Effects of Overexpression of SAP12 and SAP13 in Providing Tolerance to Multiple Abiotic Stresses in Plants

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EFFECTS OF OVEREXPRESSION OF SAP12 AND SAP13 IN PROVIDING TOLERANCE TO MULTIPLE ABIOTIC STRESSES IN PLANTS

A Dissertation Presented
by
PARUL RANA TOMAR

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

Doctor Of Philosophy

September 2015

Plant and Soil Sciences
EFFECTS OF OVEREXPRESSION OF SAP12 AND SAP13 IN PROVIDING TOLERANCE TO MULTIPLE ABIOTIC STRESSES IN PLANTS

A Dissertation Presented

by

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ACKNOWLEDGMENT

First of all, I gratefully acknowledge the invaluable guidance and indispensable support provided by Dr. Om Parkash Dhankher, my research advisor. He generously spared his time as and when I required his advice on research problems. I feel deeply indebted to him for all the excellent opportunities, expert advice and ample support he provided.

The members of my advisory committee Dr. Om Parkash Dhankher, Dr. Samuel Hazen and Dr. Michelle DaCosta who evinced great deal of interest in my research and offered valuable suggestions, constructive criticism as and when they felt it appropriate. Besides this, they also allowed me to use their facilities whenever I needed. I thankfully acknowledge their contributions and support.

Many a times I had the opportunity to discuss research related scientific problems with my colleagues Chuanxin Ma, Hesham Abdullah, Kenny Ablordeppy, Parisa Akbari, Juliette Humer and Ryan Reynolds in the lab. I really owe affectionate thanks to all of them for the love, support and genuine advice they shared. Chuanxin Ma specifically had a great deal in advising and discussing the scientific problems. I would also like to thank my seniors Sudesh Chikkara, Bibin Paulose, Aniruddha Dixit, Kareem Mosa and Kundan Kumar who advised and guided me in my initial years of PhD.

I take this opportunity to express my gratitude to my department, Stockbridge School of Agriculture, for the financial support and scientific advice, without which it would have not been possible for me to render, take this programme.

I have profound regards and deep sense of gratitude for my parents, my husband Anand and his parents for all the unconditional love, kind support and care they blessed me with
during my degree. I could not have done this without their emotional and physical support. Also, my two-year-old son Agastya who was conceived and born during the course of my study has been a great stress buster and source of inspiration.
ABSTRACT

EFFECTS OF OVEREXPRESSION OF SAP12 AND SAP13 IN PROVIDING TOLERANCE TO MULTIPLE ABIOTIC STRESSES IN PLANTS.

SEPTEMBER 2015

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Environmental stresses are the one of the main reasons for the decline of crop production worldwide. In the past years, a major focus has been on improving plant species and their tolerance towards these stresses but not much has been achieved because of the limited knowledge of the gene/network of genes that might be involved in providing tolerance to such multiple abiotic stresses. Recently, members of Stress Associated Protein (SAP) family in plants have been shown to impart tolerance to multiple abiotic stresses. There are 14 SAP genes in Arabidopsis thaliana and these proteins contain A20, AN1 and C2H2 zinc finger domains. AtSAP13, a member of the SAP family, carries two AN1 zinc finger domains and an extra Cys2-His2 domain. AtSAP13 showed differentially regulation in response to multiple abiotic stresses such as toxic metals arsenic (As), cadmium (Cd), and zinc (Zn), drought, and salt. When overexpressed in Arabidopsis and Brassica juncea, it showed strong tolerance to these stresses. However, the mode of action of this SAP member in providing tolerance to multiple abiotic stresses is largely
unknown. *In-silico* analysis of the promoter sequences upstream of ATG start codon of *AtSAP13* using PLACE database predicted the presence of various abiotic stress related *cis* regulatory elements. We hypothesized that the expression of *AtSAP13* gene might be regulated via the interaction of *cis*-elements present in the *AtSAP13* promoter with abiotic stress related *trans* factors via protein-DNA interactions under different abiotic stresses. Through yeast one hybrid assay (Y1H), we have proved this hypothesis and identified several transcription factors such as DREB, ERE, ZIP, HSE etc that are interacting with the *AtSAP13* promoter. These interactions were analyzed through Electrophoretic mobility shift assay (EMSA) to understand the molecular and biochemical functioning of *AtSAP13*.

Further, *Camelina sativa*, a member of *Brassicaceae* family and closely related with Arabidopsis, has been proposed as an ideal biofuel crop. In order to improve it’s adaptability to wider geographical ranges and marginal land, we have characterized and overexpressed endogenous *SAP13* in *C. sativa* in providing tolerance to various stresses. We have identified and cloned *CsSAP13* in *C. sativa*. Resulting transgenic plants showed enhanced biomass and seed yield under multiple abiotic stresses. The knowledge and information gained here will not only be applied on agricultural crops that will be better able to withstand such abiotic stresses and still produce sustainable yield but will also help to grow crops for food and biomass production on barren lands, thus making them more cultivable over time. Therefore, the proposed research could have a significant impact on global food security, biofuel production, and human and environment health enhancement.
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CHAPTER 1
INTRODUCTION

1.1 Effects of abiotic stresses on plants

In a specific environment, variations in non-living factors can cause an adverse effect on living organisms and this phenomenon has been termed as an abiotic stress\(^1\). Drought, high salinity, extreme temperatures, and heavy metals and metalloids such as Arsenic (As), Cadmium (Cd), Nickel (Ni), Mercury (Hg), Zinc (Zn), Lead (Pb), Chromium (Cr), Copper (Cu), and Selenium (Se) are the various types of abiotic stresses, which together with biotic stresses severely affect the plant growth. Additionally, because of global climate change, the effects of the environmental stresses on crops will only worsen in the coming years. Global agricultural production and food security are thus at serious risk due to these stresses\(^2\). It has been estimated that a 30-50% decrease in crop production worldwide occurs due to abiotic stresses\(^3\). Agriculture alone consumes more than 70% of the fresh water consumption, which is the major reason for the decline in the underground water table. Other than that, abiotic stresses such as salinity, heat and cold induce water deficiency leading to a decrease in the available ground water. With the decrease in water table, metals that are naturally found in underground water increase in concentration, finally entering the food chain affecting human health\(^3\).

Salts are naturally present in all soil types, most of which are essential plant nutrients. However, when concentration of soluble salts such as sodium chloride (NaCl) increase in soil and water, salinity (salt stress) occurs\(^{111}\). Salt concentration in soil
increases because of human intervention with activities such as mining, irrigation and also due to natural processes like inadequate rainfall and/or weathering of mineral rocks such as in arid or semi-arid areas. World's agricultural land of about 7% is affected by salinity in soil and water. On an average, 30% of the agricultural land irrigated with salt concentrated water is considered economically unproductive. Inside a plant, higher salt concentration can cause various stresses such as hyperosmotic, oxidative and hyperionic largely due to the increased accumulation of sodium (Na\(^+\)) ions. Elevation of Na\(^+\) to toxic levels in plant leaves leads to membrane damage, production of reactive oxygen species (ROS), and photosynthesis inhibition, further leading to cell death which ultimately affects the crop yield. To avoid oxidative damage, plants use three genes – \textit{SOS1}, \textit{SOS2} and \textit{SOS3} of the Salt Overly Sensitive (SOS) pathway to control the transport of excess Na\(^+\) into the vacuoles.

Plant tissues maintain gradients of water potential favorable for water influx. Upon water stress, osmotic potential often decreases in plant tissues leading to a decrease in turgor pressure which further halts cell expansion and division, decreasing photosynthesis, translocation of nutrients, respiration and other mechanisms. Severe water stress can lead to decrease in leaf’s relative water content (RWC), decrease in root length causing plants to wilt permanently, shutting down of metabolic processes eventually leading to plant death. Under osmotic stress, Plants perceive drought and trigger the production of phytohormone abscisic acid (ABA), which shuts down the stomata to minimize water usage and reduces gas exchange. Upon production of ABA, ABA-independent and ABA-dependent regulatory systems in the plant activate stress
ABA-responsive genes\textsuperscript{9}. ABA-responsive gene expression is regulated by a number of known transcription factors such as DREB2A/2B, AREB1, RD22BP1 and MYC/MYB\textsuperscript{10,11,12}. In response to water stress, plants also accumulate osmolytes in the cytosol, which are low molecular weight osmotically active compounds. Simple or complex sugars, polyols, inositols, sugar alcohols etc are all different types of osmolytes. Presence of osmolytes in the cell cytosol helps decrease the osmotic potential by a net accumulation of cellular solutes. This process is called osmotic adjustment (OA)\textsuperscript{1}. The main role of osmotic adjustment is to maintain appropriate turgor pressure during water deficits, which in turn is essential for maintenance of turgor-related processes such as stomatal regulation\textsuperscript{1}.

Apart from drought and salinity, heavy metals and metalloids mentioned above are inorganic pollutants and are present as positive or negatively charged ions in the soil\textsuperscript{13}. They are termed as heavy metals because of their density higher than 5 g cm\textsuperscript{-3}\textsuperscript{14}. Some out of these have biological importance such as iron (Fe), molybdenum (Mo) and manganese (Mn). They are classified as important micronutrients. Others such as Zn, Ni, Cr, Co, and Cu can be toxic in higher concentration. Heavy metals and metalloids like As, Hg, Cd, and Pb are considered toxic to plants, animals and microorganisms\textsuperscript{15,16,17}. These heavy metal pollutants, originate from mining activities, industrial effluents and wastes, sewage sludge, pesticides use, city waste etc. and affect the ecosystem\textsuperscript{18,19}.

Among the naturally existing heavy metals in soil and water, As which is present in various concentrations and forms, poses a big threat to crop production, human and animal health. It is a carcinogen and has been shown to cause major health problems such
as cancer of the liver, kidney and lungs. Cadmium easily enters the food chain affecting human health when absorbed and transported through various calcium and iron transporters. Sulfhydryl homeostasis and enzyme inhibition have been seen in animal and mammalian cells due to Cd. Cadmium is known to cause health problems such as renal tubular disease. Lead enters the plant mostly through apoplastic pathway or via Ca\(^+\)-permeable channels in the roots that are exposed to the contaminant. High concentration of Pb accumulation in plant cells disrupts various physiological, morphological and biochemical functions such as cell membrane and DNA damage, lipid peroxidation, ROS production, seed germination, plant growth, photosynthesis, ATP production inhibition and many more. Lead can damage the developing brain and nervous system in humans especially in young children. It can also cause vision and hearing loss, reproductive failure in adults along with many more side effects (http://www.epa.gov/superfund/lead/health.htm).

Mercury has strong affinity for thiol group and nitrogen ligands and thus gets absorbed through various ionic channels binding to the cell walls and membranes of plants and microorganisms. Mercury also impairs neurological function and development especially in fetuses and young children along with damage to cognitive thinking, language, vision impairment, hearing loss along with other detrimental health effects.

Since all these above mentioned stresses go hand in hand, it has thus become absolutely essential to understand the responses of plant to such abiotic stress. Plants under such stresses induce various biochemical and physiological changes. In order to tolerate these stresses and survive, at the molecular level, plants perceive the stress and
then transduce the signal, enabling the production of various regulatory elements upstream of stress-inducible genes such as transcription factors (TFs). These series of events lead to up or down regulation of stress responsive genes which further lead to production of stress protective proteins\textsuperscript{20,30,31}. At physiological levels, these stresses cause cellular damage by either binding to the sulfhydryl groups of various enzymes and proteins such as TFs or by inducing oxidative stress such as lipid peroxidation through production of ROS and free radicals\textsuperscript{32,33}. In response to ROS production, plants produce antioxidants such as tocopherols, glutathione, and ascorbate, which can reduce ROS such as superoxide anions (O$_2^-$) to a less harmful state by accepting electrons from them\textsuperscript{34}. They also produce antioxidant enzymes such as peroxidases (POD), catalases (CAT), ascorbate peroxidases (APX) and superoxide dismutases (SOD) as a defense mechanism. SOD is capable of converting O$_2^-$ to hydrogen peroxide (H$_2$O$_2$) and other enzymes such as CAT, APX, etc. and POD then converts H$_2$O$_2$ into H$_2$O and O$_2$\textsuperscript{35,36}.

Detoxification of metals and metalloids in non-hypertolerant plants can be achieved with the help of certain ligands such as thiols in glutathione (GSH) and Phytochelatins (PCs). Highly reactive specie of arsenite (AsIII) and Cd$^{2+}$ show great affinity for these low molecular weight chelators\textsuperscript{37}. When absorbed by the plant, As in the form of arsenate (AsV) as phosphate analog is first reduced to a more toxic form AsIII by the plants followed by binding with peptides with thiol reactive groups such as $\gamma$-glutamylcysteine ($\gamma$-EC), glutathione (GSH), and phytochelatins (PC)\textsuperscript{38,39,40,41}. Once bound, the metal peptide complexes can be further sequestered into plant vacuoles through glutathione-conjugating pumps (GCPs)\textsuperscript{42,43,44,45,46}. Recently, two vacuolar PC
transporters AtABCC1 and AtABCC2 in Arabidopsis, were shown to play a role in the tolerance and transport of PC bound metalloids such as As(III)-PC into the vacuole\textsuperscript{47}. GSH (Glu-Cys-Gly), which is a tripeptide, is synthesized by gamma-glutamylcysteine synthetase (\(\gamma\)-ECS) and glutathione synthetase (GS) whereas enzyme phytochelatin synthetase (PCS) synthesizes phytochelatins\textsuperscript{38}.

1.2 Arsenite-Inducible RNA-Associated Proteins and Stress-Associated Protein Family

Among the various heavy metals and metalloids mentioned above, As poses a big threat to plants, animals and humans around the world. It is a carcinogen and has been shown to cause major health problems including but not limiting to cancer of the liver, kidney and lungs\textsuperscript{28}. High levels of As have been reported in the underground drinking water and food crops such as rice (\textit{Oryza sativa}) grown in many parts of southeast Asia especially India and Bangladesh where this staple food is widely irrigated using As contaminated ground water subjecting millions of people to arsenic poisoning risk\textsuperscript{48}. Multiple stress pathways get activated once a cell has been exposed to As stress. As a result many unknown genes, along with known stress associated genes, get induced whose role in defending against As stress is poorly understood.

Genes encoding Arsenite-inducible RNA associated proteins (AIRAPs), which are highly cysteine- and histidine-rich, were first identified in \textit{Caenorhabditis elegans} and mouse cells. These AIRAPs has been shown to protect cells against the toxicity caused by AsIII\textsuperscript{49}. Stresses such as the As toxicity cause proteins to mis-fold, leading to an increase in polyubiquitylation and finally allowing aggregation in the cells\textsuperscript{50,51,52}. Stanhill \textit{et al.}\textsuperscript{53} showed that the AIRAP’s protect the cells such as those of \textit{C. elegans} and
mouse cells against such toxicities by associating tightly with the 19S cap of the 26S proteasome altering their biochemical properties and increasing the breakdown of the aggregated polyubiquitinated proteins. Thus AIRAP has been shown to counteract stress-induced proteotoxicity by adapting to the cell’s core protein degradation machinery\textsuperscript{53}.

Using the BLAST algorithm, four AIRAP homologous proteins with unknown functions were identified from the \textit{Arabidopsis} protein database\textsuperscript{54}. These AIRAPs were recently designated as Stress-Associated Proteins (SAPs) and they are differentially regulated by various abiotic stresses in plants\textsuperscript{48,55,56,57}. On overexpression in tobacco, one of these genes from rice, \textit{OsiSAP1}, carrying zinc finger (ZnF) domains showed tolerance to stresses such as cold, salt, and dehydration\textsuperscript{48}.

While searching for the paralogs and orthologs of the newly identified SAP, 14 and 18 genes were identified in Arabidopsis and rice genomes respectively, encoding hypothetical proteins whose functions were unknown\textsuperscript{56}. These homologous proteins were shown to carry A20 and/or AN1 ZnF domains. Primary response ZnF protein gene A20 was first identified in human umbilical vein endothelial cells as a gene that gets induced by cytokine\textsuperscript{58}.

Presence of seven Cys2/Cys2 finger motifs at the C-terminus is a typical characteristic of the A20 ZnF domain\textsuperscript{59} whereas; presence of multiple Cys2-His2 finger motifs is a characteristic of the AN1 ZnF domain. Specific arrangements of the Cys- and His-residues in these ZnF domains also gives rise to typical metal-binding domains\textsuperscript{59}.

During the past few years, several SAP proteins have been characterized from different systems carrying either the A20/AN1 domains or even both. At the C-terminal,
extra Cys2-His2 domains has also been found in some SAPs. The interactions between these ZnF domains have been shown to play a role in regulating the immune responses when plants are under various abiotic stress conditions\textsuperscript{56,58,60,61,62,63}.

Using phylogenetic and computational methods, Jin et al\textsuperscript{64} divided the ZnF AN1 genes from \textit{A. thaliana}, \textit{Populus trichocharpa}, \textit{O. sativa}, \textit{Chlamydomonas reinhardtii} and \textit{Zea mays} into two types. Type I members had one of each A20 ZnF domain and one AN1 ZnF domain on the N and C-terminus, respectively. Type II members had two AN1 ZnF domains on N-terminus and one/two Cys2-His2 type ZnF domains on their C-terminus. Out of these, SAPs from Arabidopsis were shown to carry the following ZnF domains (Figure 1.1).

\textbf{Figure 1.1} Arrangement of the ZnF domains of Arabidopsis SAP family members. (Adapted from Vij and Tyagi, 2006 and Jin et al, 2007). Not drawn to the scale

Recently, several SAP genes were identified and characterized form different plant species. One such SAP gene from rice namely \textit{ZFP177/OsSAP9} (subsp. japonica) carrying A20/AN1 ZnF domains showed an increased tolerance to H\textsubscript{2}O\textsubscript{2} when overexpressed in tobacco. Greater tolerance to stresses caused due to high and low

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\textsuperscript{56,58,60,61,62,63}
temperature was also observed in these transgenic plants. However, when overexpressed, an increased sensitivity to dehydration and salt stress was also seen\textsuperscript{65}. Another SAP family gene from rice known as \textit{OsiSAP8} was characterized as a single copy gene with an A20 ZnF domain at its N-terminus and an AN1 ZnF domain at its C-terminus\textsuperscript{55}. OsiSAP8 was induced under stresses like heavy metals, cold, submergence, drought, and salinity along with hormones such as ABA in homologous system such as \textit{O. sativa}. Under these abiotic stresses, it was also induced in a heterologous system such as \textit{Nicotiana benthamiana}\textsuperscript{55}. Just like \textit{OsiSAPI}, it was thought to play a role in the initial stages of stress response signal transduction through A20/AN1 protein-protein interactions. A yeast two-hybrid analysis also showed that the A20 domain interacts with itself as well as with the AN1 domain but there is no interaction of the AN1 domain with itself\textsuperscript{55}.

\textit{OsDOG} (\textit{O. sativa} dwarf rice with overexpression of gibberellin-induced gene) is another A20/AN1 ZnF protein. It was named so based on the phenotype that appeared in the transgenic rice lines and played a novel role in plant growth regulation\textsuperscript{66}. This gene negatively regulated GA-mediated cell elongation in rice. \textit{OsDOG} contain one A20-type ZnF at its N terminus and one AN1-type ZnF at its C terminus. Any defect or mutation associated with GA synthesizing enzymes or affecting its signaling pathway results in a typical dwarf phenotype\textsuperscript{67}. On supplementing GA, the expression of OsDOG increased in rice whereas when treated with paclobutrazol (PAC), which is a GA synthesis inhibitor, the mRNA level of OsDOG decreased. Transgenic lines of rice overexpressing OsDOG
showed a dwarf phenotype, whereas the RNAi lines were quite similar to wild type (WT)\textsuperscript{66}.

Overexpression of a halophyte grass \textit{Aeluropus littoralis} stress associated protein; A\textsc{i}SAP, which contains an A20 and an AN1 conserved domains, in tobacco showed increased tolerance to salt, drought, osmotic, cold, and heat stress\textsuperscript{68}. It was also reported that in the A\textsc{i}SAP transgenic lines, transcripts of eight stress-induced genes were also high compared to wild type. Recently, \textit{Medicago truncatula} stress associated protein (\textit{MtSAP1}) isolated from the seed embryos, was also shown to contain an A20 and an AN1 domain. It was demonstrated that in the embryo axis, \textit{MtSAP1} gene gets expressed and accumulates proteins under cold and hypoxia, ABA and during desiccation but was down regulated during imbibition\textsuperscript{69}. During late stages of seed maturation, tolerance to low oxygen availability and desiccation provided by Mt\textsc{i}AP\textsc{1} was also confirmed. RNAi inactivation of Mt\textsc{i}AP\textsc{1} in transgenic lines showed lost expression of Mt\textsc{i}AP\textsc{1}, confirming that Mt\textsc{i}AP\textsc{1} was required for embryo development and for storage globulin proteins, vicilin and legumin accumulation\textsuperscript{69}.

\textit{AtSAP5}, another such member of the Arabidopsis SAP family proteins, codes for a protein that contains both A20 and AN1 ZnF motifs and has been shown to have E3 ubiquitin ligase activity through an \textit{in vitro} E3 ubiquitin ligase activity assay\textsuperscript{70}. Through this assay, it was demonstrated that \textit{AtSAP5} AN1 ZnF domain was found to be enough for \textit{in vitro} E3 ligase activity, whereas A20 domain was found capable of auto-ubiquitination\textsuperscript{70}. It has been shown further that \textit{AtSAP5} when expressed ectopically in
cotton not only improved its carbon gain and productivity by providing tolerance against heat and drought stress but also increased the viability of the leaf and protection of the seedling growth against these stresses. In these transgenic cotton plants, expressions of four genes normally induced during drought and heat stresses were seen to be high even under non-stressed conditions. After four days of drought stress, photosystem (PS) II complexes were shown to be protected against photodamage in the AtSAP5 expressed cotton as compared to wild type plants\(^71\).

Recently, \textit{AtSAP10}, carrying an A20 and an AN1 domain at the N- and C-terminal respectively was also characterized\(^57\). AtSAP10 showed differential expression under heavy metals, drought, salt and heat stresses. When overexpressed, AtSAP10 provided tolerance to high temperature and heavy metals such as Ni, Mn and Zn and also showed to accumulate significant concentrations of Ni and Mn in both roots and shoots\(^57\). Further, \textit{AtSAP12} under salt and cold stress was also shown to be upregulated within six hours of treatment\(^72\).

Another Arabidopsis SAP, namely SAP11, was also shown to provide tolerance to heavy metals. Transcript levels of AtSAP11 were differentially regulated with several fold increase in transcripts seen in response to AsV, AsIII, Cd, Zn and drought treatment. Transcript levels were seen to go down in shoots on providing salt and heat stress, and in both roots and shoots on providing cold stress. Moderate to enhanced tolerance of AtSAP11 overexpression lines was also seen on AsIII, Zn and PEG-8000 treatments\(^109\).

1.3 Project Aims
In the recent past years, a lot of focus has been on improving plant species and their tolerance towards these stresses but not much has been achieved because of the limited knowledge of the gene/network of genes that might be involved in providing such tolerance to multiple stresses. With global warming along with the increase in world population, predicted decline in available irrigation water, increasing need for biofuel production and decrease in arable land due to activities such as non-sustainable farming, soil erosion, urbanization and others, it has become utmost important to gain further knowledge and information on SAPs and their mechanism to provide tolerance to abiotic stresses. The knowledge will not only be applied on agricultural crops to engineer crops that will be better able to withstand such abiotic stresses and still produce sustainable yield but will also help to grow crops for food and biomass production on barren uncultivable lands. Therefore, the proposed research could have a significant impact on global food security, human health enhancement and the environment.\textsuperscript{73,74,75}

\textit{AtSAP13} (At3G57480) carrying two AN1 domains and an extra Cys2-His2 domain is shown to be induced in response to multiple abiotic stresses such as toxic metals (As, Cd, Zn), drought, and salt.\textsuperscript{56} However, the mode of action of this SAP member in providing tolerance to multiple abiotic stresses is largely unknown. \textit{In silico} analysis of the \textit{AtSAP13} promoter sequence predicted the presence of various abiotic stress related \textit{cis} regulatory elements. We hypothesized that the expression of \textit{AtSAP13} might be regulated via the interaction of \textit{cis}-elements present in the \textit{AtSAP13} promoter with abiotic stress related trans factors via protein-DNA interactions under different abiotic stresses. Our main objective is to understand the molecular and biochemical
functioning of AtSAP13 that will ultimately lead to understand in general the SAPs biochemical and molecular mechanism of tolerance to multiple abiotic stresses. Further, C. sativa, a member of Brassicaceae family and closely related with Arabidopsis, has been proposed as an ideal biofuel crop\textsuperscript{76,77}. In order to improve adaptability to wider geographical ranges and marginal land, we aim to characterize and overexpress endogenous SAP13 in C. sativa in providing tolerance to various stresses. We have identified and cloned CsSAP13 in C. sativa. Resulting transgenic plants will be analyzed for enhanced biomass; seed and oil yield under multiple abiotic stresses.

In order to understand more about plant SAP function, I propose the following specific aims:

**Specific Aims**

1. Characterization and mode of action of AtSAP13 in providing tolerance to abiotic stresses.
   
   1.1. Overexpression of AtSAP13 in Arabidopsis.
   
   1.2. Inactivation of AtSAP13 and AtSAP13AN1 in Arabidopsis using RNAi approach.
   
   1.3. Y1H assay for analysis of AtSAP13 promoter interactions with abiotic stresses related TFs.

2. Confirming the overexpression and RNAi knockdown of AtSAP12 and analysis of transgenic plants for various heavy metal stress tolerance.
   
   2.1. Overexpression of AtSAP12 in Arabidopsis.
   
   2.2. Inactivation of AtSAP12 in Arabidopsis using RNAi approach.
3. Overexpression of *AtSAP13* in other crops (*B. juncea*) for enhancing tolerance to multiple abiotic stresses.

CHAPTER 2
MATERIALS AND METHODS

2.1 Plant materials and growth conditions

2.1.1 Arabidopsis plants grown in plates

*A. thaliana* ecotype Columbia wild type and transgenic seeds were surface sterilized first for five minutes with 70% (v/v) ethanol, then by two separate washes with 30% (v/v) sodium hypochlorite (The Clorox Company, Oakland, CA) each lasting for 15 minutes followed by five minutes each of five washes with deionized autoclaved water. Solid media containing half strength Murashige and Skoog (MS) medium with vitamins (Murashige & Skoog, 1962) (PhytoTechnology Laboratories, KS, USA), supplemented with 0.8% w/v Phytoblend agar (Caisson Laboratories, UT, USA), and 1% w/v sucrose was prepared, pH of the media was adjusted to 5.7 before autoclaving and then poured onto petri plates. Sterilized seeds were placed on these plates, vernalized at 4°C for 24 hours and then transferred to a growth chamber (cycling 16 hours/8 hours, day/night period at 22°C and 18°C, respectively). Plates were first grown horizontally till seeds start to germinate and then grown vertically for 21 days.

2.2 Abiotic stress treatments

2.2.1 Growth and analysis of Arabidopsis plants on As, Cd, Zn, salt and drought

Sterilized seeds of wild type and transgenic lines were placed on half strength MS plates containing previously optimized 30 µM sodium arsenite (NaAsO₂), 75 µM cadmium chloride (CdCl₂), 500 µM zinc sulfate (ZnSO₄) along with no metal controls. Plants were
grown for 3-weeks and the phenotype were analyzed for root length and shoot biomass. The experiment for each set up was at least repeated twice and the data has been included in the appendix section of the document.

For drought and salt stress, plants were subjected to drought and salt stress by supplementing half strength MS media petriplates with D-Mannitol (Sigma Aldrich) and sodium chloride (Fisher Scientific), respectively, at a concentration of 100mM each. D-mannitol, which is an osmolyte, (normally produced in plants under drought stress) mimics drought conditions by lowering the water potential of the media, making it harder for the plants to absorb water. Water potential for 100mM mannitol was -0.25MPa calculated using van’t Hoff equation ($\pi = I \times M \times R \times T$) where $\pi$ is media’s osmotic potential, $i$ is the van’t Hoff’s factor for solute (1 for mannitol), $M$ is the solution’s molarity, $R$ is the gas constant ($=0.0083$ MPa g/L$^{-1}$ K$^{-1}$), $T$ is the temperature in Kelvin (298K)$^{116}$. Electrical conductivity (EC) for 100mM NaCl concentration was 9.8 dS/m$^{117}$. Plates were kept horizontal for the first five-six days till the seeds start to germinate and then were arranged vertically and allowed to grow for 21 days in a growth chamber set at 16 hours/8 hours, day/night period at 22°C and 18°C, respectively. After 3 weeks, Plants were harvested, weighed, and their root lengths were measured. The experiment for each set up was at least repeated twice and the data has been included in the appendix section of the document.

2.2.2 Growth and analysis of Brassica plants on As, Cd, Zn, drought and salt

*B. juncea* wild type and transgenic seeds were surface sterilized first for five minutes with 70% (v/v) ethanol, then by one 10 minute wash with 0.2% (v/v) silver nitrate
(Fisher Scientific) followed by five minutes each of five washes with deionized autoclaved water. Solid media containing half strength MS medium with vitamins (Murashige & Skoog, 1962) (PhytoTechnology Laboratories, KS, USA), supplemented with 0.8% w/v Phytoblend agar (Caisson Laboratories, UT, USA), and 2% w/v sucrose was prepared, pH of the media was adjusted to 5.7 and autoclaved. Optimal concentrations of metals, NaCl and D-mannitol were determined subjecting WT Brassica plants to a range of lower and higher concentrations and selection of concentration was done based on growth of the plants and toxicity effects. Media was then supplemented with optimized 70 µM NaAsO$_2$, 100 µM CdCl$_2$, 500 µM ZnSO$_4$ before pouring onto magenta boxes along with no metal controls. Plants were also exposed to short-term drought and salt stress by supplementing half strength MS media in the magenta boxes with 200 mM D-mannitol and 150 mM sodium chloride respectively. Sterilized seeds, 15 of each line were then germinated in the boxes with four replicates and transferred to a growth chamber (16 hours/8 hours, day/night period at 22°C and 18°C, respectively). Plants were grown for a period of 21 days after which germination rate was calculated, shoot and root biomass tissues were harvested separately, weighed and analyzed. The experiment for each set up was at least repeated twice and the data has been included in the appendix section of the document.

### 2.2.3 Growth and analysis of Camelina plants on As, Cd, Zn, and salt

*C. sativa* wild type and transgenic seeds were surface sterilized first for 10 minutes with 70% (v/v) ethanol, then by two separate washes with 25% (v/v) sodium hypochlorite (The Clorox Company, Oakland, CA) each lasting for 15 minutes followed by five minutes each of five washes with deionized autoclaved water. Solid media containing
half strength MS medium with vitamins (Murashige & Skoog, 1962) (PhytoTechnology Laboratories, KS, USA), supplemented with 0.8% w/v Phytoblend agar (Caisson Laboratories, UT, USA), and 3% w/v sucrose was prepared, pH of the media was adjusted to 5.7 and autoclaved next. For toxic metal stress, various concentrations of metals, 25 µM NaAsO$_2$, 75 µM CdCl$_2$, 500 µM ZnSO$_4$ was added before pouring onto magenta boxes. Plants were also exposed to short term salt stress by supplementing half strength MS media in the magenta boxes with 150 mM sodium chloride. 15 sterilized seeds of each line were then germinated in the boxes with four replicates and transferred to a growth chamber (16 hours/8 hours, day/night period at 22°C and 18°C, respectively). Plants were grown for a period of 21 days after which germination rate was calculated and shoot and root biomass tissue weight were analyzed separately.

2.3 Metal uptake and accumulation study

Followed by germination and growth on 1/2X MS solid media plates, 14 days old wild type Arabidopsis seedlings were transferred to vermiculite and Brassica and Camelina were germinated directly in the vermiculite. Plants were grown for 14 days in the greenhouse temperature conditions set at 25 °C, 74% relative humidity and 16 hours/8 hours, day/night period) After 14 days, roots of the plants were cleaned with tap water to remove all the vermiculite and then placed in glass bottles with 100 ml half strength Hoagland’s solution (1.63 gms of Hoagland’s powder purchased from PhytoTechnology Laboratories dissolved in 1 L deionized water, pH 5.7). Flat glass containers containing 600 ml of half strength Hoagland’s solution were used for Arabidopsis metal uptake study instead of glass bottles. Holes were drilled in the lids of the glass bottles and glass containers to put plants in each hole with shoot staying up and roots dipping in the media.
Plants were acclimatized for a period of seven days. After seven days, old hydroponic solution was replaced by the fresh solution. Next, plants were treated with As, Cd and Zn (at concentrations mentioned above) carrying hydroponic solution along with control treatment with no metals for a total period of five days. At the end of five days, root and shoot tissues were harvested, washed thoroughly with deionized water and dried in the oven at 65°C for three days and digested with nitric acid (HNO₃) for one hour at 115°C Celsius heating digestion block. Digested samples were diluted and were analyzed by inductively coupled plasma mass spectroscopy (ICP-MS, Agilent 7500ce).

2.4 Long-term abiotic stress treatments

2.4.1 Long-term drought stress treatment in crop plants

2.4.1.1 Drought stress in Brassica

Plants were subjected to drought stress in the greenhouse. Transgenic and wild type *B. juncea* seeds were grown directly in vermiculite for 14 days in the greenhouse at 25 °C, 74% relative humidity and 16 hours/8 hours, day/night period. After 14 days, four replicates each of wild type and two transgenic lines were transferred to 8 inches sand pots mixed with 25% garden soil and labeled as ‘control’ and ‘drought’ treatment. Plants were allowed to acclimatize for another 10 days during which all control and treatment pots were watered well (250 mls). Complete randomized block design (CRBD) was used to separate the replicates of each line under each treatment in blocks, with treatments assigned at random within blocks. Fertilizer was applied to all the pots once/week. Treatment was then started where pots labeled ‘control’ were watered with 250 mls of water on need basis, however, watering of the pots labeled ‘drought’ was withheld till
drought symptoms starting appearing and water withholding was continued for a period of 10 days. At the severe stage of drought (around 7\textsuperscript{th} day of water withholding), net photosynthetic assimilation, transpiration rate and stomatal conductance were measured. ‘Drought’ treatment plant pots were re-watered with 75 ml water after implying 10 days of drought stress to look at recovery of the WT and transgenic plants. Net photosynthetic assimilation, transpiration rate and stomatal conductance were again measured around fifth day of recovery. ‘Control’ pots were watered with 250 mls of water and ‘drought’ labeled pots were watered with only 75 mls of water on every second day till they matured. At maturity, shoots were harvested and number of seed pods/plant, number of seeds in pods, total seed weight and total shoot biomass was calculated and analyzed through one-way analysis of variance (one-way ANOVA) followed by least significant difference (LSD) test.

2.4.1.2 Drought stress in Camelina

This study was again carried out in the greenhouse. Transgenic and wild type camelina seeds were grown directly in vermiculite for 14 days in the greenhouse at 25 °C, 74% relative humidity and 16 hours/8 hours, day/night period. After 14 days, five replicates each of wild type and two transgenic lines were transferred to 8 inches sand pots mixed with 25% garden soil. Plants were allowed to acclimatize for another 10 days during which all pots were watered well (250 mls). Complete randomized block design was used to separate the replicates of each line under each treatment in blocks, with treatments assigned at random within blocks. Fertilizer was applied to all the pots once/week. Following the experimental plan designed by Dr. DaCosta/Dr. Paulose (contact directly for personal communications) five pots were labeled as ‘D1’, which served as the control
in this experiment and were well watered with 250 mls of water. Five pots were labeled as ‘D2’, which were watered with reduced amount of 150 mls and five pots were labeled as ‘D3’, which served as the drought treatment and were watered with only 75 mls of water during the course of this study. D1, D2 and D3 labeled pots were watered with 250mls, 150mls and 75mls of water every two days for the first four weeks and then every alternate day. At the moderate and severe stages of drought, net photosynthetic assimilation, transpiration rate and stomatal conductance were measured. Plants were grown till the end of their life cycle at which shoots were harvested and number of seed pods per plant, number of seeds in pods, total seed weight and total shoot biomass was calculated and analyzed through one-way ANOVA followed by LSD.

2.4.2 Long term salt stress treatment in crop plants

2.4.2.1 Salt stress in Brassica

For salt stress, Brassica plants were grown in the 8-inch pots in the greenhouse. Transgenic and wild type *B. juncea* seeds were grown directly in vermiculite for 14 days in the greenhouse at 25 °C, 74% Relative humidity and 16 hours/8 hours, day/night period. After 14 days, four replicates each of wild type and two transgenic lines were transferred to sand pots mixed with 25% garden soil and labeled as ‘control’ and for ‘salt’ treatment. Complete randomized block design was used to separate the replicates of each line under each treatment in blocks, with treatments assigned at random within blocks. Plants were allowed to acclimatize for another 10 days during which all control and treatment pots were watered well (250 mls). Fertilizer was applied to all the pots.
once/week. Treatment was then started where ‘control’ labeled pots were well watered (250mls) however ‘salt’ labeled treatment pots were watered with 250mls of water containing 150mM dissolved NaCl. Pots were watered with regular water and 150mM salt water on need basis, starting with every couple of days when the plants were young and small, followed by every alternate day once they grew bigger. At the moderate and severe stages of salt stress, net photosynthetic assimilation, transpiration rate and stomatal conductance were measured. All the pots were continued to water with 250 mls of regular water in control pots and 250 mls of 150mM salt water in the treatment pots till they matured. At maturity, shoots were harvested and number of seed pods/plant, number of seeds in pods, total seed weight and total shoot biomass was calculated and analyzed through one-way ANOVA followed by LSD.

2.4.2.2 Salt stress in Camelina

Long-term salt stress study for C. sativa was also carried out in the pots in the greenhouse. Transgenic and wild type Camelina seeds were grown directly in vermiculite for 14 days in the greenhouse at 25 °C, 74% Relative humidity and 16 hours/8 hours, day/night period. After 14 days, five replicates each of wild type and two transgenic lines were transferred to sand pots mixed with 25% garden soil and labeled as ‘S1’ (for 100mM salt treatment) and ‘S2’ (for 150mM salt treatment). Complete randomized block design was used to separate the replicates of each line under each treatment in blocks, with treatments assigned at random within blocks. Plants were allowed to acclimatize for another 10 days during which all control and treatment pots were watered well (250 mls). Fertilizer was applied to all the pots once/week. D1 labeled pots from the drought stress study served as the controls and watered with 250 mls of regular water and S1 and S2
treatment pots were watered with 250mls of water containing 100mM and 150mM NaCl concentrations, respectively every two days for the first four weeks and then every alternate day. At the moderate (2\textsuperscript{nd} week of salt treatment) and severe stages (4\textsuperscript{th} week of salt treatment) of salt stress, net photosynthetic assimilation, transpiration rate and stomatal conductance were measured. Plants were grown till the end of their life cycle at which shoots were harvested and number of seed pods per plant; number of seeds in pods, total seed weight and total shoot biomass was calculated and analyzed through one-way ANOVA followed by LSD.

2.5 Physiological analysis

2.5.1 Measurement of net photosynthetic assimilation, stomatal conductance, and transpiration rate

Infrared gas exchange analyser Li-Cor 6400 (Li-Cor, Inc., Lincoln, NE) was used to measure the movement of CO2 and water on a portion of a leaf enclosed in a broadleaf chamber where the CO2 concentration (400 umol/mole), humidity (above 50%), flow rate (200 umol) and light intensity (750 umol) were controlled. The 5\textsuperscript{th} or 6\textsuperscript{th} broad and mature leaf from top was used and the instrument calculated the net rates of photosynthetic assimilation, transpiration and stomatal conductance. Measurements were performed on five replicates per treatment and were taken once per week between 10 am to 2 pm at moderate and severe stress conditions.
2.5.2 Relative water content (RWC)

To measure the hydration status of the control and treatment plant leaves, Barrs and Weatherly’s (1962) protocol\textsuperscript{78} (with minimal changes) to calculate RWC was used as an indicator. Approximately 50 mgs of fresh mature leaf was excised from the plant and immediately weighed (fresh weight) followed by immersion in water in a closed petri plate. After 24 hours, leaf sample was taken out of water, blotted dry and weighed again (turgid weight). Leaf samples were then dried for at least three days in an incubator set at 65°C. After three days, leaf samples were weighed again (dry weight). Formula: \((FW–DW)/(TW–DW)\times 100\) was used to calculate RWC.

2.5.3 Chlorophyll Measurement

Lichtenthaler method\textsuperscript{79} of quantifying chlorophyll was modified slightly to estimate chlorophyll content in our samples. Approximately, 50 mgs of fresh and healthy leaf tissue was cut and immediately immersed in 10 mls of 90% (v/v) ethanol. Samples were covered with aluminum foil to protect against light and kept in dark for three days. On the 3\textsuperscript{rd} day, absorbance of supernatant was measured at 663 and 645 nm by UV through spectrophotometer (Spectronic Genesis 2, ThermoFisher Scientific). Chlorophyll content was determined using the following equations, \(Chla = 13.36A\text{663} – 5.19A\text{645}\), \(Chlb = 27.43A\text{645} – 8.12A\text{663}\), and total chlorophyll = Chla + Chlb.

2.5.4 Physical parameters of plant growth

Plant height from the apical of the plant to the base of above soil plant, total number of mature leaves, width of the most mature and expanded leaf and total number of branches
were also counted as morphological parameters to see differences between control and treatment groups.

2.6 Molecular techniques

2.6.1 Cloning of *AtSAP13* for overexpression in Arabidopsis

*AtSAP13* gene was previously cloned between *NcoI/XhoI* restriction sites of plant binary vector pBIN19 under *Actin2* gene promoter and terminator expression cassette (*ACT2pt*) by Anirudha Dixit in Parkash laboratory (Figure 2.1A).

2.6.2 Cloning of *AtSAP12* for overexpression in Arabidopsis

*AtSAP12* gene was previously cloned between *NcoI/XhoI* restriction sites of plant binary vector pBIN19 under *ACT2pt* expression cassette by Anirudha Dixit in Parkash laboratory.

2.6.3 Cloning of *AtSAP13* for overexpression in Brassica

*AtSAP13* gene was previously cloned between *NcoI/XhoI* restriction sites of plant binary vector pCAMBIA 1300 under *ACT2pt* expression cassette by Evan Vaine in Parkash laboratory.
Figure 2.1 Cloning of *AtSAP13* and *AtSAP12* genes for overexpression. (A) *AtSAP13* gene cloned in pBIN19 under *ACT2pt* expression cassette. (B) *AtSAP12* gene cloned in pBIN19 under *ACT2pt* expression cassette. (C) *AtSAP13* gene cloned in pCAMBIA1300 under *ACT2pt* expression cassette.

2.6.4 Making RNAi constructs for *AtSAP12*, *AtSAP13AN1* and *AtSAP13* genes

*AtSAP12*, *AtSAP13* gene specific primers were designed and used to amplify 147 bp region of the 3’ UTR sequences of *AtSAP13* and 210 bp region of the 3’ UTR sequences of *AtSAP12* to make RNAi constructs for knocking down the expression of these genes (Table 2.1). In order to knockdown SAP13, I also used the conserved AN1 domain region for making RNAi construct. Primers were designed using 129 bp of AN1 domain specific sequence from one of the *AtSAP13 AN1* domain. Stem–loop RNAi transcript was achieved by inserting a 3’UTR sense fragment between *PstI/ HindIII* restriction sites on one side of a 1,000 bp β-glucuronidase spacer region present in a modified pBSK vector and inserting a 3’tUTr antisense sense fragment amplified and cloned between
*NotI/BamHI* restriction sites on the other side. pBSK vector was then restricted at *NcoI/HindIII* to release the entire RNAi construct which was further cloned into the binary vector pCambia1300 for plant transformation (Figure 2.2A-B).

The RNAi constructs were expressed under the *ACT2pt* expression cassette. Resulting RNAi constructs (AtSAP13Ri, AtSAP12Ri and AtSAP13AN1Ri) were transformed into the C58 strain of *Agrobacterium tumefaciens*. Agrobacterium-mediated

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**Figure 2.2** Cloning of *AtSAP13*, *AtSAP12* and *AtSAP13AN1* genes for RNAi knockdown

(A) RNAi construct used for knocking down *AtSAP13*, *AtSAP12* and *AtSAP13AN1* genes

(B) RNAi map used to construct *AtSAP13*, *AtSAP12* and *AtSAP13AN1* knockdown lines
vacuum infiltration method of transformation was used to transform Arabidopsis wild
type plants and after screening the transformed plants, hygromycin (15mg/L) resistant
homozygous seed lines were selected to perform metal tolerance and uptake study.

2.6.5 Cloning of the AtSAP13 promoter fragments

A 704 bp fragment of Arabidopsis SAP13 (accession no. At3G57480) promoter sequence
was obtained from TAIR and was fragmented in two sequences of 300-400 bp each.

TOPO entry vector (Invitrogen) was used for cloning. Special primers were designed for
each sequence with at least 25 bp overlapping sequences (Table 2.1). CACC needed for
TOPO cloning was added to each of the forward primer at the 5’end. AtSAP13 promoter
fragments were amplified using these primers from Arabidopsis genomic DNA. PCR
conditions used were: 98°C for 30 secs (1 cycle); 98°C for 10 secs, 55°C for 1 min, 72°C
for 45 secs (36 cycles); and final extension at 72°C for 10 mins. PCR amplification was
carried out using Phusion (New England Biolabs) according to manufacturer instructions.
Amplified PCR fragments were gel purified using gel and DNA recovery kit from Zymo
Research. The product was quantified and then mixed with the TOPO vector for
recombination and finally transformed into chemical competent E. coli cells supplied
with the kit (Top10). Transformed reaction was plated on Kanamycin selection plates.
Colonies were checked for insert by PCR using promote-specific primers and sequences
were confirmed before proceeding to the next step.

Using Gateway cloning technology (Invitrogen), which promotes site-specific
recombination, promoter fragments were fused to LacZ reporter gene by performing an
LR recombination reaction using "LR Clonase" enzyme (Invitrogen) between the TOPO
entry clone and pGLAC destination vector that carries the LacZ gene to obtain an
expression vector. *pGLAC* vector carrying the *AtSAP13* promoter fragment was again transformed into chemical competent *E. coli* cells (Top10) on ampicillin and chloramphenicol selection and presence of clones were confirmed with PCR using promoter specific primers. Plasmid was isolated from the positive colonies. This plasmid with promoter fragment was then linearized (*NcoI* restriction enzyme) for recombination reaction with yeast vector *YM4271* and integrated into the host yeast strain through yeast lazy bones transformation method.

### 2.6.6 Cloning of *C. sativa* SAP13 gene under *ACT2* Promoter

Camelina is a hexaploid and thus each gene has three copies. Sequences of the *C. sativa* SAP13 gene copies were obtained from Camelina database ([http://www.cameleonadb.ca/](http://www.cameleonadb.ca/)) by blasting Arabidopsis SAP13 sequence as a query against the Camelina genome. In the database, a *CsSAP13* sequence has missing nucleotides from the C-terminus. Therefore, it was decided to amplify from 3’UTR extending into C-terminus of *CsSAP13*. Forward and reverse primers were designed (Table 2.1) to PCR amplify *CsSAP13* coding region of the most conserved sequence of SAP13 gene copy using Camelina leaf cDNA as a template. PCR conditions used were: 94°C for 2 mins (1 cycle); 94°C for 45 secs, 55°C for 1 min, 72°C for 45 secs (36 cycle); 72°C for 10 mins for final extension. *ExTaq* polymerase (TaKara) was used to perform PCR amplification according to the manufacturer’s instructions. *CsSAP13* PCR fragments was amplified and ligated separately into a linearized *pGEM-T* easy vector with a single 3’-terminal T overhangs at both ends (promega). Ligated product was transformed in JM109 competent cells (promega). Transformed culture was plated on LB/ampicillin/IPTG/X-gal plates. White transformed colonies were restricted to test for the genes inserted, sequenced and plasmid
was obtained (Zymo mini-prep kit).

Camelina promoter was also amplified using ACT2 gene specific primers (Table 2.1) from the promoter region. ACT2p promoter fragment was next cloned in the multiple cloning site of pCambia DS-Red vector between Ascl/SalI-HF restriction sites. Colonies were restricted to see if they were positive for ACT2p and once confirmed, CsSAP13 was cloned next to ACT2p in pCambia-DS Red vector (Figure 2.2 A) in multiple cloning site between Spe1-HF/Bbvcl (Figure 2.3).

![Diagram of pCambia RedSeed vector](image)

**Figure 2.3** Cloning of CsSAP13 gene. 
(A) pCambia DS-Red vector map used for overexpressing CsSAP13 under Act2 promoter in *C. sativa*, (B) Overexpression of CsSAP13 in *C. sativa*

Transformed culture was plated on kanamycin LB plates and colonies obtained
were checked to confirm the presence of CsSAP13. pCambia DS-Red vector carrying ACT2p and CsSAP13 was then transformed into GV3101 agrobacterium tumefaciens. Agrobacterium-mediated vacuum infiltration method of transformation was used to transform Camelina wild type plants, which were in their early flowering stage with this construct. Act2p/CsSAP13 transformed Camelina plants were raised in the greenhouse and mature brown pods were harvested 50-60 days after transformation. T0 seeds were screened using DsRed filter for the presence of transgenic seeds emitting red fluorescence due to the expression of DsRed reporter gene in which Act2p/CsSAP13 were cloned. The transgenic seeds were obtained and grown in soil to raise next generation T1 seeds. T1 seeds were again screened using DsRed filter for the presence of DsRed gene and eight lines showing 75% DsRed were selected to get next generation T2 homozygous seeds. After screening T2 lines, three 100% DsRed seed lines have been selected to carry out metal tolerance and uptake study.

2.7 Plant transformation

Gene constructs of Arabidopsis, and Brassica were transferred into C58 and of Camelina in GV3101 Agrobacterium tumefaciens strains80. Using the method described by Bechtold & Pelletier, 199881, vaccum infiltration was used as a strategy to transform wild type Arabidopsis flowering plants, Brassica callus (Tissue culture performed by Dr. Chikkara) and Camelina flowering plants respectively, with the constructs of interest.

2.8 Screening and selection of Arabidopsis homozygous seed lines

After Agrobacterium infiltration, the resulting T0 seeds were screened on for the presence
of transgene. For *AtSAP13* and *AtSAP12*, 24 seedlings showing resistance to the selection marker were selected and grown in soil to raise next generation T₁ seeds. Transgenic seeds of T2 lines were screened on ½ MS media containing desired antibiotic for 3:1 (resistant: sensitive) Mendelian segregation ratio to identify lines containing single insertion of T-DNA. qPCR was performed on selected transgenic Arabidopsis lines to confirm the overexpression or down regulation of the gene of interest.

### 2.9 Confirmation of gene overexpression by qRT-PCR

Overexpression and down regulation of wild type and transgenic seedlings was carried out through qRT-PCR. Plants were grown for a minimum of 14 days and then harvested to isolate RNA. Plant tissue from the half strength MS media plates were cleaned with water, frozen in liquid nitrogen and finally stored at -80°C till further use. The root and shoot tissues were then pulverized separately using mortar and pestle. 120 mg of grounded tissue was used to isolate total RNA using RNeasy plant mini kits (Qiagen). RNA concentration was quantified using NanoDrop spectrophotometry (ThermoScientific) and was used to perform first-strand cDNA synthesis using a ThermoScript RT-PCR kit (Invitrogen). For qRT-PCR analysis, sense and antisense qPCR primers specific to the gene of interest were designed using the 3’ UTR of the gene (Table 2.1). ACT2 gene from the desired plant specie, which is a housekeeping gene, was used as an internal control (Table 2.1). 100 ng/µL of cDNA was used as a template and qRT-PCR reactions were performed using absolute Blue qPCR SYBR Green Mix (Thermo Fisher Scientific) in Mastercycle® realplex (Eppendorf AG). Total reaction volume was 20 microlitres. Following qPCR conditions were used – 95°C for 15 min (1 cycle), 95°C for 15 secs, 55°C for 30 secs, 72°C for 1 min (31 cycles), 95°C for 15 sec,
55°C for 15 sec, melting curve 20 mins, 95°C for 15 sec. Relative gene expression level was further calculated using relative quantity \((2^{-\Delta \Delta Ct}}\) method).

2.10 Identification and cloning of the TFs interacting with *AtSAPI3* promoter.

2.10.1 Y1H assay and selection of interacting TFs

Once the DNA bait (promoter)::reporter construct was integrated into the specific yeast strain, bait containing yeast colonies were selected on SD-Ura media as the DNA bait::reporter (*LacZ*) construct carries a wild-type marker gene (*URA3*). Colonies obtained were tested for self-activation, phenomenon where a promoter fragment without interacting with TFs is able to drive the expression of *LacZ* reporter gene. Self-activation test helps to eliminate constructs that are not using TFs for the activation of the reporter genes. Single non-self active yeast colony was used for further screening with the TF library. If activated, yellow color was produced from the interaction due to activation of *LacZ* gene, which produces β-galactosidase, which further hydrolyzes the substrate ONPG (*ortho*-Nitrophenyl-β-galactoside) into galactose and *ortho*-nitrophenol. The latter produced yellow color and was used as indicator for self-activation. Interactions between *cis*-regulatory DNA elements from the gene promoter generally termed as “DNA bait” and TFs normally termed as “protein preys” which also carry a heterologous transcription activation domain are identified next through Y1H screen (Figure 2.4). One non-self active colony was selected and its culture was mixed with the library of about 1800 Arabidopsis TFs and screened for activation through Y1H assay. If there was an interaction between TFs and promoter fragment, the heterologous activation domain activates reporter gene expression, thus resulting in the identification of interacting TFs.
2.10.2 Cloning of the candidate TFs.

Wild type Arabidopsis cDNA was used as a template to amplify the selected TFs using gene specific primers designed manually. PCR conditions used were: 94°C for 2 mins; 94°C for 45 secs, 58°C for 1 min, 72°C for 45 secs (36 cycles); and final extension at 72°C for 10 mins. PCR amplification was carried out using *ExTaq* polymerase (TaKara) according to the manufacturer’s instructions. Amplified PCR fragments was purified using Zymo PCR purification kit. PCR purified product was restricted using site specific restriction enzymes along with restriction of the *His*-tag containing vector pET23b_hisSUMO (vector is from Dr. Peter Chen laboratory) and then gel purified (Zymo gel and DNA recovery kit). Gel purified vector and insert were then ligated and kept overnight at 16°C. Ligated product was purified and transformed into DH5A competent cells on selection media. Colonies obtained were cultured, plasmid DNA was obtained and sequenced for confirmation of the sequences of each of the four
transcription factors. These four constructs were individually transformed next into Rosetta-B competent cells to express proteins for all the four genes.

2.11 Induction and purification of TF proteins in Rosetta-B competent cells.

Colonies obtained after transforming Rosetta-B competent cells were restricted with specific enzymes to check if they were positive for the TFs and then one colony from each of the four TFs were inoculated in overnight cultures with ampicillin. Overnight cultures were inoculated into 1L LB cultures for protein induction and purification.

Lysozyme and bug buster protein extraction reagent from Novagen were used to lyse the cells. Cells were then homogenized and centrifuged to obtain supernatant which was then passed through Ni column, washed several times to finally obtain eluent fraction that would contain the soluble protein. Pellet obtained was also incubated and dissolved in binding buffer (5mM imidazole, 500mM NaCl, 20mM Tris-HCl pH 7.9) and 6M urea. Centrifugation was next performed to obtain the supernatant, which was mixed with His-bind resin in a column which was previously equilibrated with sterilized water, charge buffer (50mM NiSO4) and binding buffer containing 6 M urea. Flow thru was collected and the column was then washed with binding buffer containing 6 M urea and 20mM imidazole buffer (20mM imidazole, 500mM NaCl, 20mM Tris-HCl pH 7.9) containing 6 M urea and finally with elute buffer (500mM NaCl, 1M imidazole, 20mM Tris-HCl pH 7.9) containing 6 M urea. Flow thru from each of the above-mentioned steps and the final eluent fractions were collected. All the fractions were mixed with 6X Laemelli buffer and run on a SDS-PAGE gel. The fraction that carried the most soluble protein eluted from the pellet was then subjected to dialysis with a urea gradient (6 M, 4 M, 2 M, 0 M) to
obtain urea free protein.

2.12 Electrophoretic mobility shift assay (EMSA)

Using the Bradford method, concentrations of the proteins were determined. Next step was to perform an EMSA – interaction of the protein (TF) and DNA (promoter). With some modifications to Hazen lab’s protocol, a 15ng of amplified promoter DNA and 300ng of each of the purified proteins were incubated together with buffer made up of 137mM NaCl, 4.3 mM Na₂HPO₄, 2.7 mM KCl, and 1.5mM KH₂PO₄, making a total volume of 10 ml with water. The mixture was incubated for 30 mins at room temperature. Reaction was then loaded on 1% agarose gel alongside unbound DNA and protein to see a shift in the bound fragment.

2.13 Statistical Analysis.

For each petriplate plant growth assay described above, growth measurements were performed using groups of 10 plants. Analysis of root and shoot samples were carried out by averaging the means from three to four replicates. In all the figures, standard error of the mean has been represented. Experimental set up for each treatment was repeated to validate the results. Student t-test is applied to calculate significant difference between control and tested groups. p < 0.01 is considered as highly significant labeled as **, and p < 0.05 is considered significant labeled as *. One-way ANOVA followed by LSD test was also used to determine the statistical significance of some parameters among the treatments. The values of each assay followed by different letters are significantly different at p < 0.05.
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*Table 2.1* Set of primers used in this study.
CHAPTER 3
CHARACTERIZATION AND MODE OF ACTION OF \textit{AtSAP13} IN PROVIDING TOLERANCE TO ABIOTIC STRESSES.

3.1 Introduction

SAPs has been shown be involved in providing tolerance to abiotic stresses\textsuperscript{30,56}. \textit{A. thaliana} has a total of 14 SAP genes, of which proteins of AtSAP1 through AtSAP10 carry both the A20 and the AN1 ZnF domains whereas AtSAP11 through 13 code for proteins with two AN1 ZnF domains and AtSAP14 code for protein containing a single AN1 domain\textsuperscript{56}. \textit{AtSAP13} (AT3G57480) belongs to type IIA AN1 zinc finger family, carries two AN1 domains at the N- terminus and one C2H2 domain at the C- terminus (Figure 1.1) and codes for a 250 amino acids protein sequence. Previous analysis showed a close phylogenetic relationship between the AN1 domains of AtSAP13 protein sequence with \textit{C. elegans} and mouse cell AIRAP protein sequences. AIRAPs have been shown to protect the cells such as those of \textit{C. elegans} and mouse cells against such toxicities by associating tightly with the 19S cap of the 26S proteasome altering biochemical properties and increasing the breakdown of the aggregated polyubiquitinated proteins thus providing tolerance against proteotoxicity\textsuperscript{53}. Thus, this close relationship between AN1 domains of AtSAP13 protein sequence with \textit{C. elegans} and mouse cell AIRAP protein sequences led to the hypothesis that the AN1 domain carrying \textit{AtSAP13} might be providing tolerance to arsenite and other abiotic stresses similar to AIRAP.

In this study, we have characterized \textit{AtSAP13} for it’s role in providing tolerance to abiotic stresses by overexpression, RNAi knockdown and further attempted to understand the mode of action of \textit{AtSAP13}.
3.2 Results

3.2.1 Confirming the overexpression of *AtSAP13* in transgenic lines

*AtSAP13* was previously overexpressed in Arabidopsis under the control of a constitutively expressed *ACT2pt* cassette in Parkash’s laboratory. Seeds of kanamycin resistant T₁ transgenic lines were screened for Mendelian segregation ratio of 3:1 (resistant: sensitive) to identify transgenic lines with single T-DNA insertion which were grown further to obtain the next generation T₂ homozygous seeds. Four *AtSAP13* transgenic lines (8, 9, 11 and 13) were selected and overexpression of *AtSAP13* was analyzed using qRT-PCR using the procedure described under ‘material and method’ section. *AtSAP13* mRNA transcript levels in lines 8, 9 and 11 were increased more than 70-folds, whereas line 13 showed lower levels of expression compared to wild type (Figure 3.1).

![Figure 3.1 qPCR analysis of AtSAP13 overexpression lines. The means are averaged from three replicates and the error bars correspond to standard error of mean. Transcription levels of SAP13 in each line followed by single asterisks (*) are significantly different as compared to WT at p<0.05.](image)
3.2.2 Analysis of AtSAP13 transgenic plants for tolerance to various heavy metals

Sensitivity/tolerance phenotypes of AtSAP13 lines 8, 9 and 11 on NaAsO$_2$ (30 µM), CdCl$_2$ (75 µM) and ZnSO$_4$ (500 µM) were analyzed by sterilizing the seeds using the method described in the section 2.1.1 and then growing the seeds on ½ MS plates vertically. After 21 days, root length was measured for all the plants and shoot biomass was collected and analyzed. This experimental set up was repeated to validate the results (see Appendix A1 and A2).

AtSAP13 transgenic lines, when grown on media supplemented with AsIII, Cd and Zn, showed enhanced tolerance to these metals. On media supplemented with AsIII and Cd, plants of transgenic lines had fully developed green leaves and roots whereas wild type plants showed stunted growth and chlorosis in the leaves of plants exposed these metals. These overexpression lines attained about 25%-50% higher shoot biomass compared to wild types on NaAsO$_2$ and CdCl$_2$ (Figure 3.2 A and B). On media containing Zn, there was no significant difference in biomass accumulation in transgenic lines compared to wild type controls. On media containing AsIII, Cd and Zn, roots of transgenic plants showed comparatively longer roots with lateral branching, however, roots of wild type plants were smaller with less branching (Figure 3.2 A, B and C). Both wild type and transgenic lines on Zn were severely stressed with pale leaves and stunted growth; however, significant difference in tolerance between them was still observed. No phenotypic difference was observed between wild type and transgenic Arabidopsis seedlings on the control media.
Because of the observed strong tolerance to heavy metals such as As, Cd, and Zn,
provided by AtSAP13 overexpression, we analyzed these plants for the accumulation of metals in shoot and root tissue. Two selected lines (#8, 9) of the AtSAP13 transgenic Arabidopsis and wild type plants were grown and exposed to metals hydroponically and elemental analysis was estimated for As, Cd and Zn content in the roots and shoots. When exposed to 30 µM As for five days, both AtSAP13 overexpressing lines had accumulated about 30% more As in roots as well as in shoots compared to wild type plants (Figure 3.3A and 3.3B). When grown on 75 µM Cd, the AtSAP13 overexpressing lines showed no difference in Cd accumulation in their shoots or roots as compared to the wild type plants (Figure 3.3A and 3.3B). When analyzed on 500µM Zn, AtSAP13 transgenic line 8 had accumulated about 25% more Zn in roots but line 9 showed no difference in accumulation in roots compared to the wild type plants. These transgenic lines also showed no difference in Zn accumulation in shoots compared to wild type plants (Figure 3.3A and 3.3B).

**Figure 3.3** Accumulation of As, Cd, and Zn in Arabidopsis SAP13 overexpression lines. Total As, Cd and Zn accumulation in (A) roots and (B) shoots of WT and two AtSAP13 overexpression lines grown on 1/2 MS hydroponics medium containing 30 µM Sodium arsenite, 75 µM cadmium chloride and 500 µM zinc sulfate for 5 days. Data are averaged from three replicates of each. One-way ANOVA followed by LSD test was also used to determine the statistical significance of the values of metal accumulation. The values of
each assay followed by different letters are significantly different at p < 0.05.

### 3.2.4 Analysis of AtSAP13 transgenic lines for tolerance to salt stress

To study if AtSAP13 also provides tolerance to salt stress, Arabidopsis wild type and AtSAP13 overexpression lines were grown side by side on ½ x MS media containing previously optimized 100 mM salt (NaCl) concentration. After 21 days, shoot biomass was harvested and root length was calculated. No phenotypic difference was observed between wild type and transgenic Arabidopsis seedlings on the control media. However, leaves of wild type Arabidopsis seedlings were smaller and shriveled compared to the leaves of transgenic lines 8 and 9, which were comparatively bigger, broader and greener accounting for about 30%-60% shoot biomass gain. No significant difference was observed between wild type plants and #11. Root lengths of wild type and transgenic plants also showed no significant difference (Figure 3.4). This experimental set up was repeated to validate the results (see Appendix A3).

(A)
3.2.5 Analysis of AtSAP13 overexpression lines for tolerance to drought stress

AtSAP13 overexpression lines were also analyzed for their tolerance/sensitivity to D-mannitol according to section 2.2.1 in the ‘Material and Method’. D-mannitol is known to impart drought stress in plants. Analysis of transgenic and wild type control plants showed no significant difference in growth in response to mannitol exposure (Figure 3.5).

Figure 3.4 Arabidopsis SAP13 overexpression lines on NaCl (100mM). (A) Photographs of plants showing Phenotypic difference, and (B) Fresh shoot biomass of AtSAP13;8, AtSAP13;9, and AtSAP13;11 overexpression lines grown on 1/2x MS plates containing 100 mM NaCl for 21 days. Data are averaged from four replicates of 10 plants each. Values of fresh biomass and root length followed by double asterisks (**) are highly significant at p ≤ 0.01.
3.2.6 Analysis of AtSAP13 and AtSAP13 AN1 RNAi knockdown lines on multiple abiotic stress tolerance

Gene knockdown construct of AtSAP13 was constructed using RNA interference (RNAi) to test for a role in providing abiotic stress tolerance. It was believed that if its AtSAP13 mRNA expression were down regulated, it would not be able to provide tolerance to these abiotic stresses thus confirming its role. As mentioned above, several SAP genes from various plant species have been shown to provide tolerance to various abiotic stresses. For knocking down the expression of AtSAP13, we made two RNAi constructs—one using the gene-specific 3’ UTR of AtSAP13 (AtSAP13Ri) and second by using the conserved AN1 domain (AtSAP13AN1Ri).

Transgenic T₂ seeds of RNAi lines for both constructs were screened on ½ x MS media containing hygromycin for 3:1(resistant: sensitive). Mendelian segregation ratio to identify homozygous lines containing single insertion of T-DNA. qPCR was performed on several transgenic Arabidopsis lines of each of the two constructs. Following three

Figure 3.5 Phenotypic expression of Arabidopsis SAP13 overexpression lines on D-mannitol (100mM).
lines of each of the constructs were analyzed further (AtSAP13AN1; 2, AtSAP13AN1; 16, AtSAP13AN1: 24 for AtSAPI3AN1Ri and AtSAP13; 14, AtSAP13; 16, AtSAP13; 20 for AtSAPI3Ri).

Compared to wild type controls, selected RNAi lines of AtSAP13 and AtSAP13AN1 showed 1.5 to 2 folds reduction in mRNA transcript expression (Figure 3.6).

**Figure 3.6** qPCR analysis of RNAi lines (A) AtSAP13 and (B) AtSAP13AN1. The means are averaged from three replicates and the error bars correspond to standard error of mean. Transcription levels of SAP13 in each line followed by single asterisks (*) are significantly different as compared to WT at p<0.05.
3.2.6.1 Analysis of *AtSAP13* and *AtSAP13AN1* RNAi lines for tolerance to heavy metals, salt or drought stresses

Seeds of the selected *AtSAP13* and *AtSAP13AN1* RNAi homozygous lines were grown on various toxic metals (As, Cd, Zn) and under salt and drought stress condition to obtain their sensitivity/tolerance phenotypes. Compared to wild type controls, *AtSAP13* and *AtSAP13AN1* RNAi lines showed no significant difference in the growth pattern under these stresses (Figure 3.7)
(B)  

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<td>100 mM D-Mannitol</td>
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(C)  

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<td>500 µM Zn</td>
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3.2.7 Y1H assay for Analysis of *AtSAP13* promoter interactions with abiotic stresses related TFs

Based on the *in-silico* analysis of the promoter sequences upstream of ATG start codon of *AtSAP13*, we hypothesized that various *cis*-elements identified using PLACE database\(^83\) might be interacting with abiotic stress-related *trans*-factors via protein-DNA interactions and hence, they regulate the expression of *AtSAP13* gene under multiple abiotic stresses. In order to prove this, we performed protein-DNA interaction using Y1H.

**Figure 3.7 (A-B)** Phenotypic analysis of Arabidopsis SAP13 and (C-D) SAP13AN1 RNAi lines on heavy metals 30 µM As, 75 µM Cd and 500 µM Zn, NaCl (100mM) and D-mannitol (100mM).
Using Y1H system, interaction of the first promoter fragment and TF library yielded 18 interacting TFs which included ERE (ethylene responsive element), bZIP (basic leucine zipper), NAC ((NAM/ATAF/CUC family) transcription factors as few of the common ones (Table 3.1). No interactions between the second promoter fragment and TFs were observed in Y1H screen.

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<td>At5g05410</td>
<td>AP2-EREBP</td>
<td>DREB2A; DNA binding / transcription activator/ transcription factor</td>
</tr>
<tr>
<td>At4g01120</td>
<td>bZIP</td>
<td>GBF2 (G-Box binding factor 2); DNA binding / sequence-specific DNA binding / transcription factor</td>
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<td>At3g20310</td>
<td>AP2-EREBP</td>
<td>ERF7 (Ethylene responsive factor 7); DNA binding / protein binding / transcription factor/ transcription repressor</td>
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<td>At1g22190</td>
<td>AP2-EREBP</td>
<td>ERF058 ethylene-responsive transcription factor/AP2 domain-containing transcription factor, putative</td>
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<td>At4g38900</td>
<td>bZIP</td>
<td>Basic-leucine zipper (bZIP) transcription factor family protein</td>
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<td>At3g16770</td>
<td>AP2-EREBP</td>
<td>Ethylene-responsive transcription factor RAP2-3 (EBP)/ATEBP (Ethylene responsive element binding protein)</td>
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<td>AP2-EREBP</td>
<td>ERF10 (ERF domain protein 10); DNA binding</td>
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<td>AP2-EREBP</td>
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<td>NAC</td>
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<td>GcBP</td>
<td>DNA-binding storekeeper protein-related</td>
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<td>AP2-EREBP</td>
<td>ERF11 (ERF domain protein11); DNA binding / transcription factor/ transcription repressor</td>
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<td>AP2-EREBP</td>
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<td>AP2-like ethylene-responsive transcription factor AIL6 (Aintegumenta like 6); DNA binding / transcription factor</td>
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<td>AP2-EREBP</td>
<td>Arabidopsis thaliana AP2-like ethylene-responsive transcription factor</td>
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<td>AT2G44840</td>
<td>ERTF</td>
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<tr>
<td>AT1G74930</td>
<td>ERTF</td>
<td>Arabidopsis thaliana Ethylene responsive transcription factor ERF018</td>
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Table 3.1 List of candidate TFs obtained from Y1H analysis of AtSAP13 promoter fragments
3.2.7.1 Cloning, induction and purification of TF proteins

Based on the unknown function and relevance of other similar TFs towards abiotic tresses, four candidate TFs mentioned in table 3.2 (AT4G01120, AT1G74930, AT1G47870 and AT5G53950) out of the 18 TF hits from the YIH assay were selected and cloned in the *His*-tag containing vector pET23b_hisSUMO (vector is from Dr. Peter Chen laboratory) using the protocol mentioned in the 2.10.2 section. The four constructs were then individually transformed into Rosetta-B competent cells, induced and their respective proteins were further expressed and eluted according to the section 2.11 described under ‘Material and Method’. Eluent fractions of protein carrying the *His*-tag from the supernatant and pellet were mixed with Laemelli buffer and were then analyzed on SDS-PAGE gel. Two TFs, At4g01120 and At1g74930 did not get induced. Induced proteins of TFs At1g47870 and At5g53950 from the cell pellets were purified (Figure 3.8A) following denaturing 6M urea treatment and dialyzed according to a protocol (followed from Schnell lab) mentioned in the material and method section.

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<th>Size in base pairs</th>
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<td>1083bp</td>
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<td>At1g74930</td>
<td>21.5</td>
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<tr>
<td>At5g53950</td>
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<tr>
<td>At1g47870</td>
<td>43.5</td>
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**Table 3.2** Highlighted candidate TF chosen for protein purification and EMSA

The four constructs were then individually transformed into Rosetta-B competent cells, induced and their respective proteins were further expressed and eluted according to the section 2.11 described under ‘Material and Method’. Eluent fractions of protein carrying the *His*-tag from the supernatant and pellet were mixed with Laemelli buffer and were then analyzed on SDS-PAGE gel. Two TFs, At4g01120 and At1g74930 did not get induced. Induced proteins of TFs At1g47870 and At5g53950 from the cell pellets were purified (Figure 3.8A) following denaturing 6M urea treatment and dialyzed according to a protocol (followed from Schnell lab) mentioned in the material and method section.

3.2.7.2 Interaction of the candidate TFs with *AtSAPI3* promoter fragment using EMSA.

Using the Bradford method, concentration of the proteins was determined to be around
130mg/L. To look at the interaction of the protein (TF) and DNA (promoter), EMSA was performed next following the protocol mentioned in the section 2.12. No shift in the band due to the binding of the DNA and protein was observed on the agarose gel (Figure 3.8 B and C).

**Figure 3.8** Purification and confirmation of interaction of candidate TFs with *AtSAP13* promoter  
(A) SDS-PAGE showing protein of the TFs eluted from the insoluble fraction and (B-C) EMSA performed for the two transcription factors and promoter fragments.
3.3 Discussion

In the present study, we characterized the AtSAP13 gene as well as tried to study the mode of action of AtSAP13 gene for its role in providing tolerance to multiple abiotic stresses. We showed that AtSAP13 when overexpressed provided significant tolerance to heavy metals namely As, Cd and Zn and also accumulated significant concentrations of some of these metals in their shoot and root tissues. We speculate that the presence of putative heavy metal-binding motifs in the conserved stretches of cys- and his- residues of the two AN1 domains and one C2H2 domain could be the reason for this significant metal tolerance and accumulation\(^5\). Presence of heavy metal-binding motifs in the conserved cys- and his- residues of zinc RING finger domains were also seen for AIRAP genes in *C. elegans* and mouse cells\(^4\). In the recent years, two members of the HMA (Heavy Metal ATPase) family from Arabidopsis - AtHMA2\(^8\) and AtHMA4\(^8\) have been shown to better tolerate metal stresses such as that of Zn and Cd due to the presence of multiple metal binding sites comprising of cys- and his- residues in a conserved format at the C-terminus. Also, in Parkash lab, AtSAP11 another member of the SAP family with a ZnF domain arrangement very similar to that of AtSAP13 was previously characterized. *AtSAP11* carries two AN1 and two C2H2 ZnF domains with similar conserved cys- and his- residue metal binding motif pattern and provided moderate tolerance to As and Zn\(^1\). This discovery further confirms our beliefs of *AtSAP13* might be providing metal tolerance due to the presence of metal binding motifs in its ZnF domains.

*AtSAP13* also provided significant tolerance to salt stress. As mentioned earlier,
using the PLACE database\textsuperscript{83}, an \textit{in silico} analysis of the \textit{AtSAP13} promoter sequence upstream to ATG identified many abiotic and biotic stress associated \textit{cis} elements such as DREB\textsuperscript{86}, ERE\textsuperscript{87}, ZIP\textsuperscript{88}, HSE\textsuperscript{89}, etc. Interaction between these \textit{cis}-elements and associated TFs could be leading to the activation of stress-associated genes to protect against salt stress. Also, Yamaguchi-Shinozaki and Shinozaki\textsuperscript{90} previously discovered a novel \textit{cis}-element in the promoter of \textit{RD29A} gene from Arabidopsis and Tobacco, a dehydration-responsive element (DRE). Under dehydration and high salinity conditions, this \textit{cis}-element containing a 9 bp sequence TACCGACAT, was shown to be involved in the first response of RD29A gene\textsuperscript{90}. It is also possible that \textit{AtSAP13} is providing tolerance to salt stress via interaction with the 26S proteasome complex as under such stress conditions, proteins get misfolded, polyubiquitinated and aggregated in the cells\textsuperscript{51,52}. Stanhill \textit{et al.}\textsuperscript{53} showed that the homologs of SAPs, AIRAP’s protect the cells such as those of \textit{C. elegans} and mouse cells against arsenite toxicity by associating tightly with the 19S cap of the 26S proteasome and increasing the breakdown of the aggregated polyubiquitinated proteins. However, further research needs to be performed to find out exactly which interactions could be providing salt tolerance.

\textit{AtSAP13} and \textit{AtSAP13AN1} RNAi transgenic lines did not show any difference on any of the heavy metals (As, Cd and Zn) or other abiotic stresses which included salt and drought stress. As significant decrease in the mRNA transcripts of these constructs was observed with qRT-PCR, we speculate that the reason behind for no tolerance to these stresses mentioned above could be due to functional redundancy in the AtSAP family since Arabidopsis contains 14 SAP genes. It is also possible that even though transcript level reduction was significant, it might not be enough to show difference in tolerance
when compared to wild type plants.

From the *AtSAP13* promoter fragment and TF library interactions, 18 TFs were identified through Y1H assay. Most of these proteins are related to abiotic stresses in plants. Majority of the identified TFs belong to APETALA2-Ethylene Responsive Factor (AP2-ERF TF) family. This family of proteins is known to activate stress responsive genes under abiotic stress conditions such as dehydration and salt, by binding to specific *cis*-elements such as DREs present in their promoters\(^{115}\). Few other TFs identified were bZIPs (basic leucine zipper), which also respond to stresses like low temperatures and dehydration by binding to ABRE, G-box, C-box like *cis*-elements\(^{115}\). The second SAP13 promoter fragment did not show any interaction with the TF library maybe because certain plant genes do not interact and work well in yeast system or because fragmentation of some genes such as *AtSAP13* promoter in a certain way can cause problems in the Y1H system.

While trying to purify the candidate TF proteins, different competent cells and modifications of IPTG-induction duration, concentration of IPTG, temperature for induction were tried, however, TFs At4g01120 and At1g74930 did not get induced. Also, induced proteins of TFs At1g47870 and At5g53950 were repeatedly going into the pellet and not staying in the soluble supernatant. In order to purify induced protein from the cell pellets, 6M urea treatment followed by dialysis of protein was thus used to get the denatured protein into the soluble form. Once proteins for TFs At1g47870 and At5g53950 were eluted from the pellet, they were used to confirm the interaction between the promoter fragment and the TF protein through EMSA. However, no binding between the promoter DNA and TF proteins were seen, therefore no shift in the bands on
agarose gel was observed. There are two possibilities for not seeing any interaction between TFs and promoter fragments on performing EMSA. First, the purification process with urea treatment changed the conformation of protein, which could have affected its binding sites, making it an inactive protein. Hence no binding between the DNA and protein was observed. Second, the conditions in EMSA provided were not appropriate for the binding reaction.

To conclude, *AtSAP13* when overexpressed in Arabidopsis, showed significant tolerance to As, Cd, Zn and salt in young seedlings. No difference in wild type and transgenic plant phenotypes were obtained when treated with D-mannitol, which mimics drought stress. RNAi knockdown lines of *AtSAP13* and *AtSAP13AN1* also did not show any change in growth and phenotypes of the plants when exposed to As, Cd, Zn, salt and D-mannitol. Based on these promising results obtained from experiments performed on young seedlings, it is concluded that *AtSAP13* might be directly involved in providing tolerance to certain heavy metal stresses as well as some other abiotic stresses and could potentially serve in developing abiotic resistant crops after more detailed greenhouse stress studies on mature plants.
CHAPTER 4

CONFIRMING THE OVEREXPRESSION AND RNAi KNOCKDOWN OF AtSAP12 AND ANALYSIS OF TRANSGENIC PLANTS FOR VARIOUS HEAVY METAL STRESS TOLERANCE.

4.1 Introduction

Another SAP family member from Arabidopsis, namely AtSAP12 (AT3G28210) belongs to TypeIIB AN1 ZnF family carries two AN1 domains at the N-terminus (Figure 1.1) and codes for a 187 amino acids protein sequence. Previous analysis showed a close phylogenetic relationship between the AN1 domains of AtSAP12 protein sequence with C-elegans and mouse cell AIRAP protein sequences. This close relationship led to the hypothesis that the AN1 domain containing AtSAP12 might also be providing tolerance to AsIII and other abiotic stresses similar to AIRAP. AIRAPs have been shown to protect the cells such as those of C. elegans and mouse against such toxicity by associating tightly with the 19S cap of the 26S proteasome, altering their biochemical properties and increasing the breakdown of the aggregated polyubiquitinated proteins thus providing tolerance against proteotoxicity.

In this study, we have confirmed the overexpression and RNAi knockdown of AtSAP12 and it’s role in providing tolerance to abiotic stresses.

4.2 Results
4.2.1 Confirming the overexpression of \textit{AtSAP12} in transgenic Arabidopsis lines

Overexpression of \textit{AtSAP12} in Arabidopsis was also done previously in our laboratory. \textit{AtSAP12} overexpression lines were characterized for the role of \textit{AtSAP12} in providing tolerance to abiotic stresses including heavy metals. Seeds of kanamycin resistant T\textsubscript{1} transgenic lines were screened for Mendelian segregation ratio of 3:1 (resistant: sensitive) to identify transgenic lines with single T-DNA insertion which were grown further to obtain the next generation T\textsubscript{2} homozygous seeds. Two transgenic lines of \textit{AtSAP12} (57 and 63) were selected and overexpression of AtSAP12 was analyzed using qRT-PCR using the procedure described under ‘material and method’ section. The mRNA transcript levels of AtSAP12 overexpressing lines 57 and 63 were significantly increased and showed overexpression in seedling tissue compared to wild type (Figure 4.1). AtSAP12 line 63 showed more than 134-folds higher levels of transgene expression whereas line 57 showed 72-fold higher levels of transgene expression compared to wild type.

![Figure 4.1](image)

**Figure 4.1** qPCR analysis of \textit{AtSAP12} overexpression lines. The means are averaged from three replicates and the error bars correspond to standard error of mean. Transcription levels of SAP12 in each line followed by single asterisks (*) are significantly different as compared to WT at p<0.05.

4.2.2 Analysis of \textit{AtSAP12} overexpression lines for metal tolerance on As\textsubscript{III}, and Zn:
Sensitivity/tolerance phenotypes of lines *AtSAP12* 57, and 63 on NaAsO$_2$ (30 µM), CdCl$_2$ (75 µM) and ZnSO$_4$ (500 µM) were performed. Surface-sterilized seeds of each line along with wild type Arabidopsis seeds were then germinated and grown vertically for 21 days on half-strength MS agar media. After three weeks, plants were harvested and shoot biomass and root lengths were determined. This experimental set up was repeated to validate the results (see Appendix A4 and A5).

Significant difference in the shoot biomass, averaging about 40%, of transgenic lines *AtSAP12*; 57 and *AtSAP12*; 63 on AsIII compared to wild type plants was obtained (Figure 4.2). Plants of transgenic lines had fully developed green leaves and roots whereas wild type plants showed stunted growth. Also, significant difference in root length compared to wild type was also seen on AsIII (Figure 4.2). No phenotypic difference was observed between wild type and transgenic Arabidopsis seedlings on the control media.

Plants of both wild type Arabidopsis as well as transgenic lines on Zn were severely stressed with pale leaves and stunted growth, however, difference in root length between them was still observed (Figure 4.2). On Zn, there was no significant difference in shoot biomass in transgenic lines compared to wild type controls. No phenotypic difference was observed between wild type and transgenic Arabidopsis seedlings on the control media.
Figure 4.2 Arabidopsis AtSAP12 overexpression lines grown on 30 µM AsIII, and 500 µM Zn.

(A) Phenotypic expression, (B) Fresh shoot biomass and (C) root length of AtSAP12 #57, and #63 overexpression lines grown on ½ x MS plates containing 30 µM AsIII, and 500 µM Zn for 21 days. Data are averaged from four replicates of 10 plants each. Values of fresh biomass and root length followed by double asterisks (**) are highly significant at p ≤ 0.01.
4.2.3 Accumulation of As, Cd and Zn in AtSAP12 transgenic lines.

Since AtSAP12 overexpression lines showed strong tolerance to heavy metals such as As, and Zn, we analyzed these plants for the accumulation of these metals in their shoot and root tissues. Two AtSAP12 transgenic lines (#57, 63) and wild type plants were grown and exposed to metals hydroponically and elemental analysis was estimated for As, Cd and Zn content in the roots and shoots. When exposed to 30 µM As for five days, both AtSAP12 overexpressing lines showed significant accumulation of As in roots as well as in shoots compared to wild type plants (Figure 4.3A and 4.3B). However, when grown on 75 µM Cd, the AtSAP12 overexpressing #63 accumulated significantly less Cd in its root and shoot compared to the wild type plants (Figure 4.3A and 4.3B). However, AtSAP12 overexpressing #57 accumulated significantly less Cd in its shoot but showed no difference in accumulation in roots compared to the wild type plants (Figure 4.3A and 4.3B). Cadmium accumulation result is contradictory to what we observed while checking for Cd tolerance and thus needs to be repeated to confirm the results. When exposed to 500µM Zn, compared to wild type plants, both AtSAP12 overexpressing lines showed significantly higher accumulation of Zn in shoots with #63 accumulating significantly more Zn compared to #57. No difference in root Zn accumulation was witnessed in the two transgenic lines as compared to wild type plants (Figure 4.3A and 4.3B).
Figure 4.3 Accumulation of As, Cd, and Zn in Arabidopsis SAP12 overexpression lines. Total As, Cd and Zn accumulation in (A) roots and (B) shoots of WT and two AtSAP12 overexpression lines grown on 1/2 MS hydroponics medium containing 30 µM Sodium arsenite, 75 µM cadmium chloride and 500 µM zinc sulfate for 5 days. Data are averaged from three replicates of each. One-way ANOVA followed by LSD test was also used to determine the statistical significance of the values of metal accumulation. The values of each assay followed by different letters are significantly different at p < 0.05.

4.2.4 Analysis of AtSAPI2 overexpressing plants for tolerance to Cd, salt or drought stress

AtSAPI2 overexpression lines were also analyzed for their tolerance/sensitivity to Cd, NaCl, D-mannitol according to section 2.2.1 in the ‘Material and Method’. No significant difference in growth of wild type and transgenic plants was observed in response to these stresses (Figure 4.4).
Figure 4.4 Photographs of Arabidopsis AtSAP12 overexpression lines on Cd (75 μM), NaCl (100mM), and D-mannitol (100mM).

4.2.5 Knocking down Arabidopsis SAP12 through RNAi for deciphering its role in multiple abiotic stress tolerance

RNAi knockdown construct of AtSAP12 was also constructed to test for it’s role in providing abiotic stress tolerance. As mentioned above, several SAP genes from various plant species have been shown to provide tolerance to various abiotic stresses. It was believed that if its mRNA expression were down regulated, it would not be able to provide tolerance to these abiotic stresses thus confirming their role.
*AtSAP12* gene specific primers were designed and used to clone the constructs as described in the section 2.6.1 of ‘Material and Method’. Transgenic seeds of T2 *AtSAP12* RNAi lines were screened on $\frac{1}{2}$ x MS media containing hygromycin for 3:1 (resistant: sensitive) Mendelian segregation ratio to identify homozygous lines containing single insertion of T-DNA. qPCR was performed on several transgenic Arabidopsis lines of the RNAi construct. Following three *AtSAP12* RNAi lines of were analyzed further (# 3, 7, and 8). Compared to wild type controls, the expression of *AtSAP12* in the selected RNAi lines showed 1.5 to 2-fold reduction in the levels of mRNA transcript (Figure 4.5).

**Figure 4.5** qPCR analysis of RNAi lines of *AtSAP12*.
The means are averaged from three biological replicates and the error bars correspond to standard error of mean. Transcription levels of SAP12 in each line followed by single asterisks (*) are significantly different as compared to WT at p<0.05.

4.2.6 Analysis of *AtSAPI2* RNAi knockdown plants for tolerance to heavy metals, salt, and drought stresses

Seeds of the three selected *AtSAPI2* homozygous lines (3, 7, and 8) were grown on media supplemented with various toxic metals (As, Cd, Zn) and under salt and drought stress conditions to obtain their sensitivity/tolerance phenotypes. Compared to wild type controls, *AtSAPI2* RNAi showed no significant difference in the growth pattern under
these stresses (Figure 4.6)

(A)  

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**4.3 Discussion**

*AtSAP12* RNAi transgenic lines did not show any difference on any of the heavy metals (As, Cd, Zn) or other abiotic stresses which included salt and drought stress. As significant decrease in the mRNA transcripts of these lines expressing RNAi construct were seen by qRT-PCR, we speculate that the reason behind no tolerance to any of these stresses mentioned above could be due to functional redundancy in the AtSAP family similar to the results of AtSAP13 RNAi as discussed in Chapter 3. Also, significant reduction in transcript levels may not be enough to show difference in tolerance when compared to wild type plants.

In the present study, we confirmed the overexpression of SAP12 gene from *A. thaliana*. We showed that *AtSAP12* when overexpressed provided significant tolerance.
and accumulation of heavy metals. Similar to *AtSAP13*, *AtSAP12* might be providing tolerance and showed accumulation of these heavy metals due to the presence of putative heavy metal-binding motifs in the conserved cys- and his- residues of the two AN1 domains\(^59\). AIRAPs from *C.elegans* and mouse cells also showed the presence of similar metal binding sites in their ZnF domains, which carry cys- and his- residues\(^53\). Previously, tolerance to heavy metals Zn and Cd was also seen in two members of the HMA family from Arabidopsis, namely AtHMA2\(^84\) and AtHMA4\(^85\) due to the presence of similar metal binding domains in the cys- and his- residues. As mentioned in chapter 3, another SAP family member - *AtSAP11*\(^109\) was previously shown to have a similar metal binding motif pattern in the conserved cys- and his- residues of its two AN1 and two C2H2 ZnF domains and provided moderate tolerance to As and Zn. This is another confirmation of our beliefs that metal tolerance provided by *AtSAP12* might be due to the presence of metal binding motifs in its ZnF domains\(^109\).

Another possibility for tolerance and accumulation to As and Zn could be similar to AIRAPs. Just like AIRAPs provided tolerance and protected *C. elegans* and mouse cells against AsIII toxicity, *AtSAP12* might also be interacting with the 26S proteasome complex. This close interaction could be increasing the breakdown of misfolded and aggregated polyubiquitinated proteins yielded due to heavy metal stress\(^51,53,52\). However, further research needs to be performed to find out exactly which interactions could be providing this tolerance.

To conclude, *AtSAP12* when overexpressed in Arabidopsis, showed significant tolerance to As and Zn in young seedlings. No difference in wild type and transgenic plant phenotypes were obtained when treated with Cd, salt or drought stress. RNAi
knockdown lines of \textit{AtSAP12} also did not show any change in growth and phenotypes of the plants when exposed to As, Cd, Zn, salt and D- mannitol. Hence \textit{AtSAP12} might be directly involved in providing tolerance to certain heavy metal stresses but differently than \textit{AtSAP13}. This difference in tolerance to different heavy metals in \textit{AtSAP12} and \textit{AtSAP13} might be due to the presence of the different ZnF domains as \textit{AtSAP12} contains only two ANI domains and no C2H2 domain. In conclusion, \textit{AtSAP12} has great potential to be used in developing stress resistant crops. To do so, more extensive greenhouse stress studies need to be conducted on mature plants to confirm the role \textit{AtSAP12} in stress tolerance.
CHAPTER 5
OVEREXPRESSION OF \textit{AtSAP13} IN \textit{BRASSICA JUNCEA} FOR ENHANCING TOLERANCE TO MULTIPLE ABIOTIC STRESSES.

5.1 Introduction

The Indian mustard (\textit{Brassica juncea}) belongs to the \textit{Brassicaceae} family. The seed oil is used for food as cooking oil as well as for biofuel production. Seed residues from brassica are used in cattle feed and in fertilizers. Brassica is a high biomass and an important oil seed crop with 28.6\% and 45.7\% varying oil content\textsuperscript{91}. Because of its biomass and natural tolerance to toxic metals because of high sulfur contents, it is being heavily used for phytoremediation of lands contaminated with heavy metals\textsuperscript{92}. Since Brassica is close to Arabidopsis as they both belong to Brassicaceae family, it was decided to study if \textit{AtSAP13} results observed in Arabidopsis can be successfully translated into major crop plants.

As mentioned in chapter 3, \textit{AtSAP13} (AT3G57480) has been shown to provide tolerance to heavy metals As, Cd and Zn along with salt stress. Arabidopsis is a model system plant and it is harder to perform drought and salt stress assays on it under true field and greenhouse conditions. Therefore, it was decided to use a crop plant such as Brassica to study abiotic stresses in greater detail.

Thus, we transformed \textit{B. juncea} and analyzed the resulting transgenic plants for tolerance to abiotic stresses including heavy metals, drought and salt.
5.2 Results

5.2.1 Confirming the overexpression of AtSAP13 in B. juncea.

AtSAP13 was overexpressed in B. juncea under ACT2pt expression cassette, similar to what was used for Arabidopsis, using somatic embryogenesis method in Parkash laboratory. Three homozygous transgenic lines (1-1, 9-3, 4-2) were selected and overexpression of AtSAP13 in Brassica was confirmed by qRT-PCR using the procedure described under ‘Material and Method’ section.

The mRNA transcript levels of AtSAP13 overexpressing Brassica lines 1-1, 9-3, and 4-2 were significantly increased and showed overexpression in seedling tissue compared to wild type controls (Figure 5.1). Brassica AtSAP13 lines 1-1 and 9-3 showed more than 11-fold higher levels of transgene expression, whereas line 4-2 showed 3-fold higher levels of transgene expression compared to wild type.

![Figure 5.1](image)

Figure 5.1 qRT-PCR analysis of Brassica AtSAP13 overexpression lines. The means are averaged from three replicates and the error bars correspond to standard error of mean. Transcription levels of AtSAP13 in each line followed by single asterisks (*) are significantly different as compared to WT at p<0.05.
5.2.2 Analysis of overexpressed AtSAP13 Brassica homozygous lines on AsIII, Cd and Zn media:

AtSAP13 overexpressing Brassica lines 1-1, 9-3, and 4-2 along with wild type were grown on media supplemented with NaAsO$_2$ (70 μM), CdCl$_2$ (100 μM), ZnSO$_4$ (500 μM). Four replicates of each transgenic line along with wild type control for each treatment were obtained by sterilizing the seeds using the method described in the section 2.1.1 and then growing the seeds on ½ x MS plates vertically. After 21 days, root biomass and shoot biomass was collected and analyzed. This experimental set up was repeated to validate the results (see Appendix A6 and A7).

For AsIII treatment, wild type Brassica plants had stressed curled leaves with thin etiolated stems whereas transgenic plants were healthy with broad green leaves. Analysis of shoot biomass showed a weight gain of about 100% for lines 1-1 and 9-3 and nearly 200% for line 4-2 compared to wild type plants (Figure 5.2). Fresh root biomass of lines 1-1, 4-2, and 9-3 on arsenite treatment was also significantly higher compared to wild type plants. No phenotypic difference was observed between wild type and transgenic Brassica seedlings on the control media.

![Image](image.png)
For Cd treatment, wild type brassica plants had curled and small size leaves whereas transgenic plants were healthy with broad green leaves. Transgenic lines attained significantly higher biomass compared to controls. Shoot biomass weight gain for line 4-2 and 9-3 was average 30% higher compared to wild type plants (Figure 5.3 A, C and D). Fresh root biomass of lines 4-2, and 9-3 on cadmium treatment was also significantly higher compared to wild type plants. However, line 1-1 showed no change in shoot and root biomass compared to the wild type plants. Also, no phenotypic difference was observed between wild type and transgenic Brassica seedlings on the control media without any metals. This experimental set up was repeated to validate the results (see Appendix A8 and A9).

For Zn treatment, similar to plants for Cd treatment, wild type Brassica plants had
leaves that were small in size compared to plants of the transgenic lines. Shoot biomass weight gain of about 20% for line 1-1 and about 80% for lines 4-2 and 9-3 was seen compared to wild type plants. Fresh root biomass of transgenic lines 1-1, 4-2, and 9-3 were significantly higher compared to wild type plants (Figure 5.3 B, C and D). No phenotypic difference was observed between wild type and transgenic Brassica seedlings on the control media. This experimental set up was repeated to validate the results (see Appendix A8 and A9).

(A)

½x MS

WT 1-1 4-2 9-3

½x Cd 100µM

(B)

½x MS

WT 1-1 4-2 9-3

½x Zn 500µM
5.2.3 Accumulation of As, Cd and Zn in Brassica AtSAP13 transgenic lines.

Because of the observed strong tolerance to heavy metals such as As, Cd, and Zn, provided by Brassica AtSAP13 overexpression, we analyzed these plants for the accumulation of metals in shoot and root tissue. Three lines (#1-1, 4-2, and 9-3) of the AtSAP13 transgenic Brassica and wild type plants were grown and exposed to metals hydroponically and elemental analysis was estimated for As, Cd and Zn content in the roots and shoots. When exposed to 70 μM As and 500μM Zn for five days, both Brassica AtSAP13 overexpressing lines showed no difference in accumulation of metals either in shoots or in roots compared to wild type plants (Figure 5.4A and 5.4B). When grown on
100 µM Cd, the Brassica AtSAP13 overexpressing #1-1 had at least 25% more Cd accumulation in its roots but #4-2 and 9-3 showed no difference in accumulation of Cd in their roots compared to the wild type plants. Also, no difference in Cd accumulation in the shoots of these transgenic lines was seen compared to the wild type plants (Figure 5.4A and 5.4B).

![Figure 5.4](image)

**Figure 5.4** Accumulation of As, Cd, and Zn in Brassica *AtSAP13* overexpression lines. Total As, Cd and Zn accumulation in (A) roots and (B) shoots of WT and three Brassica *AtSAP13* overexpression lines grown on 1/2 MS hydroponics medium containing 70 µM sodium arsenite, 100 µM cadmium chloride and 500 µM zinc sulfate for 5 days. Data are averaged from at least three replicates of each. One-way ANOVA followed by LSD test was also used to determine the statistical significance of the values of metal accumulation. The values of each assay followed by different letters are significantly different at p < 0.05.

### 5.2.4 Analysis of Transgenic Brassica for salt and drought stress tolerance in tissue culture media

Brassica transgenic lines overexpressing AtSAP13 were subjected to salt stress and drought stress in tissue culture medium to check if AtSAP13 imparts tolerance to these stresses. These stresses were implied using NaCl for salt and D-mannitol for mimicking drought stress, respectively. Brassica wild type and *AtSAP13* transgenic plants were grown on ½ x MS media containing NaCl concentrations at 100mM and 150mM and
previously optimized D-mannitol concentration at 200mM. Seeds were germinated on these media and plants were allowed to grow for 21 days. At harvest, shoot and root biomass was harvested separately and analyzed.

No obvious phenotypic differences between the wild type and transgenic plants were observed except that the wild type plants were small compared to the transgenic plants of each line. At 100mM NaCl concentration, there was a 60% shoot biomass gain observed in transgenic line 1-1 and more than 200% for line 4-2 and 9-3 over wild type plants. At 150mM NaCl concentration, a gain of about 400% in shoot biomass of the transgenic lines over wild types plants was observed (Figure 5.5A and B). Significant difference was also observed in root biomass of all the transgenic lines on 150mM salt concentration compared to wild type. However only lines 1-1 and 9-3 showed significant root biomass difference at 100mM salt concentration compared to the wild type. Also, both the concentrations of salt (100mM and 150mM) affected the seed germination rate of the wild type Brassica plants with 150mM affecting more severely than the 100mM concentration (Figure 5.5C). Whereas all the transgenic lines at both 100 and 150 mM NaCl had significantly higher germination rate compared to the wild type Brassica plants (Figure 5.5C).
(A) ½x MS

½x Salt 100 mM

½x Salt 150 mM

WT  1-1  4-2  9-3

(B) Root biomass (g/10 plants)

CONTROL  S100  S150

WT  Sap13 1-1  Sap13 4-2  Sap13 9-3

(C) Shoot biomass (g/10 plants)

CONTROL  S100  S150

WT  Sap13 1-1  Sap13 4-2  Sap13 9-3
Figure 5.5 Brassica transgenic lines overexpressing AtSAP13 grown on 100 mM and 150 mM NaCl. 
(A) Photographs showing phenotypic differences, (B) Fresh shoot biomass and (C) Fresh root biomass of lines 1-1, 9-3 and 4-2 (D) germination rate of transgenic lines 1-1, 9-3 and 4-2 overexpression lines grown on ½ x MS media containing 100 mM and 150 mM NaCl for 21 days. Data are averaged from four replicates of 10 plants each. Values of fresh shoot and root biomass followed by double asterisks (**) are highly significant at p ≤ 0.01; Values of fresh shoot and root biomass followed by single asterisk (*) are significant at p ≤ 0.05.

When treated with 200 mM D-mannitol to apply drought stress, fresh shoot biomass of lines 1-1, 4-2, and 9-3 were significantly higher with about 13% biomass gain compared to wild type plants. Wild type Brassica plants on D-mannitol treatment were stunted and more stressed compared to the plants of the transgenic lines (Figure 5.6). No phenotypic difference was observed between wild type and transgenic Brassica seedlings on the control media. This experimental set up was repeated to validate the results (see Appendix A10).
5.2.5 Drought and salt stress tolerance studies in Greenhouse conditions

Drought and salt stress tolerance study was also carried out on Brassica transgenic lines overexpressing AtSAP13 in a long-term experiment set up under greenhouse conditions.
Experiment was performed as described under sections 2.4.1.1 and 2.4.2.1 of ‘Material and Methods’ for drought and salt stress tolerance experimental designs, respectively. Plants were grown till maturity and harvested at the end of their life cycle.

After acclimatizing Brassica wild type and transgenic plants for a period of ten days, they were subjected to reduced watering and water withholding to imply drought stress. Wild type and transgenic Brassica plants in control group (well watered controls) showed no change in phenotype throughout the treatment. However in the treatment group, around fifth day of water withholding, wild type Brassica showed leaf wilting earlier than transgenic lines (Figure 5.7A). By the end of the tenth day of water withholding (severe stress), wild type plants showed complete wilting and dryness. After the 10th day of drought stress, 75 mls of water was re-applied to the treatment pots (WT and transgenic) to assess their recovery. By the 5th day of recovery with water, one out of four wild type plants failed to show any signs of recovery even after addition of water while the other three recovered but the recovery and growth of these plants was slow and stunted (Figure 5.7B). Whereas, transgenic plants showed moderate wilting by the tenth day of drought stress and when assesses for recovery, showed complete recovery when re-watered (Figure 5.7C).

After recovery from drought stress, morphological parameters were analyzed where height of the plant, width and length of the most expanded mature leaf of the transgenic plants were found to be significantly greater compared to the wild type Brassica plants (Figure 5.7 D, E, and F).

There were no significant differences found in photosynthetic assimilation,
stomatal conductance and transpiration rate of the plants due to severe wilting between wild types and transgenics in the first set of readings taken after around seven days of water withholding. However, after recovering the plants from drought treatment, photosynthetic assimilation of the AtSAP13 Brassica transgenic plants accounted for about 250%, stomatal conductance accounted for about 700%, and transpiration rate of about 300% gain compared to the wild type Brassica plants (Figure 5.7 G, H, and I).

At maturity, all the four replicates of wild type and transgenic Brassica plants were harvested to look at differences in total shoot biomass, total seed weight, weight of 100 seeds, total number of pods per plant and average number of seeds per five pods per plant. About 33% of total shoot biomass gain for both the transgenic lines and about 75% gain in average number of seeds per pod for line 4-2 were observed for transgenic lines over the wild type brassica plants (Figure 5.7 J, and K). However, line 9-3 showed a difference in average number of seeds per pod compared to wild type but the difference was not significant enough. Similar pattern was also observed for lines 4-2 and 9-3 when analyzed for total seed weight. Line 4-2 showed significant difference in total seed weight compared to wild type plants however line 9-3 did not (Figure 5.7 L). No change in total number of seedpods per plant was observed between wild types and transgenics (Figure 5.7 M). Seed weight of 100 seeds was also analyzed for the two transgenic lines where line 9-3 showed significant difference compared to wild type whereas line 4-2 did not show significant difference. (Figure 5.7 N)
**C**

Comparing plant growth under different conditions:

- **WT**
- **#4-2**
- **#9-3**

### Control

- Plant height (cm)
- Leaf width (cm)
- Leaf length (cm)

### Drought recovery

- Photosynthetic assimilation (% baseline)

**D**

**Plant height (cm)**

- Control: WT, #4-2, #9-3
- Drought: WT, #4-2, #9-3

**Leaf width (cm)**

- Control: WT, #4-2, #9-3
- Drought: WT, #4-2, #9-3

**Leaf length (cm)**

- Control: WT, #4-2, #9-3
- Drought: WT, #4-2, #9-3

**Photosynthetic assimilation (% baseline)**

- Control: WT, #4-2, #9-3
- Drought: WT, #4-2, #9-3
- Recovery: WT, #4-2, #9-3

**G**

**Photosynthetic assimilation (% baseline)**

- Reading 1: WT, #4-2, #9-3
- Reading 2: WT, #4-2, #9-3
For salt stress, after acclimatizing Brassica wild type and transgenic plants for a period of ten days in the pots (see material and method section), they were subjected to water containing 150 mM NaCl to imply salt stress. Control wild type and transgenic Brassica plants showed no change in phenotype over a period of two weeks. However, at the end of the third week and six rounds of irrigation with salt water, all the wild type and transgenic plants showed stress symptoms and flowered early. By the sixth week and 12 rounds of watering with NaCl, plants finally died with immature seed pods on them.

Based on the phenotype, at moderate and severe salt stress stage, no difference in stomatal conductance and transpiration rate of wild type and transgenic plants were observed. Lines 4-2 and 9-3 however, showed significant difference among themselves when analyzed for stomatal conductance but compared to the wild type plants, the difference was not significant enough. Only transgenic line 9-3, in comparison to wild
type plants, showed significant difference in photosynthetic assimilation at the moderate
stage of salt stress (Figure 5.8 A, B, and C).

Once the plants died completely, they were harvested to look at differences in
total shoot biomass, total seed weight per plant, total number of pods per plant and
number of seeds per pods. No difference in total shoot biomass and total number of pods
per plant was seen between wild type and transgenic plants except for the significant
difference observed between the two transgenic lines on total number of pods per plant
analysis. However, both transgenic lines, 4-2 and 9-3 showed significant difference in
total seed weight compared to wild types. When analyzed for average number of seeds
per pod, line 4-2 showed significant reduction compared to the wild types, however, line
9-3 showed a difference but the difference was not significant enough compared to the
wild types (Figure 5.8 D, E, F, and G).
Figure 5.8 Brassica AtSAP13 overexpression lines subjected to 150mM salt (NaCl) stress. 
(A) Photosynthetic assimilation, (B) stomatal conductance, (C) transpiration rate, (D) total shoot biomass, (E) total seed weight, (F) average number of seed/5 pods and (G) total number of seed pods/plant. Data are averaged from four replicates of each line on each treatment. Values followed by double asterisks (**) are highly significant at $p \leq 0.01$; Values followed by single asterisk (*) are significant at $p \leq 0.05$.

5.3 Discussion

As mentioned above, *B. juncea* is a high biomass and an important oil seed crop$^{92}$ whose seed production and growth has declined over the years due to abiotic stresses, especially salinity and drought$^{93}$. Although, several studies have shown the differential regulation of gene/gene networks in Brassica in response to drought and salt stress$^{94,95,96}$ but still not much is understood about stress tolerance in Brassica. Thus, after obtaining the highly encouraging results in *AtSAP13* overexpressed Arabidopsis showing tolerance to heavy metals, salt and drought, we were interested to see if a similar effect is observed when *AtSAP13* is overexpressed in a crop plant such as Brassica. We found out that *AtSAP13* when overexpressed in Brassica provided significant tolerance to heavy metals namely
As, Cd and Zn. We speculate that the presence of the conserved cys- and his- residues in the putative heavy metal-binding motifs of the two AN1 domains and one C2H2 domain could be the reason for this significant metal tolerance and accumulation. As mentioned in chapter 3, recent findings have shown that the presence of multiple metal binding sites comprising of cys- and his- residues in the conserved ZnF domains might be protecting against metal stresses. This discovery further confirms our beliefs that presence of multiple metal binding motifs in its ZnF could be a reason for increased metal tolerance and accumulation provided by \textit{AtSAP13}.

In Arabidopsis, \textit{AtSAP13} was shown to be providing significant tolerance to salt mainly NaCl stress and drought through D-mannitol. In the past, strong tolerance to salt and drought stresses have been shown in SAP family members from rice namely \textit{OsiSAP8} and from \textit{A. littoralis}, \textit{AlSAP} due to the interaction of their ZnF domains. It is possible that the tolerance provided by \textit{AtSAP13} towards salt and drought was also due to a similar interaction between its ZnF domains. However, further research needs to be performed to find out exactly which interactions could be providing this tolerance.

Abiotic stresses such as salt and drought can lead to oxidative damage such as lipid peroxidation in plants. This damage further causes damage to the cell membrane and cell organelles. This coupled with stomatal closure to reduce gas and water exchange and disturbed or reduced enzyme activity such as that of CO\textsubscript{2} fixation affects various plant mechanisms that help to judge the growth of the plant in general. These mechanisms include photosynthetic assimilation, stomatal conductance, and transpiration rate. Other
morphological parameters that in general determine plant health, growth and overall yield and which get affected under abiotic stresses include leaf length, leaf width, plant height, total shoot biomass, seed weight, total number of seeds per plant and total number of pods per plant. Analyzing these parameters under drought stress and at maturity will give a better understanding of how plants behave under such abiotic stress conditions.

Under drought stress, drastic differences were observed in photosynthetic assimilation, stomatal conductance and transpiration rate of the *AtSAP13* transgenic lines compared to the wild type plants. It was also seen that the transgenic plants were better able to maintain normal growth, development and functioning under drought stress conditions compared to the wild type plants. This could be attributed to the ability of *AtSAP13* in providing tolerance to abiotic stresses such as salt and drought due to the presence and/or interactions of its ZnF domains. Also, interaction of SAP13 protein with 26S proteasome complex as discussed above could also be another reason for this tolerance\textsuperscript{53}, which needs further evaluation. However, inconclusive differences were observed between the transgenics and wild types under salt stress because of the early drying of the plants due to toxic salt concentrations, which needs to be redone with appropriate salt stress conditions.

To conclude, *AtSAP13* when overexpressed in Brassica, showed significant tolerance to heavy metals such as As, Cd and Zn in young plants. It also showed tolerance to drought and salt in short and long term experimental setups. Hence, *AtSAP13* might be directly involved in providing tolerance to certain heavy metal stresses as well as some abiotic stresses such as salinity and dehydration. These results clearly show that AtSAP13 and its homologs will be highly useful in engineering crops for enhanced
tolerance to multiple abiotic stresses. However, to develop such crops, the role of AtSAP13 in providing stress tolerance needs to be extensively studied on mature crops under greenhouse conditions.
6.1 Introduction

Another close relative of Arabidopsis, *Camelina sativa* (Camelina), which has a hexaploid genome, often referred to as ‘false flax’ or ‘gold of pleasure’, is an oilseed crop of the genus Cruciferae and is a rising biofuel crop. Camelina is native to southeastern Europe and southwestern Asia and is also naturalized in northern states of America.

It has been proposed by the Department of Energy (DOE) as a dedicated biofuel crop to produce biodiesel from seed oil. Seed oil content in Camelina has been estimated between 36-47% and along with more than 90% unsaturated fatty acid profile makes it an extremely important plant for fuel industry. Recently, there has been a tremendous interest in Camelina as a renewable biofuel crop. US navy has successfully tested their fighter jets with 50:50 blend of Camelina oil with jet fuels. Several commercial airlines such as Virgin Atlantic have also successfully tested the viability of Camelina oil for jet fuels. Also, Camelina meal is considered a desirable animal feed due to its additional high levels of essential fatty acids such as α-linolenic acid and linoleic acid, which corresponds to about 32-40% of total oil content.

It not only is a low agronomic input crop but also requires low cost for oil production due to its reduced need of fertilizers and pesticides. Furthermore, Camelina has a short life cycle of about 100 days, which makes it useful for cultivation in
regions that have short growing seasons. Also in some other regions, double crop can also be obtained for producing more oils and seed meal. Camelina has not only been shown to be resistant to a number of pathogens and diseases\textsuperscript{104} but also been found to be tolerant to abiotic stresses such as drought\textsuperscript{105}.

Due to all the above-mentioned aspects and its close relationship with well-studied Arabidopsis, it is essential to understand Camelina genome further and thus develop it to generate an abiotic stress tolerant crop. Therefore, in order to increase Camelina production on marginal, nutrient poor soil, the aim here is to characterize the \textit{SAP13} homolog gene from Camelina and overexpress \textit{CsSAP13} to increase the tolerance to various abiotic stresses.

\section*{6.2 Results}

\subsection*{6.2.1 Identification, cloning and confirmation of the overexpression of \textit{CsSAP13}.}

Sequence of one of the \textit{C. sativa SAP13} gene copies was obtained from Camelina database (http://www.camelinadb.ca/) by blasting Arabidopsis \textit{SAP13} sequence as a query against the Camelina genome. In the database, a \textit{CsSAP13} sequence has missing sequences from the C-terminus. Therefore, using Camelina leaf cDNA as a template, amplification from 3'UTR extending into C-terminus of \textit{CsSAP13} was performed using primers mentioned in table 1. Sequencing was performed on the obtained amplified sequence and also matched against Arabidopsis \textit{SAP13} gene sequence using ClustalW software to check for similarities between the two. \textit{CsSAP13} protein sequence was found to be highly similar to the Arabidopsis SAP13 sequence with conserved \textit{cys-} and \textit{his-}
residues. The other two gene copies of SAP13 from Camelina genome were also matched with Arabidopsis SAP13 sequence, of which only one was found to be highly similar and the third sequence had a lot of mismatches. Camelina CsSAP13 amino acid sequences are highly similar to AtSAP13 (Figure 6.1). CsSAP13, similar to AtSAP13 also contain 2 AN1 domains and one C2H2 domain (Figure 6.1).

Figure 6.1 Alignment of Camelina and Arabidopsis SAP13 genes carrying AN1 and C2H2 ZnF domains. The conserved cysteine and histidine residues of the two AN1 ZnF domains are marked with black asterisk and the conserved cysteine and histidine residues of the C2H2 ZnF domains are highlighted with red asterisk.

Cloning of CsSAP13 gene and ACT2 promoter was performed as mentioned in section 2.6.6 of ‘Material and Methods’. Act2p/CsSAP13 construct (Figure 6.2) was transformed in Camelina using flower dip method. Transformed Camelina plants were raised in the greenhouse and mature brown pods were harvested 50-60 days after transformation. T₀ seeds were screened using DsRed filter for the presence of transgenic
seeds emitting red fluorescence due to the expression of \textit{DsRed} reporter gene in which \textit{Act2p/CsSAP13} were cloned (Figure 6.2A). Transgenic seeds were obtained and grown in soil to raise next generation T\textsubscript{1} seeds. T\textsubscript{1} seeds were again screened using DsRed filter for the presence of \textit{DsRed} gene and eight lines showing 75\% DsRed were selected to get next generation T\textsubscript{2} homozygous seeds (Figure 6.2B).

\textbf{Figure 6.2} Analysis of \textit{CsSAP13} transformed Camelina seeds (A) T\textsubscript{0} \textit{CsSAP13} transgenic seeds under DsRed filter, (B) Homozygous \textit{CsSAP13} seeds under DS-Red filter.

After screening T\textsubscript{2} lines, out of several 100\% DsRed seed lines obtained, three best homozygous lines (2, 8, and 13) were selected based on their qRT-PCR expression profile. qRT-PCR was performed as mentioned under ‘Material and Method’ section. Transcript levels of \textit{CsSAP13} overexpressing Camelina lines 2, 8, and 13 were
significantly increased and showed overexpression in seedling tissue compared to wild type (Figure 6.3). All the three Camelina CsSAP13 lines showed more than 6-fold higher levels of transgene expression compared to wild type.

![Graph showing qPCR analysis of Camelina CsSAP13 overexpression lines.](image)

**Figure 6.3** qPCR analysis of Camelina CsSAP13 overexpression lines. The means are averaged from three replicates and the error bars correspond to standard error of mean. Transcription levels of CsSAP13 in each line followed by single asterisk (*) are significantly different as compared to WT at p<0.05.

### 6.2.2 Analysis of CsSAP13 overexpressing *C. sativa* lines for AsIII, Cd and Zn tolerance

In order to evaluate the role of *CsSAP13* in providing tolerance to toxic metals, T2 homozygous Camelina lines (2, 8, and 13) overexpressing CsSAP13 were analyzed on media supplemented with NaAsO₂ (25 µM), CdCl₂ (75 µM), ZnSO₄ (500 µM). After sterilizing the seeds using the method described in the section 2.1.1, seeds of each transgenic lines and WT control, were grown vertically on ½ MS plates supplemented with appropriate levels of toxic metals. After 21 days, root biomass and shoot biomass was collected and analyzed.
For AsIII treatment, plants of wild type Camelina were severely stressed, barely had any leaves and grew with stunted and thin stems. Whereas, plants of transgenic lines were healthy with broad green leaves. Transgenic lines attained at least 100% or more shoot biomass for lines 2, 8 and 13 compared to wild type plants (Figure 6.4). No change was observed in fresh root biomass between the wild type and transgenic lines on AsIII. No phenotypic difference was observed between wild type and transgenic Camelina seedlings on the control media.

For Cd treatment, there were no obvious shoot biomass or phenotypic differences between wild type and transgenic Camelina plants on Cd treatment except for line13, which grew taller than the other transgenic and wild type plants. However, fresh root biomass of lines 2, 8 and 13 on Cd treatment was significantly higher compared to wild type plants (Figure 6.4). Also, no phenotypic difference was observed between wild type and transgenic Camelina seedlings on the control media.

For Zn treatment, no difference in shoot biomass between wild type and transgenic Camelina plants were seen. However, fresh root biomass of lines 2, 8 and 13 on Zn treatment was highly significant compared to wild type plants (Figure 6.4). Also, no phenotypic difference was observed between wild type and transgenic Camelina seedlings on the control media.
6.2.3 Accumulation of As, Cd and Zn in Camelina CsSAP13 transgenic lines.

Because Camelina CsSAP13 overexpression lines provided strong tolerance to heavy metals such as As, Cd and Zn, we analyzed these plants for the accumulation of metals in shoot and root tissue. Two lines (#8, 13) of the CsSAP13 transgenic Camelina and wild type plants were grown and exposed to metals hydroponically and elemental analysis was estimated for As, Cd and Zn content in the roots and shoots. When exposed to 25 μM As for five days, CsSAP13 overexpressing lines accumulated at least 25% more As in root tissues and at least 38% less As in shoot tissues compared to wild type plants (Figure 6.5A and 6.5B). When grown on 75 μM Cd, the CsSAP13 overexpressing lines showed no difference in Cd accumulation in their roots or shoots as compared to the wild type plants (Figure 6.5A and 6.5B). On 500 μM Zn, CsSAP13 overexpressing #13 accumulated about 32% less Zn in its root, however, #8 showed no difference in
accumulation compared to WT plants. These two transgenic lines also showed no
difference in accumulation of Zn in shoots compared to WT plants (Figure 6.5A and
6.5B).

**Figure 6.5** Accumulation of As, Cd, and Zn in Camelina CsSAP13 overexpression lines. Total As, Cd and Zn accumulation in (A) roots and (B) shoots of WT and two Camelina CsSAP13 overexpression lines grown on 1/2 MS hydroponics medium containing 25 µM sodium arsenite, 75 µM cadmium chloride and 500 µM zinc sulfate for 5 days. Data are averaged from at least three replicates of each. One-way ANOVA followed by LSD test was also used to determine the statistical significance of the values of metal accumulation. The values of each assay followed by different letters are significantly different at p < 0.05.

6.2.4 Salt stress tolerance in CsSAP13 overexpressing Camelina in tissue culture media

Camelina CsSAP13 transgenic lines were also subjected to salt stress in a short-term experiment on tissue culture media to check for their tolerance. NaCl was used to imply this stress. Camelina wild type and CsSAP13 transgenic plants were grown on ½ MS media containing NaCl concentration at 150mM. After 21 days, shoot biomass and root biomass was harvested and analyzed.

Camelina wild type and transgenic seedlings on the control media showed no
phenotypic difference. On media containing NaCl, wild type plants were severely stressed. WT plants barely had any leaves and stems were extremely stunted and thin, whereas transgenic lines were grew healthy with green leaves and normal stems (Figure 6.6A). At 150 mM NaCl concentration, transgenic lines 2 and 8 attained around 400% and line 13 attained around 900% more shoot biomass, respectively, compared to wild type plants (Figure 6.6B). No difference was observed in root biomass for all the transgenic lines on 150 mM salt concentration compared to wild type (data not shown). Also, 150mM concentration of salt severely affected the germination rate of the wild type Camelina plants but not the CsSAP13 transgenic lines. On the above-mentioned salt concentration, all the transgenic lines had significant to highly significant germination rate compared to the wild type Camelina plants (Figure 6.6C).
Figure 6.6 Camelina CsSAP13 overexpression lines on 150mM NaCl. (A) Phenotypic expression, (B) Fresh shoot biomass of lines 2, 8 and 13 (C) germination rate of overexpression lines grown on ½ x MS media containing 150mM NaCl for 21 days. Data are averaged from four replicates of 10 plants each. Values of fresh shoot and root biomass followed by double asterisks (**) are highly significant at p \( \leq 0.01 \); Values of fresh shoot and root biomass followed by single asterisk (*) are significant at p \( \leq 0.05 \).

6.2.5 Long term drought and salt stress tolerance assay in greenhouse conditions

Drought and salt stress tolerance study was also carried out on Camelina CsSAP13 transgenic lines in a long-term experiment set up under greenhouse conditions. Experiment was performed as described under sections 2.4.1.2 and 2.4.2.2 of ‘Material and Methods’ for drought and salt stress tolerance experimental designs respectively. Plants were grown to maturity and harvested at the end of their life cycle.

After acclimatizing Camelina wild type and transgenic plants to the new environment for a period of ten days, they were subjected to three different water volumes – D1 pots were watered with 250 ml water, D2 with 150 ml and D3 with only 75 ml water to imply no, moderate and severe drought stress, respectively. Plants were grown and harvested at maturity. D1 wild type and transgenic Camelina plants, which served as the controls, showed no change in phenotype. D2 pots of wild type and transgenic Camelina plants, which were watered with 150 ml also showed no phenotypic
difference. Wild type Camelina and transgenic plants when subjected to reduced watering (75 ml) in D3 labeled pots, by the end of second week after acclimatizing, started showing moderate stress signs such as the reduced turgidity of the attached leaves but still no obvious phenotypic change among them. By the fourth week of treatment, it was observed that the leaves of the wild type Camelina plants under continuous reduced watering, wilted severely several hours faster than the transgenic plants. However, despite of plant types, all the plants exhibited wilting by late afternoon. Plants in D1, D2 and D3 treatments pots were watered as and when needed but treated equally.

Morphological parameters were analyzed during the course of experiment where plant height, width, and length of the most expanded mature leaf of the transgenic plants, and number of branches per plant were measured. Two sets of readings for these parameters were taken in week two (reading 1) and in week four (reading 2) of drought stress respectively (Figure 6.7 B, C, D and E). Significant differences were observed in terms of leaf width and leaf length for line 13 under severe drought stress compared to other transgenic lines and WT control. Significant differences were also observed for line 8 and 13 for plant height where line 8 grew significantly shorter and line 13 grew significantly taller compared to wild type plants under moderate drought stress. Line 13 also had significantly greater number of branches per plant under severe stress (Figure 6.7 B, C, D and E). However, line 13 was in general found to be growing better in terms of number of branches per plant (Figure 6.7 C).

Two sets of readings for photosynthetic assimilation, stomatal conductance and transpiration rate were taken in week two (reading 1) and in week four (reading 2) of drought stress respectively. For Photosynthetic assimilation and transpiration rate
measurement, there were no significant differences found due to moderate drought (D2) stress between wild types and transgenics analyzed initially in the 2nd week of water stress (Figure 6.7 F and H), except for lines 8 and 13 which showed significant differences in stomatal conductance with line 13 being more significant than line 8 compared to wild type plants under D2 treatment (Figure 6.7 G). Under D3 treatment of moderate stress stage, line 13 showed significant difference in terms of stomatal conductance compared to wild types, however, line 8 showed a difference but the difference was not significant enough. At the severe wilting stage (D3-2), photosynthetic assimilation of CsSAP13 in line 13 accounted for about 67%, stomatal conductance and transpiration rate accounted for about 100% gain for each compared to the wild type Camelina plants (Figure 6.7 F, G, and H). Line 8 under D2 severe stress stage (D2-2) showed about 16% greater photosynthetic assimilation, 25% gain for stomatal conductance and about 14% greater transpiration rate (Figure 6.7 G).

Total chlorophyll contents were also significantly increased in line 13 at severe drought stress (D3) compared to wild type plants. No change in total chlorophyll content was seen at moderate (D2) or severe stress (D3) in line 8 (Figure 6.7 I). Further, line 8 showed a significant decrease in relative water content (RWC) of the plant leaves from the reduced water pots under moderate (D3) stress, whereas line 13 showed a difference but the difference was not significant enough when compared to wild type plants. No change was seen in RWC for lines 8 and 13 at the severe stress stage (Figure 6.7 J).

At maturity, all five replicates of wild type and transgenic Camelina plants were harvested and differences in total shoot biomass, total seed weight, seed weight of 100 seeds, total number of pods per plant and average number of seeds per pods were
analyzed. Transgenic lines 8 and 13 showed a significant increase in the average number of seeds per pods from the pots that were placed under drought stress compared to wild type plants. No change was seen between wild type and transgenic plants in control and reduced water pots. Seed weight of 100 seeds was also analyzed under which line 8 showed significant increase D1 and D2 sets compared to line 13 and wild types. However, there was no difference in 100 seed weight under D3 stress treatment. There was no change between wild types and transgenics in any of the treatments when analyzed for total shoot biomass, total seed weight and total number of pods per plant (Figure 6.7 K, L, M, N and O).
(A) Before treatment

(B) Initial stage

(C) Severe stress

(B) Severe stress

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Leaf width (cm)

[Bar chart showing leaf width measurements with different readings and various treatments indicated by letter groups (a, b, c, etc.).]

Leaf length (cm)

[Bar chart showing leaf length measurements with different readings and various treatments indicated by letter groups (a, b, c, etc.).]

Photosynthesis (baseline+)

[Bar chart showing photosynthesis measurements with different readings and various treatments indicated by letter groups (a, b, c, etc.).]

Transpiration rate (baseline+)

[Bar chart showing transpiration rate measurements with different readings and various treatments indicated by letter groups (a, b, c, etc.).]

Total chlorophyll (baseline+)

[Bar chart showing total chlorophyll measurements with different readings and various treatments indicated by letter groups (a, b, c, etc.).]
Figure 6.7 Camelina CsSAP13 overexpression lines subjected to drought stress (D1, D2 and D3).

(A) severe stress – after four weeks of reduced watering, (B) plant height, (C) total number of branches, (D) leaf width, (E) leaf length, (F) photosynthetic assimilation, (G) stomatal conductance, (H) transpiration rate, (I) total chlorophyll, (J) relative water content, (K) total seed weight, (L) total shoot biomass, (M) total number of seed
pods/plant, (N) average number of seed/pod and (O) seed weight of 100 seeds. Data is normalized against the controls for each line and are averaged from five replicates of each line on each treatment. Values followed by double asterisks (**) are highly significant at $p \leq 0.01$; Values followed by single asterisk (*) are significant at $p \leq 0.05$.

For salt stress, after acclimatizing to the new environment for a period of ten days, Camelina wild type and transgenic plants were subjected to 250 ml of water containing NaCl at concentrations of 100 mM (S1) and 150 mM (S2) to imply moderate and severe salt stress. By the end of second week after acclimatizing, plants from control as well as treatment started showing moderate stress signs such as reduced plant height and reduced leaf length. This quickly turned to severe wilting and extreme dryness by the fourth week of treatment. By the end of the 4th week, wild type and transgenic plants under 150 mM salt concentration (S2) did not survive to reach maturity and died early with immature pods and seeds. Control wild type and transgenic Camelina plants without salt stress showed no change in phenotype.

Morphological parameters were analyzed during the course of salt stress experiment where plant height, width and length of the most expanded mature leaf of the plants, number of branches per plant were measured (Figure 6.8 B, C, D, and E). Two sets of readings for these parameters were taken in week two (reading 1) and in week four (reading 2) of drought stress respectively. When exposed to higher salt concentration (S2-150 mM NaCl), significant differences were observed for lines 8 and 13 in terms of number of branches per plant under moderate stress. Line 13 was also observed to have significantly greater number of branches under 100mM S1 severe stress stage. However, line 13 was in general found to be growing better compared to wild type and line 8 was in general found to be growing shorter compared to wild type and line13 in each treatment
and controls in terms of plant height (Figure 6.8 B and C). Significant differences were also observed in terms of leaf width and length for line13 plants under 100 mM NaCl concentration under moderate and severe stress stage (Figure 6.8 D and E).

Two sets of readings for photosynthetic assimilation, stomatal conductance and transpiration rate were taken in week two (reading 1) and in week four (reading 2) of drought stress respectively. Under moderate salt stress, gain in photosynthetic assimilation for CsSAP13 overexpression lines 8 and 13 accounted for above 12% in S1 treatment (100 mM NaCl), about 15% for line 8 in S2 treatment-150 mM NaCl. Line 13 under S2 treatment showed a difference but the difference was not significant enough compared to wild type plants (Figure 6.8 F). Stomatal conductance for line 13 accounted for 57% in S2 plants compared to the wild type Camelina plants (Figure 6.8 G). S1 plants subjected to severe stress showed 60% and 180% gain in photosynthetic assimilation and stomatal conductance, respectively, for line 13 compared to WT controls. Further, S1 transgenic lines 8 and 13 showed 62% and 150% gain in transpiration rate, respectively, compared to controls (Figure 6.8 F, G, and H).

No change in total chlorophyll was seen at moderate and severe stress under S1 treatment (Figure 6.8 I). Also, no significant difference was seen in RWC of lines 8 or 13 compared to wild type Camelina plants at the moderate or severe stress stage (Figure 6.8 J).

At maturity, all the five replicates of wild type and transgenic Camelina plants were harvested and were analyzed for differences in total shoot biomass, total seed weight per plant, 100 seed weight, total number of pods per plant and average number of
seeds per pods. An increase in the total seed weight was seen for line 13 compared to the wild types under S1 treatment however, it was not significant enough (Figure 6.8 K). Transgenic line 13 under S1 treatment also showed significant increase compared to the wild type and line 8 plants in the average number of seeds per pod (Figure 6.8 N). No difference in total shoot biomass as well as average number of seed pods was seen between wild type and transgenic plants under S1 treatment (Figure 6.8 L and M). Line 8 showed significant difference when analyzed for 100 seed weight under control and treatment pots compared to wild types and line 13 (Figure 6.8 O). Because plants under S2 treatment died prematurely, hence, no readings for these parameters were taken for this set.
Figure 6.8 Camelina CsSAP13 overexpression lines subjected to 100mM (S1) and 150mM (S2) of salt stress. (A) severe stress – after four weeks of salt water addition, (B) plant height, (C) total number of branches, (D) leaf width, (E) leaf length, (F) photosynthetic assimilation, (G) stomatal conductance, (H) transpiration rate, (I) total chlorophyll, (J) relative water content, (K) total seed weight, (L) total shoot biomass, (M) total number of seed pods/plant, (N) average number of seeds/pod and (O) seed weight of 100 seeds. Data is normalized against the controls for each line and are averaged from five replicates of each line on each treatment. Values followed by double asterisks (**) are highly significant at $p \leq 0.01$; Values followed by single asterisk (*) are significant at $p \leq 0.05$.

6.3 Discussion

*C. sativa* is an oil seed crop that is getting popular for its short life cycle, low agronomical input requirement, recently sequenced genome, and most importantly, its biomass content and potential for being used as a biofuel crop\textsuperscript{106}.

Not much is known about the genes and pathways that get involved in the stress response of Camelina to abiotic stresses and oil biosynthesis pathways. Hence, after obtaining results showing strong tolerance to multiple abiotic stresses in Arabidopsis and Brassica overexpressing *AtSAP13*, we wanted to see if *SAP13* from Camelina genome also responds to abiotic stresses like the Arabidopsis *SAP13* gene.
The presented results here showed that CsSAP13 when overexpressed in Camelina provided significant tolerance and accumulation of heavy metals namely As, Cd and Zn. Because of the high degree of homology between Arabidopsis and Camelina SAP13 genes, we hypothesize that there must be putative heavy metal-binding motifs in the conserved stretches of cys- and his- residues present in CsSAP13 similar to AtSAP13 and that could be the reason for this significant metal tolerance and accumulation. Previously, it has been shown that in Arabidopsis, presence of multiple metal binding sites comprised of conserved cys- and his- residues at the C-terminus were able to enhanced tolerance to metal stresses such as that of Zn and Cd such as in HMA family from Arabidopsis - AtHMA2\textsuperscript{84} and AtHMA4\textsuperscript{85}. Also, a number of Arabidopsis SAP genes including SAP10, SAP11 and SAP13 which also share a high degree of sequence homology with CsSAP13 and with similar conserved cys- and his- residue metal binding motif pattern, were shown to provide a similar metal stress tolerance and accumulation effect when subjected to toxic concentrations of heavy metals\textsuperscript{57,109}. These facts further reinforce our beliefs that CsSAP13 might be providing metal tolerance due to the presence of multiple metal binding motifs in its conserved domains.

CsSAP13 was also shown providing significant tolerance to salt stress in a short term study grown in $\frac{1}{2}$ x media supplemented with NaCl. Also, salt stress in Camelina was found to be inhibiting the germination rate at significantly higher rate than transgenic lines under salt stress. Similar strong tolerance to salt stress have been shown in other SAP family members from rice namely OsiSAP8\textsuperscript{55} and from *A. littoralis*, AISAP\textsuperscript{68}. These SAP genes also carry similar conserved cys- and his- residue pattern. Earlier, an *in-silico*
analysis of the AtSAP13 promoter sequence upstream to the start codon identified many dehydration and salt stress associated cis elements such as DREB\(^{86}\), ERE\(^{87}\), HSE\(^{89}\), etc, that leads us to speculate that similar interaction between the cis-elements and associated TFs could be leading to the activation of stress-associated genes to protect against salt stress in Camelina too. However, further research needs to be performed to find out exactly which protein-DNA interaction could be providing salt tolerance.

Camelina transgenic plants when exposed to drought and salt in a long term greenhouse study, the data obtained was found to be inconclusive. 150 mM salt concentration proved to be extremely toxic to the wild type and transgenic plants as a result all the plants under S2 treatment in the long term experimental set up wilted and died with immature seeds and seed pods before attaining maturity. Long term exposure to salt stress (150 mM) seems too toxic for Camelina. Therefore, salt stress conditions for Camelina need to be optimized or plants should be treated with salt stress for short term and effects should be analyzed based on the amount of salinity in the treated soil. Oxidative damage such as lipid peroxidation causes cell organelle and structural damage and occurs due to abiotic stresses\(^{97}\). Under unstressed conditions, during the process of photosynthesis, loss of water and uptake and fixation of CO\(_2\) occurs through the stomatal openings. However, when plants are subjected to stresses such as drought and salt, one of the most crucial plant stress response includes the regulation of the stomatal opening. Under such stress conditions, leaf stomatal aperture is regulated (stomatal conductance) to control the water loss from the leaf (transpiration rate) to protect plant against drought stress and consequently restricts CO\(_2\) diffusion into the leaf (photosynthetic
assimilation). A plant that is more severely affected by an abiotic stress such as drought will have its stomata shut down much more, affecting its transpiration rate and CO$_2$ uptake, than a plant that is not so much affected by the drought stress. These parameters, thus in general help to judge the growth of the plant$^{97,118}$. Other morphological parameters that in general determine plant health, growth and overall yield and which get affected under abiotic stresses include leaf length, leaf width, plant height, total shoot biomass, total number of seeds per plant and total number of pods per plant. Analyzing these parameters under drought stress and at maturity will give a better understanding of how plants behave under such abiotic stress conditions.

Under salt and drought stress, no conclusive differences were observed in photosynthetic assimilation, stomatal conductance and transpiration rate of the transgenics compared to the wild type plants. It was also seen that one of the transgenic lines (13) grew better than the wild type and other transgenic line and was able to maintain normal growth, development and functioning under these stress conditions. Second transgenic line (8) grew healthy but had a different growth pattern compared to 13 and wild type plants. Significant differences observed under various analysis carried out for drought and salt stress were random and could have risen due to a number of factors such as one of the transgenic line in general performed better than the other lines. It is possible that one line did better than the other due to pleiotropic effect of the gene being inserted in the region of the Camelina genome, which in general promotes growth of the plant. Another factor might be that individual variance of Camelina was not uniform among the treatments. Although Cs$SAP13$ overexpressed Camelina showed
significant tolerance to heavy metal stresses and salt in a short-term experiment performed under controlled laboratory conditions, it might not be able to counteract the abiotic stress caused by drought and salt in the greenhouse conditions which mimic real field conditions where a combination of factors can affect the growth and yield of a crop. Hence, this experimental set up needs to be repeated and further evaluation needs to be done to find out more about drought and salt stress tolerance in Camelina.

To conclude, *CsSAP13* when overexpressed in Camelina, showed significant tolerance to heavy metals such as As, Cd and Zn in young seedlings. It also showed tolerance to salt in short term experimental set up and demonstrated promising results in terms of providing tolerance to a number of abiotic stresses. Along with more detailed greenhouse studies on mature plants, *CsSAP13* overexpressed Camelina plants have great potential to be used as stress tolerant crop in future.
Plants are subjected to various abiotic stresses throughout their life cycle and these abiotic stresses such as heavy metal stress, drought, salinity, etc. are few of the major reasons for the decline in agricultural production worldwide. However, due to the limited knowledge available on the mode of actions of various stress-related genes, not much has been achieved in terms of improving plant species and/or increasing crop production.

Homologs of AIRAPs which have shown protection against AsIII in *C. elegans* and mouse cells were recently designated as SAPs and were found to have characteristic features of possessing either A20, AN1 or both the ZnF domains along with C2H2 domain in some of the SAPs and have shown to impart tolerance to multiple abiotic stresses in different plant species. In the recent years, a lot of work has been done on SAPs in various plant and animal species but not much is understood about the SAP family in terms of their mode of action. In this study, we have not only characterized one of the SAP genes namely *SAPI3* in Arabidopsis, but we have also tried to unravel some of the details about its mode of action in Arabidopsis through Y1H assay. We found out that *AtSAPI3* when overexpressed in Arabidopsis, provide tolerance to heavy metals such as As, Cd, and Zn and also salt. This tolerance is suspected to be either being provided by the presence of metal binding domains in the conserved *cys*- and *his*- residues of their ZnF domains or by the interaction of the *cis*- elements in the promoter of *AtSAPI3* with the stress-associated TFs or may be due to the interaction of *SAPI3* with the 26S
proteasome which could be leading to breakdown of misfolded and aggregated polyubiquitinated proteins formed due to abiotic stresses\textsuperscript{53}. When expression of AtSAP13 and one of its AN1 domains was knockdown through the RNAi approach, there was no change in tolerance to any abiotic stresses between wild type Arabidopsis plants and \textit{AtSAP13} or it’s AN1 domain probably due to functional redundancy in the AtSAP family since Arabidopsis contains 14 SAP genes. On Y1H analysis, 18 candidate TFs interacting with the \textit{cis}- elements in the promoter of AtSAP13 were identified. Out of which we tried to purify and confirm the interaction obtained through Y1H for four stress related putative TFs. However, due to the inability to confirm this interaction we put a hold on this for now. Arabidopsis is a model plant, hence we wanted to see if we could get the same tolerance to abiotic stresses when \textit{AtSAP13} is overexpressed in Brassica, which is an oil crop. Overexpressed AtSAP13 in Brassica showed tolerance to As, Cd, Zn, salt as well as drought. Homolog of \textit{AtSAP13}, \textit{CsSAP13} was also overexpressed in Camelina (another oil crop) and similar tolerance results were obtained. CsSAP13 was seen providing tolerance to heavy metals including As, Cd, Zn as well as salt. However, drought tolerance results were inconclusive and thus need to be repeated.

We also confirmed the overexpression of AtSAP12 in Arabidopsis. Overexpressed AtSAP12 was found providing tolerance to As, and Zn but not to salt, mannitol or Cd. Also, compared to wild type Arabidopsis plants, RNAi knockdown lines of \textit{AtSAP12} did not show any difference in tolerance to any abiotic stress including As, Cd, Zn, salt as well as mannitol due to functional redundancy in the AtSAP family.
In conclusion, new information on *SAP13* as well as *AtSAP12* has been obtained. This knowledge could be applied to grow crops for increased biomass and oil production such as Camelina and Brassica, which have already shown that on overexpressing SAP13 in them, they provide increased tolerance to abiotic stresses. The knowledge could also be used to engineer other agricultural crops to not just make them more tolerant to abiotic stresses but also produce sustainable yield.\textsuperscript{73,108}
## APPENDIX

### ADDITIONAL TABLES

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Table 8.1 Fresh shoot biomass of Arabidopsis SAP13 overexpression lines and wild type controls grown on 1/2MS plates containing 30 µM Sodium arsenite, 75 µM cadmium chloride and 500 µM zinc sulfate for 21 days (replicate data). Data are averaged from four replicates of 10 plants each. Values of fresh biomass followed by single asterisk (*) are significant at \( p \leq 0.05 \).

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Table 8.2 Root length of Arabidopsis SAP13 overexpression lines and wild type controls grown on 1/2MS plates containing 30 µM Sodium arsenite, 75 µM cadmium chloride and 500 µM zinc sulfate for 21 days (replicate data). Data are averaged from four replicates of 10 plants each. Values of root length followed by single asterisk (*) are significant at \( p \leq 0.05 \).

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Table 8.3 Fresh shoot biomass of Arabidopsis SAP13 overexpression lines and wild type controls grown on 1/2MS plates containing 100 mM NaCl for 21 days (replicate data). Data are averaged from four replicates of 10 plants each. Values of fresh biomass followed by single asterisk (*) are significant at \( p \leq 0.05 \).
Table 8.4 Fresh shoot biomass of Arabidopsis SAP12 overexpression lines and wild type controls grown on 1/2MS plates containing 30 µM Sodium arsenite and 500 µM zinc sulfate for 21 days (replicate data). Data are averaged from four replicates of 10 plants each. Values of fresh biomass followed by single asterisk (*) are significant at p ≤ 0.05.

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Table 8.5 Root length of Arabidopsis SAP12 overexpression lines and wild type controls grown on 1/2MS plates containing 30 µM Sodium arsenite and 500 µM zinc sulfate for 21 days (replicate data). Data are averaged from four replicates of 10 plants each. Values of root length followed by single asterisk (*) are significant at p ≤ 0.05.

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Table 8.6 Fresh shoot biomass of Brassica AtSAP13 overexpression lines and wild type controls grown on 1/2MS plates containing 30 µM Sodium arsenite for 21 days (replicate data). Data are averaged from four replicates of 10 plants each. Values of fresh shoot biomass followed by single asterisk (*) are significant at p ≤ 0.05.

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<td>Sap13 1-1</td>
<td>2.218</td>
<td>0.597*</td>
<td>0.127</td>
<td>0.016</td>
</tr>
<tr>
<td>Sap13 4-2</td>
<td>2.234</td>
<td>0.937*</td>
<td>0.040</td>
<td>0.072</td>
</tr>
<tr>
<td>Sap13 9-3</td>
<td>2.323</td>
<td>0.927*</td>
<td>0.050</td>
<td>0.050</td>
</tr>
</tbody>
</table>
### Table 8.7 Fresh root biomass of Brassica AtSAP13 overexpression lines and wild type controls grown on 1/2MS plates containing 30 µM Sodium arsenite for 21 days (replicate data).

Data are averaged from four replicates of 10 plants each. Values of fresh root biomass followed by single asterisk (*) are significant at $p \leq 0.05$.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>As III</th>
<th>SE-C</th>
<th>SE-AsIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.192</td>
<td>0.055</td>
<td>0.023</td>
<td>0.001</td>
</tr>
<tr>
<td>Sap13 1-1</td>
<td>0.199</td>
<td>0.092*</td>
<td>0.018</td>
<td>0.012</td>
</tr>
<tr>
<td>Sap13 4-2</td>
<td>0.207</td>
<td>0.127*</td>
<td>0.012</td>
<td>0.005</td>
</tr>
<tr>
<td>Sap13 9-3</td>
<td>0.215</td>
<td>0.107*</td>
<td>0.004</td>
<td>0.002</td>
</tr>
</tbody>
</table>

### Table 8.8 Fresh shoot biomass of Brassica AtSAP13 overexpression lines and wild type controls grown on 1/2MS plates containing 75 µM cadmium chloride and 500 µM zinc sulfate for 21 days (replicate data).

Data are averaged from four replicates of 10 plants each. Values of fresh shoot biomass followed by single asterisk (*) are significant at $p \leq 0.05$.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Zn</th>
<th>Cd</th>
<th>SE-C</th>
<th>SE-Zn</th>
<th>SE-Cd</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>2.819</td>
<td>0.920</td>
<td>0.418</td>
<td>0.057</td>
<td>0.015</td>
<td>0.012</td>
</tr>
<tr>
<td>Sap13 1-1</td>
<td>2.884</td>
<td>1.116*</td>
<td>0.492</td>
<td>0.118</td>
<td>0.080</td>
<td>0.023</td>
</tr>
<tr>
<td>Sap13 4-2</td>
<td>2.747</td>
<td>1.241*</td>
<td>0.702*</td>
<td>0.061</td>
<td>0.038</td>
<td>0.070</td>
</tr>
<tr>
<td>Sap13 9-3</td>
<td>3.006</td>
<td>1.221*</td>
<td>1.055*</td>
<td>0.168</td>
<td>0.071</td>
<td>0.060</td>
</tr>
</tbody>
</table>

### Table 8.9 Fresh root biomass of Brassica AtSAP13 overexpression lines and wild type controls grown on 1/2MS plates containing 75 µM cadmium chloride and 500 µM zinc sulfate for 21 days (replicate data).

Data are averaged from four replicates of 10 plants each. Values of fresh root biomass followed by single asterisk (*) are significant at $p \leq 0.05$.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Zn</th>
<th>Cd</th>
<th>SE-C</th>
<th>SE-Zn</th>
<th>SE-Cd</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.288</td>
<td>0.042</td>
<td>0.153</td>
<td>0.009</td>
<td>0.004</td>
<td>0.008</td>
</tr>
<tr>
<td>Sap13 1-1</td>
<td>0.354</td>
<td>0.067*</td>
<td>0.178</td>
<td>0.070</td>
<td>0.010</td>
<td>0.025</td>
</tr>
<tr>
<td>Sap13 4-2</td>
<td>0.274</td>
<td>0.061*</td>
<td>0.294*</td>
<td>0.014</td>
<td>0.006</td>
<td>0.015</td>
</tr>
<tr>
<td>Sap13 9-3</td>
<td>0.389</td>
<td>0.071*</td>
<td>0.251*</td>
<td>0.064</td>
<td>0.010</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>200mm Mannitol</td>
<td>SE-C</td>
<td>SE-200mM M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>---------</td>
<td>----------------</td>
<td>-------</td>
<td>------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>WT</strong></td>
<td>2.619</td>
<td>0.716</td>
<td>0.0796</td>
<td>0.0174</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sap13 1-1</strong></td>
<td>2.608</td>
<td>0.847*</td>
<td>0.1051</td>
<td>0.0472</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sap13 4-2</strong></td>
<td>2.651</td>
<td>0.949*</td>
<td>0.1113</td>
<td>0.0617</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sap13 9-3</strong></td>
<td>2.588</td>
<td>0.855*</td>
<td>0.1340</td>
<td>0.0153</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 8.10** Fresh shoot biomass of Brassica AtSAP13 overexpression lines and wild type controls grown on 1/2MS plates containing 200mM Mannitol for 21 days (replicate data). Data are averaged from four replicates of 10 plants each. Values of fresh shoot biomass followed by single asterisk (*) are significant at $p \leq 0.05$. 


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