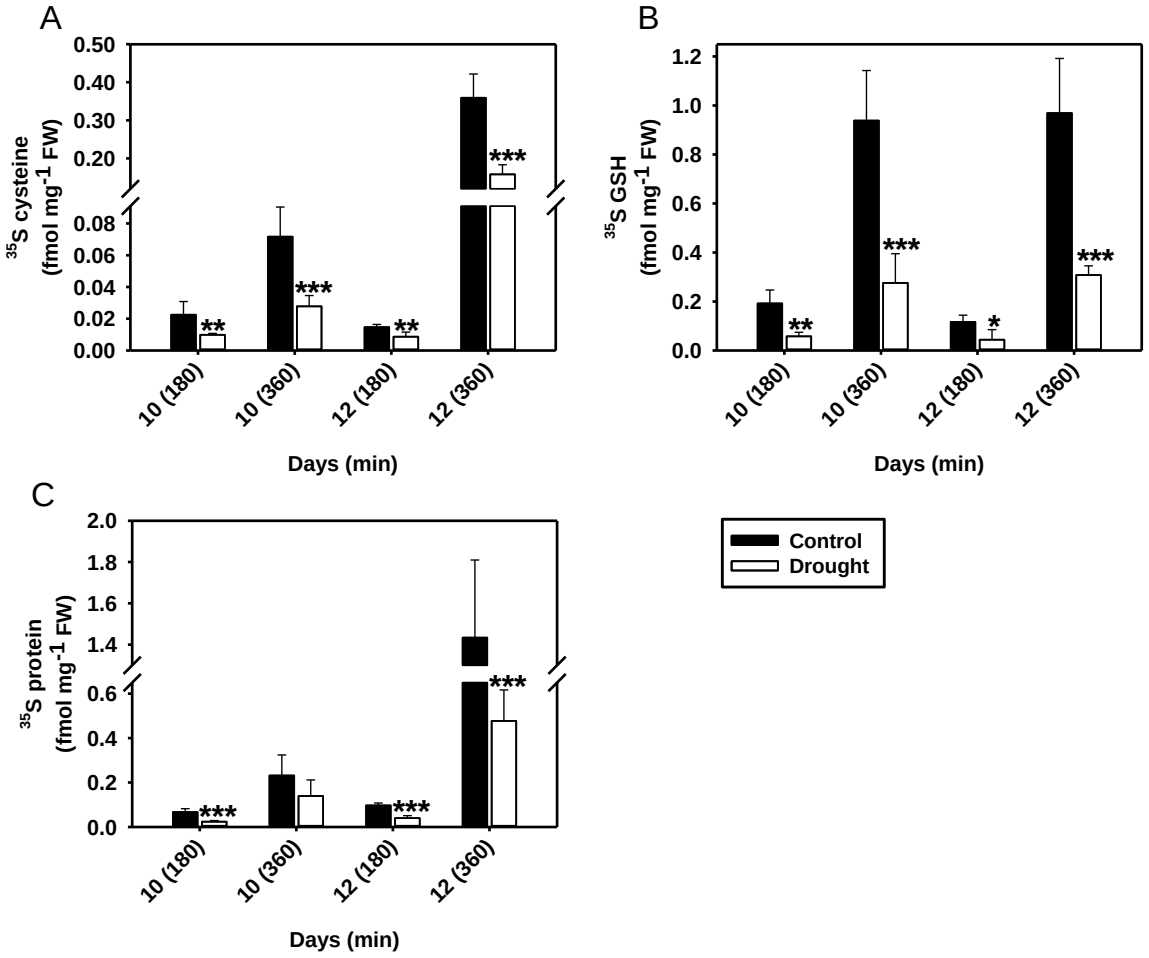

Fig. 48 Impact of drought on the incorporation of $^{35}SO_4^{2-}$ into thiol and protein *in vivo* in roots of maize

Root pieces of approximately 50 mg fresh weight (FW) of control and drought stressed plants were 1st rehydrated in dH_2O and then incubated in $^{35}SO_4^{2-}$ labeled solution for 180 and 360 min. Samples were taken at the indicated time points. Thiols were separated by HPLC while proteins were quantified by specific precipitation. The ^{35}S that was incorporated into the fractions was quantified by scintillation counting (thiol and protein n=6). Mean ± standard deviations are shown.

A more drastic reduction in the incorporation rate was observed for GSH. An average decrease of approximately 70-75 % was recorded in the incorporation of ^{35}S into GSH after 10 days and 63-75% after 12 days in drought treated roots, respectively relative to well-watered (Fig. 48B).

Consistent with the lowered thiol contents, the incorporation of ^{35}S label into protein fraction also showed a reduction in drought stressed roots compared to the control. In roots, an average reduction of approximately 60-66% was observed after 10 and 12 days of drought stress relative to control in the incorporation of ^{35}S label into protein fractions although 360 min incubation showed no significant difference after 10 days of drought stress (Fig. 48C).

3.9 Analysis of transport of $^{35}SO_4^{2-}$ from root to shoot under drought

In leaves, all metabolites of the sulfur assimilation pathway were decreased due to lower steady state level of sulfate and also reduced flux through the sulfur assimilation pathway. In contrast, in roots all metabolites were accumulated due to sufficient availability of sulfate, indicating that there might be less transport under drought that affected the sulfur assimilation pathway in leaves. In order to test this hypothesis, the transport rate of sulfate from root to shoot was investigated by injecting 1 ul of radioactive labeled $^{35}SO_4^{2-}$ into stem of control and drought stressed plants for 1 min. Stem was then cut into 4 pieces each at 3 cm distance above the injection site and the amount of radioactivity was measured in each piece.

Measurement of the amount of $^{35}SO_4^{2-}$ in each segment revealed almost no transport through the stem under drought stress. The amount of $^{35}SO_4^{2-}$ (% of total) measured after 10 days of drought was approximately 44%, (0-3cm), 23.7%, (3-6cm), 18.61%, (6-9cm) and 13.65%, (9-12cm) of the control. Similar transport rate was also observed for 12 days control plants (Fig. 49). However, after 10 and 12 days in drought almost 99.5% of $^{35}SO_4^{2-}$ was present in the first segment (0-3 cm) of stem, indicating that drought stress limits the transport of sulfate from root to shoot (Fig. 49).

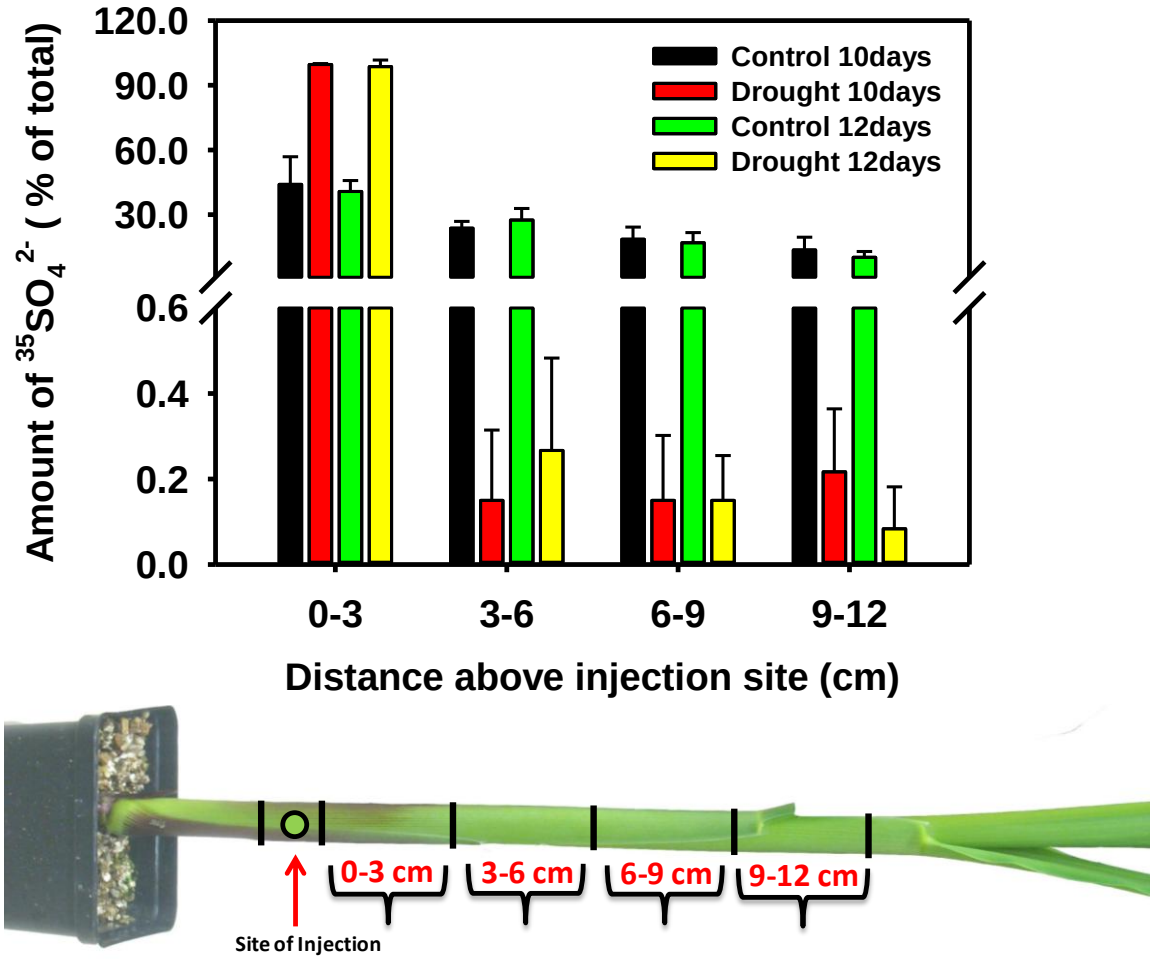

Fig. 49 Impact of drought on transport rate of $^{35}SO_4^{2-}$ from root to shoot
1ul of radioactive labeled $^{35}SO_4^{2-}$ was injected into stem of control and drought stressed plants 3-4 cm above the soil level and after 1 min of injection stem was cut into 4 pieces each at 3 cm distance above the injection site and the amount of radioactivity was measured in each piece by scintillation counting (n=6). Mean ± standard deviations are shown.

4. Discussion

Drought is one of the major abiotic stresses, causing an increased production of reactive oxygen species (ROS) that can damage the membranes and other important cellular components (Mittler, 2002). Plant regulates the level of ROS with an efficient scavenging system such as catalase (CAT), superoxide dismutase (SOD) and glutathione-ascorbate cycle (Bowler *et al.*, 1992; Willekens *et al.*, 1997, Noctor and Foyer, 1998). CATs detoxify H_2O_2 only in peroxisomes that are produced as a result of photorespiration (Mittler *et al.*, 2004; Vandenabeele *et al.*, 2004). SOD dimutates superoxide to oxygen and H_2O_2 (Bowler *et al.*, 1992). H_2O_2 is very stable ROS and move from one compartment to another easily that attacks thiol proteins, thus needs detoxification by an efficient antioxidant system in every compartment (Noctor and Foyer, 1998; Cruz de Carvalho, 2008). Glutathione-ascorbate cycle is vital that function in a well cooperative system and is located in every cellular compartment. In this cycle, oxidized glutathione (GSSG) is formed in the last step, which can then be recycled back to reduced glutathione (GSH) by glutathione reductase (GR) using NADPH as a reductant (Noctor *et al.*, 2002). Glutathione is the most abundant non-protein thiol in cells of prokaryotes and eukaryotes. The enhanced production of ROS during drought (Cruz De Carvalho, 2008) may require increased GSH production for the efficient detoxification of ROS, thus the regulation of sulfur assimilation during drought is vital due to the dependency of GSH synthesis on the sulfur assimilation pathway. This project has therefore attempted to elucidate the role and response of sulfur assimilation pathway to drought stress in maize.

The rationale of this work is that plants often experience drought that effect growth and ultimately productivity. To further analyze the production of and detoxification of ROS, a significant part of this study is devoted to investigate the possible role of GSH and GR in the last step of glutathione-ascorbate cycle under normal and water deficit condition due to elevated levels of H_2O_2 production. Furthermore, the response of the sulfur assimilation pathway was investigated at the gene and the metabolite level, which could provide useful information for future engineering or improved cultivation of plants.

4.1 Characterization of drought stress in maize

As expected, the imposition of drought for 7, 10 and 12 days to maize seedlings severely affected the shoot and root biomass. To cope with the water shortage situation, plant promotes root growth which indicates that roots grow more than the aerial parts of the plants under water stress. The inhibition of shoot growth and an increase in root to shoot

ratio is considered as an adaptive response to drought (Matthews *et al.,* 1990; Chapin, 1991; Neumann, 1995; Aachard *et al.,* 2006). This short-term adaptation includes the ability of a plant to either avoid or tolerate stress. Avoidance is accompanied by altering growth schedule such as completing life cycle or maximizing water extraction from the soil by the development of large root system. On the other hand, plants tolerate by responding at the biochemical and or morphological level to minimize the potential damage from stress (Araya, 2007; Bray, 2007).

L-Δ1-pyrroline-5-carboxylate synthetase (P5CS) is one of the key enzymes of proline biosynthesis and an increase in proline contents during drought can be used as a drought stress marker (Secenji *et al.,* 2010). A several fold increase in proline contents indicates the onset of drought-induced stress both in leaves and roots. An induction in the transcript levels of P5CS followed by proline accumulation is reported under abiotic stress in various plants (Cramer *et al.,* 2007; Szekely *et al.,* 2008; Chen *et al.,* 2009) and in maize leaves and roots upon water stress (Ober and Sharp, 1994; Alvarez *et al.,* 2008; Sicher and Barnaby, 2012). The early response of water shortage is recognized by roots due to a decrease in soil water content and subsequent stomata closure in order to reduce water loss by or via transpiration. Stomata aperture was significantly decreased even at early stage of drought. This indicates that there might be some chemical signals (reviewed by Schachtman and Goodger, 2008, Ernst *et al.,* 2010) from roots to shoot and there act to close stomata at this relatively early stage of water stress. It has been shown that stomata closure can respond faster to decrease in soil water availability rather than plant water status (Tardieu *et al.,* 1992a; Ali *et al.,* 1999). The decrease in stomata aperture limits the amount of CO_2 available for photosynthesis during water shortage (Chaves *et al.,* 2009; Erismann *et al.,* 2008; Peeva and Cornic, 2009). Besides reduction in biomass, total chlorophyll contents (a & b) were also significantly decreased compared to control plants. Drought induced reduction of pigments in maize leaves might be due to photoinhibition and photoreduction as reported for various species such as pea (Moran *et al.,* 1994, Loggini *et al.,* 1999) wheat (Nyachiro *et al.,* 2001) and barley (Guo *et al.,* 2009) upon drought.

4.2 Drought causes oxidative stress in leaves and roots of maize plants

Under normal condition, the ROS formation is at low level in plants that can be triggered by drought stress (Foyer and Noctor 2000, Miller *et al.,* 2010). The reduced CO_2 availability due to drought-induced stomata closure disturbs the internal metabolism of the cell under drought stress, thus causing oxidative stress. Based on this assumption, the

steady state level of ROS was expected to be higher under drought. 3,3'-diaminobenzidine (DAB) staining showed elevated level of ROS such as H_2O_2 during drought stress after 10 and 12 days. However, no difference was observed in DAB staining between control and drought stressed leaf disc after 7 days. Since the RWC was not changed after 7 days, thus no formation of ROS was likely to be expected. The accumulation of ROS during drought was also observed in various species (Sgherri and Navari-Izzo, 1993, 1995; Moran *et al.*, 1994; Wellburn *et al.*, 1996; Loggini *et al.*, 1999; Boo and Jung, 1999). The elevated levels of ROS can rapidly influence the redox state of GSH pool compared to ascorbate/DHA redox couple, thus GSH acts as redox sensor (Foyer and Noctor, 2011). This elevated level of H_2O_2 caused the redox state of GSH pool towards more oxidized state where more than two-fold change was observed in drought-affected maize leaves. The ratio of GSSG/GSH was also increased by more than two-fold in roots, which indicates oxidative stress both in leaves and roots. An oxidation of the GSH pool upon exposure to drought is reported in barley (Smirnoff, 1993), pine leaves (Tausz *et al.*, 2001b), rice seedlings (Sharma and Dubey, 2005) and in apple leaves (Ma *et al.*, 2011). GR catalyzes the final step of the glutathione-ascorbate cycle that keeps the redox state of GSH pool in reduced form (Foyer *et al.*, 2002). Together with high GSSG to GSH ratio, up regulation of 2-fold in *GR* expression level in leaves and roots and an increase in GR activity in leaves imply an important role of GR in ROS detoxification and maintaining reduced GSH during drought in maize. Polyethylene-glycol (PEG) imposed mild water stress in detached leaves of maize also showed significant increase in APX and GR activities due to elevated level of ROS production (Jiang and Zhang, 2002). An increase in *GR* transcript levels in grapevine and wheat (Cramer *et al.*, 2007; Secenji *et al.*, 2010) and GR activity during drought has also been reported in many species such as wheat seedlings (Bartoli *et al.*, 1999; Keles and Oncel 2002), alfalfa (Rubio *et al.*, 2002), cotton and spurda anoda (Ratnayaka *et al.*, 2003), rice (Sharma and Dubey, 2005), cowbean (Contour-Ansel *et al.*, 2006) and beans (Turkan *et al.*, 2005; Torres-Franklin *et al.*, 2007).

4.3 GSH biosynthesis is tissue specific regulated in leaves and roots under drought

GSH conjugation for detoxification of endogenous oxidized metabolites is important in maintaining redox balance (Dalton, 1995; Noctor and Foyer, 1998).

The steady state level of GSH contents in leaves was significantly decreased by 50% during drought stress compared to control. The same trend of decrease was also observed for γ-EC and cysteine. The decrease in GSH steady state level is a consequence of

decrease rate of GSH biosynthesis due to reduction in γ-EC and cysteine that are important precursors for GSH biosynthesis Our results are in agreement with previous study where a reduction in GSH concentration was reported in *Cochlearia atlantica* (62% decrease) (Buckland *et al.,* 1991), *Sporobolus stapfianus* leaves (Sgherri *et al.,* 1994b), wheat leaves (Loggini *et al.,* 1999) and rice seedlings (Sharma and Dubey, 2005) upon drought stress. In contrast to the decline in total GSH in leaves, drought induced an accumulation of GSH in grasses (Price and Hendry, 1989) and sunflower seedling (Sgherri and Navari-Izzo, 1995). In consistent with our results, Nikiforova *et al.,* (2003, 2005) reported a decrease in cysteine and GSH contents after 10 and 13 days of sulfur deficiency in Arabidopsis leaves. Similarly, a reduction in cysteine, γ-EC and GSH contents was observed in oilseed rape upon sulfate deprivation (Lencioni *et al.,* 1997). In contrast to leaves, root showed 1.75-1.82, 2.7-4 and 1.8-2.3-fold accumulation in the steady state levels of cysteine, γ-EC and GSH, respectively. The accumulation of thiols reflects the ability of a plant by keeping steady state level of these thiols at a maximum level in order to cope with the oxidative stress in roots of maize. In xylem sap of maize, cysteine was reported to be accumulated during drought (Alvarez *et al.,* 2008). Also up-regulation of *GSH1* expression level is accompanied by the higher steady state level of GSH that is feedback inhibiting GSH biosynthesis (Jez *et al.,* 2004). Despite elevated level of GSH in roots, *GSH1* transcript was also induced, indicating that this feedback inhibition is rather at the protein level. GSH binds to GSH1 and inhibits the activity or GSSG/GSH ratio regulates via oxidation that induces activity. Similar induction of *GSH1* expression level was also reported in grapevine upon drought stress (Cramer *et al.,* 2007).

4.4 Cysteine synthesis is affected by drought

A significant decrease of 25-30% was observed in OAS steady state level during drought compared to control. Further analysis of SAT activity revealed also a 50% decrease in total enzymatic activity whereas OASTL activity was unchanged in leaves. This decrease in OAS level is attributed to decrease in SAT activity under drought stress. There is also possibility that this might be due to more incorporation into cysteine thus causing lower steady state level of OAS in leaves. In roots, SAT activity was unchanged whereas OAS contents were significantly decreased. In Arabidopsis the down regulation of SAT3, the major mitochondrial isoform, using artificial micro RNA (amiRNA) approach also showed reduction in 50% SAT activity and 80% OAS level in leaves (Haas *et al.,* 2008). Similarly, T-DNA knockout mutants for SAT3 showed 80% lower SAT activity and 25%

reduced OAS levels in leaves (Watanabe *et al.*, 2008b). In contrast to SAT activity in leaves, OASTL activity was unchanged whereas in roots a decrease was observed despite elevated level of thiols. Compartment-specific antisense-mediated down regulation of OASTL activity also showed increased steady state level of cysteine and GSH in transgenic potato leaves (Riemenschneider *et al.*, 2005). In *oastlAB* mutant, with only residual OASTL activity, the steady state level of cysteine and glutathione were increased by several folds in Arabidopsis (Heeg *et al.*, 2008). Thus, elevated levels of cysteine and GSH despite decrease in OASTL activity seems to be common observation between these mutants and drought in maize and reflects the importance of these thiols for cellular redox balance and protein functions during drought stress.

Serine is the metabolic precursor of O-acetylserine (OAS) that is mainly produced during photorespiration in the mitochondrion (reviewed by Bauwe *et al.*, 2012; Eisenhut *et al.*, 2012) which is the important site for OAS synthesis (Heeg *et al.*, 2008; Haas *et al.*, 2008). Analysis of serine revealed accumulation by 2.4-to 3-fold in leaves and 1.7-to 2.9-fold in roots under drought stress. The lower SAT activity possibly contributes to the accumulation of serine due to no incorporation into cysteine during drought stress. Similar accumulation of serine by more than two-fold was reported in leaves and xylem sap of maize (Foyer *et al.*, 1998; Alvarez *et al.*, 2008) and grapevine (Cramer *et al.*, 2007) upon drought stress and in grey poplar under salts stress (Herschbach *et al.*, 2010).

4.5 Drought-induced changes of sulfate reduction pathway are differentially regulated in leaves and roots

In leaves, the expression level of *APR* was not changed, while more than 2-fold up-regulation in *ATPS* transcript of all three isoforms was observed during drought. This up-regulation and increase in the steady state level of APS indicates that ATPS is rate limiting and regulated by sulfur status. Passera and Ghisi (1982) reported an increase in ATP sulfurylase activity in both bundle sheath (58%) and mesophyll cells (94%) upon sulfur deficiency in maize leaves which reflects ATPS as regulatory enzymes of the sulfate reduction pathway in maize. The levels of *ATPS* and sulfate permease transcripts increased upon sulfur deficiency and decreased in the presence of excess reduced sulfur compounds in maize leaves (Bolchi *et al.*, 1999). The data reported here is consistent with the above mentioned study where cysteine feedback inhibits the expression of *ATPS* and sulfate permease in maize. Since in leaves of maize both cysteine and sulfate contents were decreased during drought, the expression of *ATPS* is likely to be up-regulated. Our data shows that drought adaptation includes sulfur deficiency response in leaves of maize.

Therefore, it is important to compare drought with sulfur starved condition, since sulfur limiting like response is expected in maize leaves. Hopkins *et al.,* (2004) reported an increase in *ATPS* and *APR* mRNA level under sulfur deficiency where *ATPS* expression was more abundant than *APR* in leaves of maize indicating different mode of regulation between maize and Arabidopsis where *APR* showed highest induction under sulfur starvation (Takahashi *et al.,* 1997; Vauclare *et al.,* 2002). In contrast to maize, most of the study in Arabidopsis described APR as a key regulator of the sulfur assimilation pathway and is induced upon sulfur deficiency (Takahashi *et al.,* 1997; Yamaguchi *et al.,* 1999), oxidative stress (Leustek *et al.,* 2000), or heavy metal stress (Heiss *et al.,* 1999) and exerting major control of flux over the pathway (Vauclare *et al.,* 2002). These fundamental differences indicate different regulation and coordination of sulfur reduction pathway in these species, especially in leaves.

In roots, *APR* was induced whereas all three isoforms of *ATPS* were down-regulated during drought in maize. This down-regulation in *ATPS* transcript level is attributed either to higher steady state level of cysteine or accumulation of sulfate in roots since cysteine and sulfate availability inhibits the expression of *ATPS* in maize (Bolchi *et al.,* 1999). *ATPS* and *APR* mRNA levels were induced in roots of maize under sulfur deprived condition (Hopkins *et al.,* 2004) and reduced sulfur compounds caused down-regulation in *ATPS* expression level (Bolchi *et al.,* 1999). This means that drought stress results in a different regulation than sulfate limitation or another signal overrules the *APR* induction in the drought stressed plants.

The lower steady state level of sulfide by 70-80% in leaves was observed upon drought stress compared to control. This decrease in sulfide is a consequence of lower steady state level of sulfate. In contrast to leaves, roots showed an accumulation of more than 2.5-fold. The higher steady state level of sulfate and subsequently cysteine likely caused the accumulation of sulfide since it is directly incorporated into cysteine which is one of the precursors of GSH that plays an important role in oxidative stress response. The exogenous application of hydrogen sulfide (H_2S) resulted in a decrease stomata aperture size and improved drought resistance in Arabidopsis (Jin *et al.,* 2011) and regulated the GSH metabolism by enhancing GSH1 activity and GSH contents in wheat under water shortage (Shan *et al.,* 2011). Thus, the elevated sulfide contents in roots demonstrate its potential role in the regulation of GSH metabolism in coping production of ROS and might also be important signal from roots to shoots in stomata closure during drought in maize.

3′-phosphoadenosine 5′-phosphosulfate (PAPS) acts as a sulfate donor for various molecules in the sulfotransferases (SOT)-catalysed sulfation process, producing 3′-phosphoadenosine 5′-phosphate (PAP) as a by-product (Klein and Papenbrock, 2004). Analysis of adenosine related metabolites showed different response towards drought compared to control. The steady state levels of PAPS were significantly decreased only after 12 days whereas PAP remained unchanged in leaves during drought. In contrast to leaves, PAPS was significantly increased whereas PAP was decreased in roots compared to control. Moreover, ATP and ADP were decreased by approximately by 60-74% in leaves and even more in roots compared to control. In Arabidopsis, it has been recently shown in *fry1* mutant that PAP accumulated 20-fold compared to wild type whereas 10-fold increase was observed in PAP levels in wild type when the RWC was below 40% (Estavillo *et al.,* 2011) although other adenosine were not changed significantly (APS, PAPS, PAPS, SAM, Ade, AMP, ADP, and ATP). The accumulation of PAP was not observed in our study that might be due to RWC that was 66% after 12 days of drought in maize which is consistent with Arabidopsis where the PAP contents was also not changed at this particular RWC. The accumulation in PAP in their study was recorded when the RWC was below 35%. There is also possibility that there could be different regulations between maize and Arabidopsis in response to drought.

Our results of amino acids analysis in leaves and roots are consistent with previous study where the same trends of accumulation was observed in leaves and xylem sap of maize during drought (Alvarez *et al.,* 2008; Sicher and Barnaby, 2012), although the functional significance of accumulation of these amino acids is not understandable but might have some specific function in response to drought.

4.6 Drought-induced differential regulation of sulfate in leaves and roots

Analysis of total CNS showed significant decrease in the total sulfur in leaves, whereas roots showed a significant decrease only after 12 days of drought stress. Further analysis of anions revealed that sulfate contents were also decreased by approximately 62-68% in drought stressed leaves compared to control. The reduction in total sulfur in leaves is a direct consequence of the lower steady state level of sulfate during drought stress. Similarly, a decrease in sulfate and total sulfur is reported in leaves and roots of two weeks old maize seedling upon sulfate deprivation. The sulfate contents were decreased by 70 and 95% after 2 and 10 days of sulfate deficiency, respectively whereas total sulfur showed a slight reduction after 2 days in leaves (Bouranis *et al.,* 2012). Our data also shows a reduction in sulfate contents and total sulfur that might be due to low availability

of sulfur in the shoot under drought which is consistent with the above mentioned study. Furthermore, up-regulation of more than 2-fold was observed in *Sultr1;1* expression level in leaves. This up-regulation of *Sultr1;1* in leaves further support the idea of sulfur starved condition in leaves during drought since *Sultr1;1* expression is often induced under sulfur deficiency and is responsible for uptake under stress (Takahashi *et al.,* 2000; Yoshimoto *et al.,* 2002). In addition to that, sulfate transporter was also induced by 5.4-fold during drought in grapevine leaves (Cramer *et al.,* 2007). Hopkins *et al.,* (2004) reported an increased abundance of *Sultr1;1* transcripts in leaves upon sulfur deficiency in maize.

Members of the Sultr4 group are responsible for sulfate efflux from the vacuole that accounts for 99% of total sulfate storage in the cell (Rennenberg, 1984; Bell *et al.,* 1994; Kataoka *et al.,* 2004b). An increase in the *Sultr4;1* transcript level during drought indicates diminished sulfate concentration in leaves. Together with the up-regulation in *Sultr1;1,* they support the idea of intracellular sulfate remobilization from the vacuole indicating increase demand for sulfur during drought in maize leaves. Similar induction in *Sultr4;1* expression level was reported in *Brassica oleracea*, wheat and poplar upon sulfur depletion (Koralewska *et al.,* 2007; Buchner *et al.,* 2010; Honsel *et al.,* 2012) and also up-regulation in *Sultr1;1* in poplar was observed in leaves under sulfur deficiency (Honsel *et al.,* 2012) which is consistent with our results in leaves during drought.

For the uptake of sulfate in roots, three competing reactions are important. The activation of sulfate to APS by ATP sulfurylase in order to produce enough sulfur-containing amino acids for local protein synthesis, storage of sulfate in vacuole and transport to shoots (Sorbo, 1987; Clarkson *et al.,* 1993; Bolchi *et al.,* 1999). The accumulation of sulfate in roots might be due to increased sulfate uptake for roots growth or less transport from root to shoot during drought as proven by the lack of transport of labeled sulfate in the stem of drought stressed plants. This might also be true for other anions (nitrate and phosphate) since they are also accumulated in roots. A significant increase in the expression of *Sultr1;1* was observed in roots whereas *Sultr1;2* was not significantly changed. The up-regulation in *Sultr1;1* in roots could be attributed to the following factors: (1) Less sulfate in the leaves that causes induction in *Sultr1;1* expression in roots for more sulfate uptake, and (2) Higher requirement of sulfate in roots during drought since high affinity *Sultr1;1* is induced under sulfur deficiency and responsible for sulfate uptake under stress (Takahashi *et al.,* 2000; Yoshimoto *et al.,* 2002). Similarly, in previous study, an induction in *Sultr1;1* expression level was reported in roots of maize during drought

(Ernst *et al.*, 2010). Under sulfur deficiency, *Sultr1;1* was induced in roots of maize (Hopkins *et al.*, 2004). Moreover, a down-regulation in the *Sultr4;1* transcript level in roots under drought indicates the storage of sulfate in vacuole. These results reported here clearly demonstrate no or less transport to shoot that caused storage of sulfate in the vacuole that might also contribute to reduced flux into cysteine and GSH.

4.7 Reduced flux through the pathway is a consequence of low sulfate in leaves

The lower steady state level of sulfate and subsequently cysteine and GSH in leaves indicated sulfur starved condition. Thus, flux analysis can provide better explanations for the lower steady state levels of metabolites of the sulfur assimilation pathway. In order to unravel flux during drought stress, feeding experiment was performed with radioactive labeled sulfate ($^{35}SO_4^{2-}$) to monitor the incorporation rate of ^{35}S into cysteine and GSH. A strong reduction was observed in the rate of incorporation of radioactive labeled sulfate into cysteine and GSH in leaves under drought compared to control. This decrease in the incorporation of cysteine and GSH was about 65-80%. Furthermore, the same magnitude of reduction was also observed in the incorporation of ^{35}S into protein. The reduced flux through the pathway might explain lower steady state level of cysteine and GSH that is a direct consequence of the lower sulfate availability in shoot under drought stress. The data strongly suggests that enhanced formation of ROS in leaves reflects the serious impact of drought on the sulfur assimilation pathway by limiting the availability of sulfate from roots to shoots.

4.8 Drought creates a bottle-neck in the sulfur assimilation pathway in roots

A similar feeding experiment was performed in roots to monitor the incorporation rate of radioactive labeled sulfate ($^{35}SO_4^{2-}$) into cysteine and GSH. As observed in leaves, roots also showed a reduction of approximately 50-62% in the incorporation of ^{35}S into cysteine and GSH relative to control. The data strongly in contrast to the steady state level where an increase of approximately 1.72- to 1.82-fold in cysteine and 1.8-to 2.3-fold in GSH was observed. The incorporation of ^{35}S into protein revealed more or less the same reduction as recorded for thiol in roots. The basis for reduced flux and higher steady state level is not clear, although there could be some other signals that could alter the pattern of gene expression in roots during drought or there is also possibility that the reduced flux could be due to sulfate that accumulates in the vacuole as indicated by the down-regulation in *Sultr4;1*. Comparison of fluxes revealed down in both leaves and roots that is achieved by total different concentration of the sulfur related metabolites. This

indicates that non-sulfur signals are responsible in one tissue. In previous findings a down-regulation of gene encoding SAT3 using amiRNA approach reduced OAS biosynthesis by 80% in Arabidopsis. In this mutant, strongly reduced incorporation of ^{3}H-Ser into OAS, cysteine and GSH was observed despite elevated steady state levels of cysteine and GSH (Haas *et al.*, 2008). Recently in *sir1-1* mutant with 28% SiR activity compared to wild type, the incorporation of ^{35}S into cysteine and GSH was reduced by 28-fold although the steady state levels of cysteine was increased whereas those of GSH was maintained at wild type level (Khan *et al.*, 2010). The reduced flux and higher steady state level of thiols seem to be common observation between these mutants and drought stress.

4.9 Drought limits the transport of sulfate from root to shoot in maize

The use of radioactive tracers and incorporation of these elements into living systems provides a valuable tool in the study of uptake and transport in the biological sciences (McNaughton and Presland, 1983). One of the important approach to study root to shoot transport is the supply of $^{35}SO_4^{2-}$ to root system of hydroponically grown plants for a short time during which ^{35}S is incorporated into various endogenous sulfur pool (Sunarpi and Anderson, 1996). The redistribution and movement of ^{35}S into various parts of plants can then be detected by growing plants on the unlabeled sulfate for long time. Although this method is not useful especially working with drought stress since the supply of $^{35}SO_4^{2-}$ is not possible to plants grown on soil. Thus another approach was used by injecting labeled sulfate into stem for the investigation of transport rate from root to shoot as previously performed with ^{14}C sucrose in potato (Shekhar and Iritani, 1977). The uptake of labeled ^{13}N solution by root, transport through the stem and distribution to shoot was achieved within 2 min for nitrate and 5 min for ammonium in hydroponically grown maize seedlings (McNaughton and Presland, 1983). Thus one minute was evaluated as best time for the transport experiment. Approximately 1ul of labeled sulfate ($^{35}SO_4^{2-}$) was injected into the stem of control and drought stressed plants for one min and subsequent transport was investigated above the injection site. Analysis of $^{35}SO_4^{2-}$ (% of total) in each segment of stem revealed that 44%, (0-3cm), 23.7%, (3-6cm), 18.61%, (6-9cm) and 13.65%, (9-12cm) was observed in the control plants after 1 min of injection whereas 99.5% $^{35}SO_4^{2-}$ stayed in the first segment and only 0.5% was observed in the remaining stem segment. This clearly indicates that drought limits the availability of sulfate transport from root to shoot, thus causing lower steady state levels of many

metabolites, also reduced flux through the pathway and ultimately elevated levels of H_2O_2 production in leaves.

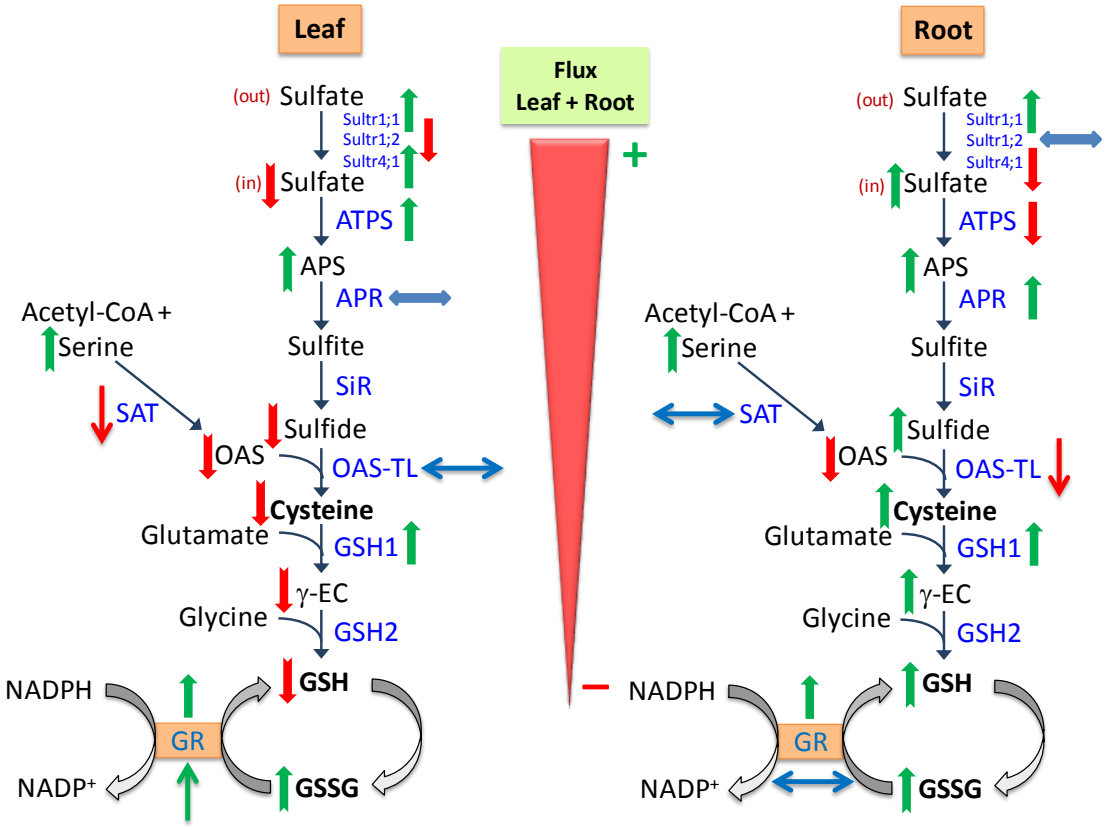

Fig. 50 Impact of drought on the expression of the primary sulfur metabolism-related genes, metabolites and enzymatic activities in maize

Differential expression of the genes, metabolites and enzymatic activities is indicated by the arrows pointing up or downward. The arrows pointing upward (green) represent significantly increased, whereas the arrows pointing downward (red) represent significantly decreased and arrows pointing on both sides (blue) represent significantly no changed in the leaves and roots of drought stressed plants compared to control.

Genes are indicated by the arrow shape ( ), metabolites ( ), enzymatic activities ( ) and flux ( ).

SULTR, sulfate transporters; ATPS, ATP sulfurylase; APS, adenosine 5'-phosphosulfate; APR, APS reductase; SiR, sulfite reductase; SAT, serine acetyltransferase; OAS-TL, O-acetyleserine(thiol)lyase; OAS, O-acetylserine; GSH, reduced glutathione; GSSG, oxidized glutathione; GSH1, γ-glutamylcysteine synthetase, and GSH2, glutathione synthetase, GR, glutathione reductase

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List of abbreviations

AcCoA	acetyl-coenzymeA
ADP	adenoseine diphospahte
APK	APS kinase
APR	adenosine-5'-phosphosulfate reductase
APS	adenosine 5'-phosphosulfate
APX	ascorbate peroxidase
A.th.	Arabidopsis thaliana
AT	Arabidopsis thaliana
ATP	adenosine triphosphate
ATPS	adenosine triphosphate sulfurylase
BASTA	glufosinate ammonium
BCIP	5-bromo-4-chloro-3-indolyl phosphate toluidin salt
Bp	basepairs
BSA	bovine serum albumin
BSCs	bundle sheath cells
BSO	L-buthionine-(S, R)-sulfoximine
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonte
Col-0	*Arabidopsis thaliana* ecotype Columbia-0
Cpm	counts per minute
CSC	cysteine synthase complex
Cys	cysteine
DAF	days after flowering
DEPC	diethylpyrocarbonate
DHA	dehydroascorbate
DHAR	dehydroascorbate reductase
DMSO	dimethyl sulfoxide
dNTP	deoxynucleotide solution mix
DTNB	5,5'dithiobis-(2-nitrobenzoic acid)
DMSO	dimethyl sulfoxide
DTT	1,4-dithiothreitol
DW	dry weight
EDTA	ethylenediamine tetraacetic acid
EGTA	glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
E_{GSH}	glutathione redox potential
FTR	ferredoxin dependent thioredoxin reductase
FW	fresh weight
GPX	glutathione peroxidase
GR	glutathione reductase
GRX	glutaredoxin
GSH	reduced glutathione
GSH1	γ-glutamylcysteine ligase
GSH2	glutathione synthetase
GSSG	glutathione oxidized, glutathione disulfide
GST	glutathione-S-transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
JA	jasmonate

kDa	kilo Dalton
Km	Michaelis constant
LC	liquid chromatography
MBB	monobromobimane
MCs	mesophyll cells
MDA	monodehydroascorbate
MDAR	monodehydroascorbate reductase
NEM	N-ethylmaleimide
NBT	nitro blue tetrazolium
NADPH	nicotinamide adenine dinucleotide phosphate
NTR	NADPH thioredoxin reductase
MOPS	3-(N-morpholino)propanesulfonic acid
NADPH	nicotinamide adenine dinucleotide phosphate
OAS	*O*-acetylserine
OAS-TL	*O*-acetylserine(thiol)lyase
OD	optical density
12-OH-JA	hydroxyjasmonic acid
PAGE	polyacrylamide gelelectrophoresis
PAP	3′-phosphoadenosine 5′-phosphate
PAPS	3′-phosphoadenosine 5′-phosphosulfate
Paraquat	N,N′-dimethyl-4,4′-bipyridinium dichloride or methylviologen
PEG	polyethylenglycol
PCR	polymerase chain reaction
PMS	phosphate buffered saline
PMSF	phenylmethanlsulphonylfluoride
PR	pathogenesis related
PVP	polyvinylpyrolidone
qRT-PCR	quantitative realtime polymerase chain reaction
ROS	reactive oxygen species
Rpm	round per minute
SAT	(serat) serine acetyltransferase
SDS	sodiumdedocylsulfate
SiR	sulfite reductase
SOD	superoxide dismutase
TAIR	The Arabidopsis information resource
TBS	tris buffered saline
TCA	trichloroacetic acid
T-DNA	transferred DNA used for insertional mutagenesis
TEMED	N,N,N',N'-Tetramethylethylenediamine
TES	N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic Acid
Tris	2-amino-2-(hydroxymethyl)propane-1,3-diol
TRX	thioredoxin
TrxR	thioredoxin reductase
TW	turgid weight
v/v	volume per volume
w/v	weight per volume
WT	wild-type
γ-EC	γ-glutamylcysteine

General Statement

I declare that I am the sole author of this submitted dissertation and that I did not make use of any sources or help apart from those specifically referred to. Experimental data or material collected from or produced by other persons is made easily identifiable.

I also declare that I did not apply for permission to enter the examination procedure at another institution and that the dissertation is neither presented to any other faculty, nor used in its current or any other form in another examination.

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