EVALUATION OF NOVEL INPUT OUTPUT TRAITS IN SORGHUM THROUGH BIOTECHNOLOGY

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EVALUATION OF NOVEL INPUT OUTPUT TRAITS IN SORGHUM THROUGH BIOTECHNOLOGY

By

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A DISSERTATION

Presented to the faculty of
The Graduate College at the University of Nebraska
In Partial Fulfillment of Requirements
For the Degree of Doctor of Philosophy

Major: Agronomy
(Plant Breeding and Genetics)

Under the Supervision of Professors Tom Clemente and Ismail Dweikat

Lincoln, Nebraska

December, 2010
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University of Nebraska, 2010

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Sorghum [Sorghum bicolor (L.) Moench] is the fifth most important cereal crop world-wide as well as an important source of feed, fiber and biofuel. It is a C4 plant and is well adapted to environments subject to high temperature and water limitation. Despite these agronomic qualities, it suffers from limitations of sensitivity to low temperature and the grain has drawbacks in relation to functionality and digestibility. The objectives of this study are: 1. To promote seed germination at low temperature and to enhance the seedling cold tolerance. 2. To enhance the grain digestibility and functionality. In an attempt to augment cold tolerance, we introduced the glycine rich RNA binding protein gene atRZ-1a from Arabidopsis, Bcl-2 mRNA sequence 725-1428 representing the 3’ non coding region of the gene from humans and rice Ca-dependent protein kinase 7 (OsCDPK7), all of which are known to improve the cold tolerance of plants. None of these genes resulted in the improvement of cold tolerance in sorghum. On the other hand ectopic expression of OsCDPK7 in sorghum led to local lesions formation in leaves. The data generated from this study indicated that constitutive modulation of the CDPK signal transduction pathway in sorghum can trigger a localized cell death response. Further we targeted improving the grain digestibility and functionality. To accomplish this objective we introduced the wheat high-molecular-weight glutenin subunits (HMW-GS) into
sorghum which are known to have significant impact on flour quality. Transgenic events expressing HMW-GS showed improvement in protein digestibility of the uncooked ground grain. In separate complementary set of sorghum transformations we introduced genetic cassettes designed to specifically down-regulate the accumulation of the sorghum alpha and gamma kafirins. Down-regulation of alpha kafirin showed presence of distorted protein bodies in the transgenic seed. The long-term goal of this project is to stack the HMW-GS trait with the modulated kafirins events, as a means to address both end-use functionality and digestibility.
Acknowledgements

First and above all I praise God, the almighty for all the opportunities in my life and standing by me at every step.

I express my sincere regards to my major advisor Dr. Tom Clemente for providing me this opportunity to work in his lab, for his continuous support, encouragement and belief in me that helped me to complete this degree. I also convey my best regards to my other major advisor Dr. Ismail Dweikat for all the technical, financial and moral support he provided me throughout the degree program. I also sincerely thank Dr. Michael E Fromm, Dr. Donald Weeks, Dr. David Holding and Dr. Tom Elthon for the critical analysis of my research and for their invaluable suggestions.

My respectful thanks are due to my parents and sisters who always inspired me to work hard. I can never put in words their sacrifice and consistent support for me. I also fall short of words to acknowledge the moral support, sacrifice and charming attitude towards life of my loving wife Gagan.

I can’t forget the most important part of my degree. Thanks to all the members of Dr. Clemente’s lab Shirley, Natalya, Arlene, Mr. Park, Saadia, Manmeet, Zhengxiang, Kaimei, Hanh, Scott, James, Karina, Forrest, Blake and all other past and present members of the lab for making my stay wonderful experience in the lab. I am also thankful a lot to Amy and Samantha for their cheerful attitude and all the help I got from them in the greenhouse.

Last but not least, I am thankful to my friends Saleem, Vikas, Fareha, Xing, Maria, Hardik, Yashi, Kamal, Wilson for their ever available support and cheer. Never forgotten are UP, Sattu and Ajay for their support in all aspects of life.
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CHAPTER ONE

Literature Review

Sorghum - Introduction

Sorghum (chromosome no. 2n=20, 7.35x10^8 bp of DNA per 1C nucleus) is a C4 crop (Xin et al, 2008) and is the 5th largest grown crop of the world (Chanapamokkhot et al, 2007). In the year of 2009 area planted for grain sorghum in the United States was 6.6 million acres that produced 383 million bushels. State-wise, the maximum area planted was in Kansas followed by Texas (http://usda.mannlib.cornell.edu/usda/nass/CropProdSu//2010s/2010/CropProdSu-01-12-2010.pdf). Internationally in 2007 the maximum area planted was in the USA followed by Nigeria and then India (http://faostat.fao.org).

Sorghum is a crop of tropical regions and thrives well in warmer climates (Anglani C, 1998). It has higher water use efficiency and grows well in marginal soils. Hence it performs well even in drought susceptible areas and is a good crop for semi arid tropics. A large proportion of the world population in Asia, Africa and in other semi arid tropics uses sorghum as a staple food crop. Sorghum is used for a variety of products like tortillas, couscous, porridges and baked goods (Anglani C, 1998).

Sorghum is an important source of energy, starch, proteins, vitamins and antioxidants (Chanapamokkhot et al, 2007). Starch and protein are the main constituents that affect quality of the grain (Taylor et al, 2006). Starch is mostly used as thickener or gelling agents while protein content is important for structure formation in the foods. It is
a good source of the B group of vitamins like thiamin, riboflavin, B6, biotin and niacin. Whole grain sorghum products are better source of these vitamins as refining leads to loss of these vitamins (Hegedus et al, 1985). Potassium and phosphorus are two major mineral elements present in sorghum (Khalil et al, 1984). Sorghum contains condensed tannins which are polyphenolic compounds and act as antinutritional factors. These bind with dietary protein and digestive enzymes to reduce their digestibility and with some minerals and vitamins to decrease their availability (Wang and Kies, 1991). These polyphenolic compounds are present in sorghums that have a pigmented testa but are absent in other genotypes that lack the pigmented testa (Anglani C, 1994).

Sorghum protein is of relatively low quality because it has low amounts of essential amino acids such as lysine, tryptophan and threonine (Badi et al, 1990). Other limitation associated with sorghum is that the wet cooking of sorghum leads to reduced digestibility which further reduces the nutrition value of the grain (Mertz et al, 1984). Sorghum proteins (kafirins) are present in the form of protein bodies where alpha kafirin is present in the center and is surrounded by beta and gamma kafirins. These protein bodies are very strong and are not easily disrupted by routine cooking methods. Moreover cooking sorghum in the presence of water results in the formation of strong bonds between beta and alpha kafirins. This prevents accessibility of enzymes to centrally located alpha kafirins, hence further reduces digestibility. Using shear forces and high pressure and temperature leads to breakage of these bodies which gives better functional properties to the dough. Still, the lack of gluten like proteins in the seed prevents attainment of similar functional properties as that of wheat dough (Chanapamokkhot and
Thongnagam, 2007). Hence, in order to expand the usage of sorghum for various applications, the end use quality of the sorghum needs to be addressed.

Sorghum is also an important energy crop for bio-ethanol production. The juice extracted from sweet sorghum stem consists of sucrose, glucose and fructose and can easily be converted to ethanol at a rate of 3500 liter/hectare to 5600 liter/hectare (Yu et al, 2010). As compared to corn, sorghum has less water requirements but suffers from lower yield and lower market prices for the grain while producing comparable per bushel ethanol yields (Paterson A H, 2008). Moreover, corn is an important food crop being used all over the world. Hence ethanol production from sweet sorghum will have less competition with food crops. Ethanol production from plants should reduce dependence on non-renewable resources of energy and sorghum is one of the candidates for bio-ethanol production.

Various genomic tools for sorghum are available which are helping to enhance the research efforts for the improvement of this crop. The genome of an inbred sorghum line, BTx623, has been sequenced and is available at http://www.phytozome.net/sorghum. A TILLING (Targeted Induced Local Lesions in Genomes) population is also available (Xin et al, 2008). This population is an important reverse genetics tool for functional characterization of the genes. With increasing world population, the water supplies are decreasing day by day. Keeping in mind that sorghum is a low input crop and with the improvement of the end use quality traits, sorghum has the potential to become a primary crop of the future.
Sorghum Seed Storage Proteins

Although sorghum is staple food for a large proportion of world population, it suffers from limitations of poor digestibility and poor bread making properties. These limitations are attributed to the molecular structure of its proteins and their assembly in the form of protein bodies. Hence these limitations need to be addressed for its improved usage in various foods and feed products. The major fraction of seed storage proteins of sorghum are known as kafirins which fall under the category of prolamins. The term prolamin was first coined by Osborne and it refers to the high content of proline and amide nitrogen which is derived from glutamine (Osborne, 1924). These two amino acids constitute about 30% of the total amino acids of kafirin fraction (Belton et al, 2006). Although kafirins are major seed proteins of sorghum, some other proteins in minor fractions are also present. These proteins include albumins (water soluble), globulins (saline soluble) and glutelins (detergent, reducing agent, alkaline pH soluble) (Jambunathan et al, 1975).

The kafirins have been classified into three groups in comparison to much studied zein proteins by composition and sequence of their amino acids, molecular weights and extractability. (Mazhar et al, 1993; Shull et al, 1991). These groups are α kafirin (molecular weights 25000 and 23000), β kafirin (molecular weights 20000, 18000 and 16000) and γ kafirins (molecular weights 28000). These proteins are present in seed in form of endoplasmic reticulum (ER) protein bodies where α kafirin is present at center surrounded by a thin layer of β and γ kafirins (Ioerger et al, 2007; Chandreshekar et al, 1999; Hamaker et al, 1995). α kafirins constitute about 80% of total kafirin and hence is the major seed storage protein of sorghum followed by γ kafirins (15%) and β kafirins.
Although sorghum proteins show a high degree of sequence homology with zein proteins of maize, these differ from zeins as kafirins have higher proportion of cross linking than zeins (Hamaker and Bugusu, 2003). Amino acid composition has shown that the β and γ kafirins have higher proportion of amino acid cysteines which is 5% in β kafirins and 7% in γ kafirins.

Shull et al (1992) showed that the α, β and γ kafirins have difference in their extractability because of the differences in their amino acid composition. α kafirins have higher percentage of hydrophobic amino acids phenylalanine, leucine and isoleucine, hence these are easily extracted in higher concentration of alcohol. β kafirins have higher percentage of the hydrophilic amino acid, glycine, and hence is easy to extract in low concentration of alcohols. γ kafirins have higher levels of the hydrophilic amino acids glycine and histidine and hence are easily extractable in further reduced concentration of alcohol.

Sorghum proteins are less digestible than those of most of other cereals (Taylor et al, 2002). MacLean et al (1981) showed that digestibility of cooked sorghum porridge is around 46% while cooked wheat, maize and rice has digestibility of about 81%, 73% and 66% respectively. Sorghum proteins are present in the form of protein bodies which are resistant to digestion. Cooking sorghum in the presence of water further reduces digestibility. It is known that β and γ kafirins have high content of sulfur containing amino acids i.e. methionine and cysteine (Shull et al, 1992). Hence it is hypothesized that when these proteins are cooked in the presence of water, these amino acids are involved in the formation of disulfide bonds between these kafirins (Rom et al, 1992; Oria et al, 1995; Hamaker et al, 1994). This results in formation of resistant protein bodies which
sustain themselves during most of cooking methods. The strong network formed on the surface of centrally located α kafirins also prevents accessibility of digestive enzymes to α kafirins. Hence these protein bodies pass undigested through the body. This hypothesis is further supported by the fact that cooking sorghum proteins in the presence of a reducing agent improves its digestibility as compared to when cooked in water alone (Hamaker et al, 1995; Oria et al, 1995). However if reduction in digestibility is the result of disulfide bond formation between β and γ kafirins alone, then there should be complete recovery of digestibility when cooked in the presence of reducing agent. But the improvement of digestibility is not complete and still many reduction resistant oligomers can be seen upon electrophoresis (Duodu et al, 2002). Two reasons have been hypothesized for this observation. First is that not all disulfide bonds could be accessible to reducing agent (Oria et al, 1995). Second is that tyrosine residues could oxidatively couple with each other and lead to formation of non disulfide inter chain bonds (Fry S C, 1982; Epstein and Lamport, 1984; Biggs and Fry, 1990). Dry heating of sorghum seed e.g. by popping or dry heating of flour does not lead to decreased digestibility of sorghum. (Duodo et al, 2001; Dahlin and Lorenz, 1993; Parker et al, 1999). This suggests that water is probably acting as a medium to facilitate the formation of indigestible complexes among proteins.

In addition to formation of disulfide bonds, some other factors are also responsible for the reduced digestibility of sorghum flour. These factors can be grouped into two categories – exogenous factors and endogenous factors. The endogenous factors include polyphenol compounds, phytic acid, cell wall components etc. while the
exogenous factors include racemization and isopeptide formation and disulfide crosslinking.

Among endogenous factors polyphenols (e.g. tannins) bind strongly to sorghum prolamins by H bonding and hydrophobic interactions and decrease the digestibility (Butler et al, 1984). Other endogenous factor i.e. phytic acid is a highly charged molecule and it forms insoluble complexes with proteins and hence reduces the digestibility (Ryden et al, 1993). Proteins are also known to be associated with various cell wall components forming indigestible complexes (Glennie C W, 1984; Bach Knudsen et al, 1985). This decreases their accessibility to digestive enzymes and hence reduces their digestibility.

Among exogenous factors, racemization is a process which converts L form of amino acids to D form of amino acids. D forms are slowly absorbed by animals. Even after absorption most of amino acids in D form are not utilized by humans (Liardon et al, 1983). Very high alkaline or acidic conditions or excessive heat during cooking are the probable reasons for this conversion. The likelihood of racemization in sorghum has not been investigated. Since normal cooking procedures for sorghum do not include above said harsh conditions, hence racemization is not expected to reduce the digestibility of sorghum.

The low proportion of essential amino acids e.g. lysine and tryptophan in the major storage protein type i.e. alpha kafirins leads to poor nutritional quality of grain (Shewry and Halford, 2003). The other drawback associated with sorghum is the lack of good bread making properties. The reason hypothesized for this is the same that the kafirin proteins are present in form of very rigid protein bodies which maintain
themselves through various food preparation methods. Hence these are not available for interaction with other components of the flour. Even if these proteins are released from protein bodies by various methods, the molecular structure of these proteins prevent them from attaining high viscoelastic properties. This reduces the scope of sorghum flour from being used in a variety of products and hence leads to reduced economic value of the crop in comparison to other crops e.g. wheat. Hence in our study, we targeted the improvement of sorghum protein digestibility and its bread making properties by modulation of its endogenous proteins and by heterologous expression of high molecular weight glutenin subunits of wheat.

**High Molecular Weight Glutenin Subunits (HMWGS) of wheat**

Wheat grain is known for its specific visco-elastic properties that help it to be processed into a variety of foods like leavened and non-leavened breads, cakes, biscuits, pasta, noodles etc. This feature is responsible for its higher economic value as compared to other crops. The presence of these viscoelastic properties in the flour has been attributed to the presence of gluten proteins which are the major seed storage proteins of wheat. Gluten determines baking quality of wheat dough as it helps to improve the water absorption capacity, cohesivity, viscosity and elasticity of the dough (Wieser H, 2007). The elasticity and extensibility of these proteins result in entrapment of CO₂ inside a network of proteins during fermentation process that gives a light porous crumb structure to the loaf.

The gluten proteins of wheat can be divided into three categories – Gliadins, Low Molecular Weight Glutenin subunits (LMW-GS) and High Molecular Weight Glutenin subunits (HMW-GS). Out of these, the HMW-GS have been attributed for the presence
of special viscoelastic properties in the wheat flour. The different HMW-GS are named based on their coding genome (A, B and D), their type (x and y) and the numbers (1-12) based on their mobility in SDS-PAGE gel (Shewry et al, 1992). HMW glutenin subunits are encoded from Glu-1 locus on long arm of chromosomes of the wheat genome i.e. 1A, 1B and 1D (Gupta et al, 1989; Payne et al, 1980). Each locus has two genes (x and y) which are tightly linked and are inherited together (Shewry et al, 1992; Payne P I, 1987). Although all hexaploid bread wheat cultivars have all 6 HMW-GS (1Ax, 1Ay, 1Bx, 1By, 1Dx, 1Dy) genes, only 3-5 are expressed in different varieties due to silencing of the other subunits. The HMW-GS named 1Ay is always silent in all bread wheat varieties (Altpeter et al, 1996). These subunits are not only having qualitative but also have quantitative effect on bread making quality of the dough (Shewry et al, 2003, Uthayakumaran et al, 2002). Payne et al (1987) studied 84 English wheat cultivars and found that better bread making quality of wheat dough is associated with HMW glutenin subunits 5 and 10 encoded by chromosome 1D.

HMW-GS have three structural domains – a non repetitive N-terminal domain, a repetitive central domain and a C-terminal domain (Wieser H, 2007). The x type subunits except Dx-5 have three cysteine residues in the N-terminal domain and a fourth in the C-terminal domain. Out of these four cysteine residues, two form intra-chain disulfide bonds and two are involved in inter-chain interaction. Subunit Dx5 has one extra cysteine residue at the start of central domain and is expected to be involved in inter-chain bonding. The Y type has five cysteines in the N-terminal domain and one in each of central and the C-terminal domain.
Initially, it was difficult to investigate the comparative effect of 1Dx5 and 1Dy10 subunits on wheat dough as these are tightly linked and are always inherited together. However, with developments in wheat transgenic technology, it became possible to genetically engineer appropriate genotypes with specific subunit genes that helped to make this investigation easier (Weeks et al, 1993). Later, various HMW glutenin subunits were studied in detail. Hence in order to investigate the role of different subunits and to improve quality of dough, either the allelic genes from cultivars have been transformed into genotypes (Altpeter et al, 1996; Alvarez et al, 2000; Barro et al, 1997), hybrid genes that translate into mutant, chimeric proteins (Blechl and Anderson, 1996) or endogenous genes in order to improve the content of a particular subunit (Barro et al, 1997) has been transformed into various wheat varieties. Studies have shown that the subunit pair 1Dx5 + 1Dy10 is associated with highest quality while subunit pairs 1Dx2 + 1Dy12, 1Dx3 + 1Dy12 and 1Dx5 + 1Dy12 are associated with poor quality. The presence of subunit 1Ax1 is superior to the null allele. Also the subunit pair 1Bx17 + 1By18 is generally superior to other alleles encoded by chromosome 1B (Shewry et al, 2003).

An appropriate ratio of Dx:Dy subunits is required for better dough quality so that dough can hydrate appropriately and become extensible (Butow et al, 2003). Reducing this ratio leads to production of weaker doughs (Gao et al, 1992; Lindsay and Skerritt, 1999). On the other hand lines expressing 1Dx5 without corresponding increase of y type subunit are not able to hydrate well and lead to production of over-strong dough probably because of increased crosslinking of polymers (Popineau et al 2001).

The different effects of x and y type subunits can be attributed to their different repetitive domain structure and to their different disulfide structure. (Wieser and
Zimmermann, 2000). It is suggested that increased amounts of Dx5 subunit lead to tightly crosslinked polymers because of the presence of additional cysteine subunit, as compared to other x type subunits, that help in increased inter molecular crosslinking. This results in limited scope of expansion of dough after absorption of water and leads to over-strong dough (Rakszegi et al, 2005; Darlington et al, 2003; Popineau et al, 2001). On the other hand, expression of subunit 1Dy10 lead to more extensible dough as compared to dough in which 1Dx5 subunit have been overexpressed (Blechl et al, 2007; Butow et al, 2003). An appropriate ratio of Dx5 and Dy10 subunits help in better interaction that leads to homogeneous protein network, hence better dough quality (Popineau et al, 2001).

Altpeter et al, (2004) expressed hybrid 1Dx5+1Dy10 subunits in rye plant. The resulting rye plants showed an increased polymerization of its seed storage proteins. Our objective is to express the above subunit pair in sorghum. Improvement of polymerization as a result of overexpression of these hybrid subunits may lead to improved bread making quality combined with high water use efficiency and low other input requirements will be a significant step toward sorghum improvement for not only the developing but also for the developed countries.

**Bcl-2 – Antiapoptotic protein family**

Bcl-2 is a family of proteins whose members are involved in the regulation of programmed cell death (PCD). In addition to PCD, they are also known to improve the stress tolerance of the plants which is one of my research objectives. Various examples are known in literature where PCD is seen when the plant or cell culture were exposed to specific kinds of stress. These examples include exposure of tobacco cell cultures to 5°C (cold stress) or to 44-48°C (heat stress) (Koukalova et al, 1997); heat shock treatment of
cucumber cotyledons to 55°C (Balk et al, 1999); application of high concentration of NaCl to barley plants (Katsuara, 1997); exposure of bobwhite wheat to high salt concentrations (Panter et al, 2004); exposure of Arabidopsis plants to high salt concentrations (Huh et al, 2002) and pathogen interactions (Wang et al, 1996). In all the above mentioned examples the exposure to stressful conditions led to death of cells showing the characteristic features of PCD.

PCD is a systematic process and cells undergoing PCD show certain hallmark features during death. These features include cell shrinkage, chromatin condensation, DNA fragmentation, internucleosomal DNA cleavage and formation of structures resembling apoptotic bodies. These features help to differentiate the cells that die because of PCD vs. those dying because of necrosis. The characteristic features of a cell undergoing apoptosis are similar in plant and animal cells although the presence of a cell wall around a plant cell results in certain differences e.g. phagocytosis of dying cells by macrophages is present in animal cells but absent in plant cells (Chen et al, 2004). Similar to animal cells, PCD in plant cells is associated with DNA fragmentation (DNA ladders) and activation of proteases.

Among the variety of research going on to understand PCD in plants and animals, a family of proteins called Bcl-2 family has been extensively studied in animals. The members of this family have the characteristic of the presence of at least one Bcl-2 homology (BH) domain. Various proteins which fall in this group are divided into three categories – antiapoptotic (typical examples include Bcl-2 and Bcl-xl) which have four BH domains (BH1 – BH4); pro-apoptotic multi-domain proteins (typical examples include bax and bak) which have three BH domains and pro-apoptotic BH3 only protein
family (Levine et al, 2008). These anti and pro apoptotic proteins continuously interact with each other to determine whether the cell should live or die. This interaction occurs by formation of heterodimers among their members which is accomplished by the insertion of BH3 domain of proapoptotic protein into a hydrophobic cleft formed by BH1, BH2 and BH3 domains of anti-apoptotic proteins. (Tsujimoto et al, 2000)

The antiapoptotic members of Bcl-2 family are known to be associated with the improvement of stress tolerance of organisms. Yeast cells harboring Bcl-2 show enhanced survival compared with wild type when both are exposed to oxidative and high temperature stress (Chen et al, 2003). Bcl-2 is known for reduced paraquat induced apoptotic cell death in mouse cells (Dickman et al, 2001 and reference therein). Although Bcl-2 did not block the production free radicals, it prevented ROS mediated PCD (Chen et al, 2003).

Although genes involved in PCD have been identified and characterized in animal cells, their homologous genes have not been identified in plant systems. It was suggested that during evolution these genes could have diverged so much that they are not easily identified by comparing their primary sequences (Li and Dickman, 2004). Transkingdom expression of these animal genes in plants caused remarkable improvement in stress tolerance.

Li and Dickman (2004) transformed tobacco with the Bcl-2 gene. The transgenic and wild type tobacco cells were then exposed to various stresses like cold, heat, menadione and hydrogen peroxide (oxidative stress). The viability of cells was evaluated by Evans blue staining. Under similar conditions the transgenic lines expressing Bcl-2 led to inhibition of cell death as compared to wild type cells. In wild type plants, the
presence of DNA laddering which is a hallmark feature of PCD indicates that cells undergo PCD rather than necrosis. Further Chen and Dickman (2004) evaluated the reaction of three independent transgenic lines expressing Bcl-2, Bcl-xl and CED-9 to chloroplast targeted herbicides. It was found that all the lines expressing the above said genes were resistant to these herbicides as compared to control lines. These herbicides are known to produce reactive oxygen species (ROS). Hence these antiapoptotic genes are expected to protect the plants from harmful effects of ROS while the exact mechanism of their action is not known. Again the death was through PCD as indicated by the presence of DNA fragmentation. Wang and coworkers (2009) expressed the CED-9 gene from Caenorhabditis elegans in tobacco plants and showed that it improves the aluminum tolerance of tobacco plants as compared to wild type plants by inhibiting aluminum induced PCD.

D-satellite RNA is a specific strain of cucumber mosaic virus (CMV) that causes disease in tomato plants. The disease development as a result of infection with this virus involves the process of PCD. Xu and coworkers (2004) expressed Bcl-xl and CED-9 genes in tomato and found increased tolerance of transgenic plants to this virus. This shows that these anti-apoptotic genes not only provide resistance against abiotic but also against biotic stress. Qiao and coworkers (2002) overexpressed the Bcl-xl and CED-9 genes in tobacco and found that the transgenic cells are not only resistant to salt, cold, wounding, UV light and paraquat treatment but the transgenics expressing Bcl-xl gene produced more vigorous rooting form the cut end of the stems than wild type plants.

Increased ROS production is one of the causes of PCD induction in plants. Increased production of ROS makes membranes more permeable to electrolytes,
especially K⁺. The disturbance of potential across membranes leads to cessation of ATP production (Shabala et al, 2007; Reed J C, 1998). Chlorophyll biosynthesis and mitochondrial function depend on proper gradient of these electrolytes across the membranes. Bcl-2 and many of its homologs have a stretch of hydrophobic amino acids at their C termini. This helps these proteins to be inserted into membranes (primarily mitochondrial membranes, nuclear membranes and endoplasmic reticulum) (Reed J C, 1998). When inserted into membranes, these proteins are expected to control the permeability of membranes of these organelles and hence maintain better electrochemical gradient across them. Hence these organelles function better even in the stress conditions and lead to cell survival.

In addition to these genes, a 3’ non coding mRNA region of human Bcl-2 gene is also known to improve the drought tolerance of transgenic tobacco plants (Awada et al, 2004). The transgenic plants containing this fragment showed considerable improvement in drought tolerance and recovery after the stressed plants were watered. We chose this DNA fragment for its expression in sorghum plant so as to evaluate its effect of stress tolerance improvement of this crop.

**Calcium Dependent Protein Kinases**

Plants are sessile organisms and their success in growth and development depends on their ability to respond to various environmental stresses. They have developed a very sophisticated signaling mechanism to transfer the environmental message to the downstream genes in order to produce a particular morphogenic response. CDPKs are some of the these important signaling molecules that are known to improve the stress tolerance of the plants. It is estimated that 1-3% of functional eukaryotic genes encode
the protein kinases (Sopory and Munshi, 1998). Plants have several classes of calcium binding proteins which include calmodulin (CaM) and CaM related proteins (Zielinski RE, 1998; Luan et al, 2002), Calcineurin B-like (CBL) proteins (Luan et al, 2002) and calcium dependent protein kinase (CDPK) (Harmon et al, 2001; Cheng et al, 2002). Among these, CDPKs are the best characterized and are responsible for most of the calcium stimulated kinase activities (Harmon et al, 2001; Cheng et al, 2002).

The activity of these calcium dependent protein kinases depends on the presence or absence of calcium ions. Hence calcium is an important signaling ion (Harper et al, 2004). While most ions freely diffuse in cells, calcium does not, providing specificity to CDPK activation. Plants store calcium in different organelles including apoplast, vacuole, nuclear envelope, endoplasmic reticulum, chloroplast and mitochondria (Bush D S, 1995; Harper 2001). The concentration of calcium in the cell changes in response to various external stimuli like light, drought, salt, injury, cold, pathogen attack and hormones (Bush D S, 1995; Poovaiah and Reddy, 1993; Sopory and Munshi, 1998; Sanders et al, 1999). The patterns of changes in calcium in cells vary in amplitude, duration, localization, frequency of calcium oscillations and the source of the influx. These specific changes lead to expression of specific downstream genes for the desired response (Ranty et al, 2006, Evans et al, 2001; Rudd and Franklin-Tong, 2001). There are a large number of CDPK isoforms in plants and each isoform is specific for a particular kind of stimuli leading to expression of precisely controlled responses (Hong et al, 1996; Lee et al, 1998). In addition, it is an important nutrient molecule and is required for proper functioning of various cellular functions and it also plays a structural role in several cellular components. Calcium accumulates as calcium pectate in the cell wall and binds
cells together. It is especially required for the growth of root and shoot tips because these tips are actively dividing by mitosis and calcium plays an important role in formation of microtubules that, in turn, plays an important role for anaphasic movement of chromosomes (Tuteja and Mahajan, 2007) as well as deposition of new cell walls in dividing cells.

The first report of CDPK was published in pea buds (Hetherington and Trewavas, 1982). The first CDPK protein was purified later by Harmon et al (1987). After that, a variety of CDPK have been reported from different plant systems including Arabidopsis, soybean, rice carrot, corn, mungbean and potato (Sopory and Munshi, 1998). The Arabidopsis genome sequence completion revealed that there are 1085 protein kinases (Hrabak et al, 2003). Among these genes, 34 encode CDPKs (Ludwig et al, 2004). Apart from plants, CDPK genes are also found in protozoans and algae. CDPK are implicated in pollen development, control of cell cycle, phytohormone signaling, light regulated gene expression, gravitropism, thigmotropism, cold acclimation, salinity tolerance, drought tolerance and response to pathogens (Tuteja and Mahajan, 2007). Certain mechanical stresses such as touch, rain and wind are also known to activate such signals.

CDPKs have a conserved structure with four domains. These domains are a variable N-terminal domain, a serine/threonine type kinase domain, an autoinhibitory junction domain and a C-terminal calmodulin-like domain which consists of EF hand calcium binding motifs (Scrasc-Field and Knight, 2003; Ludwig et al, 2004; Harper et al, 2004). Each EF hand has 13 amino acids that form a loop which is flanked by two alpha helices. Each EF hand binds to a calcium ion (Cheng et al, 2002). Different CDPK isoforms have different numbers of EF hands varying from one to four. EF hands at
position one and two have the most conserved amino acid sequence and those at position four have the least conserved amino acid sequence. Zhao et al (1994) showed that the closer the EF hand is to autoinhibitory domain, the greater is the effect of calcium regulation on its activity. Although the CDPKs display a high degree of sequence conservation, the different members of CDPK family differ from each other in terms of number of EF hands (Ullanat and Jayabaskaran, 2002). At normal cellular levels of calcium, the autoinhibitory domain binds the kinase domain. When calcium levels in the cell rise, calcium binds to an EF hand and this causes a change in the confirmation of CDPK. This confirmation change removes the inhibitory domain from the kinase domain and leads to activation of CDPK (Harmon et al, 1994; Harper et al, 1994).

The N-terminal domains of CDPK proteins are highly variable with lengths ranging from 21 to 185 amino acids and 27 out of the 34 predicted CDPKs in Arabidopsis have a predicted myristoylation site indicating a membrane association of these CDPKs. Indeed CDPKs are found to be associated with the plasma membrane (Schaller et al, 1992; Verhey et al, 1993), endoplasmic membrane (Lu and Hrabak, 2002), endosperm storage bodies (Anil et al, 2000), actin cytoskeleton system (Putnam-Evans et al, 1989), mitochondria (Pical et al, 1993) and nuclear membrane (Patharkar and Cushman, 2000). These specific localizations of these CDPKs indicate that all these different forms of CDPK may have distinct functions.

There are various reports in the literature in which different CDPKs have been linked to the adaptive processes in response to stress. Yang and coworkers (2003) showed that OsCDPK 13 expression was increased in rice plants in response to cold stress and GA treatment indicating that it is involved in plant resistance to these stresses. In a
different study when tomato plants were exposed to wounding, there was a systemic increase in the amount and activity of LeCDPK1 (Chico et al, 2002). This observation may indicate that this kinase is probably a part of plant defense response. CDPK transcripts are known to increase after exposure of Arabidopsis plants to cold, salt and drought (Urao et al, 1994; Tahtiharju et al, 1997)

CDPKs are not only activated in response to abiotic stress, but are also activated in response to biotic stress stimuli. A maize Ca dependent protein kinase (ZmCPK10) was elicited in response to inoculation of maize plants with either fungal infection or with fungal elicitors (Murillo et al, 2001). Increase in the expression of ZmCPK10 was accompanied with a simultaneous increase of some pathogen induced proteins that indicates that activation of this kinase may be a part of the defense response of the plant.

Monroy et al (1993) showed the importance of calcium in cold acclimation using alfalfa cell suspension cultures. When the culture was exposed to cold stress in the presence of antagonist of calcium dependent protein kinases or in presence of calcium channel blocker, the capacity of cells to adjust to cold stress was markedly reduced.

Saijo et al (2000) showed that overexpression of OsCDPK7 in rice plants improves the cold and salt/drought tolerance. Further, they showed that OsCDPK7 was expressed strongly in vascular tissues of crowns and roots and vascular bundles when plants were exposed to cold and drought stress (Saijo et al, 2001). These are the areas expected to show severe symptoms in response to water stress and thus, indicates a correlation between expression of these genes and increased stress tolerance. Hence, the probable role of increased OsCDPK expression could be to protect these tissues from stress. These kinase genes play an important role in improvement of stress tolerance of
plants and potentially can be utilized to improve the agronomically important crops for such traits. We expressed this rice gene in sorghum. We hypothesized that the heterologous expression of this gene could lead to improved stress tolerance of sorghum as well.

**Glycine Rich RNA Binding Proteins**

The glycine rich RNA binding proteins is an important group of proteins in plants. Although functions of most of these proteins are not known, some are known to be expressed when plants are exposed to stressful conditions. They are expected to improve the stress tolerance of the plants by controlling expression of other genes. The control of gene expression in living organisms at the post-transcriptional level can be obtained through pre-mRNA splicing; capping; polyadenylation; mRNA transport, stability and translation (Dreyfuss et al, 1993; Higgins, 1991; Simpson and Filipowicz, 1996). Various kinds of RNA binding proteins are required to execute these processes. Such RNA binding proteins have conserved RNA binding motifs such as the RNA recognition motif (RRM), glycine rich motif, arginine rich motif, RGG box, zinc finger motif, double stranded RNA binding motif, etc. (Burd and Dreyfuss, 1994). Out of these, the RNA binding proteins with RNA recognition motif are the most widely found and best characterized (Kenan et al, 1991; Burd and Dreyfuss, 1994; Alba and Pages, 1998; Lorkovic and Barta, 2002).

Glycine rich proteins (GRP) are characterized by the presence of high amounts of glycine residues. GRPs have been classified into three categories – structural proteins present in cell wall (Keller et al, 1989; Condit et al, 1990 and Oliveira et al, 1990), RNA binding proteins (Cretin et al, 1990; Nocker and Vierstra, 1993) and cytokeratin like
proteins (Rohde et al, 1990). Proteins that contain RRM in the N-terminal half and glycine rich region in C-terminal half are called glycine rich RNA binding proteins (GR-RBP) and these have been identified in many plants such as maize (Gomez et al, 1988), Arabidopsis (Carpenter et al, 1994; Nocker et al, 1993), tobacco (Hirose et al, 1993; Moriguchi et al, 1997), barley (Dunn et al, 1996, Molina et al, 1997), brassica (Bergeron et al, 1993), leafy spurge (Horvath and Olson, 1998) and Alfalfa (Ferullo et al, 1997), white spruce (Richard et al, 1999) and prunus avium (Stephen et al, 2003). The nucleic acid binding properties of some of these proteins have been documented but the functional roles of most of them have not been characterized. Some of these proteins are known to improve the stress tolerance of plants.

The *Arabidopsis thaliana* genome encodes 196 RRM containing proteins out of which 8 are classified as GR-RBPs (GRRBP-1 to GRRBP-8) (Lorkovic and Barta, 2002; Lorkovic Z J, 2009). These are known to be induced in response to various environmental stresses (Alba et al, 1994; Moriguchi et al, 1997). In addition, two more nuclear GR-RBP proteins named atRZ-1a and atRZ-1b are known that differ from GR-RBPs by the presence of a zinc finger domain between RRM and C-terminal glycine rich domain. GR-RBP 1, 2, 3, 4, 7, 8 and atRZ-1a were up-regulated when plants were exposed to cold stress. Salt stress led to high increase in the amount of GR-RBP1 and moderate increase of GR-RBP4 and GR-RBP7.

The mode of action of these GRRBPs is not known. COR6.6, COR 15a, COR47 and COR78, are some of the proteins which are known to increase in abundance when plants are exposed to low temperature stress and are known to be regulated by CBF1 (CRT/DRE (C-repeat/dehydration responsive element) binding factor-1) transcription
factors (Thomashow, 1998). Kim and Kang (2006) observed that Arabidopsis lines overexpressing atRZ-1a, it’s T-DNA tagged knock out mutants and wild type plants all have similar levels of the above stress responsive marker proteins when plants are exposed to stress. This indicates that the regulatory pathway involved in the improvement of cold tolerance by expression of atRZ-1a is different than that of CBF protein mediated pathway. Further, comparing the expression profiles using two dimensional gels, they showed that expression profile of some stress related proteins was changed while transcription level of their mRNA and their rate of degradation stayed the same under cold stress and normal conditions. This indicated that atRZ-1a may act as a protective mRNA chaperone and whose action may lead to increased amounts of the target proteins. RNA chaperones help to prevent the formation of stabilized structures of RNA and keep them in a functional state (Rajkowitsch et al, 2007 and Schroeder et al, 2004). It has been suggested that GR-RBP might act as RNA chaperones so that RNA can maintain an active state for efficient functioning (Lorkovic Z J, 2009)

The functions of most of these GRRBP proteins have not been characterized. The mRNA level of these GR-RBP is found to increase when the plants are exposed to environmental stresses like cold, wounding, water stress, phytohormones, viral infections, UV radiations and heavy metals (Sahhetto Martins et al, 2000 and references therein). It is known that increased expression of GRRBP leads to improved stress tolerance in plants. Kwak et al (2005) showed that the transcript level of GR-RBP-4 was increased in response to cold stress and decreased in response to salt/dehydration stress. Although its level increased when the plants are exposed to cold, its overexpression did not provide resistance to plants against low temperature. Kim et al, (2007b) reported that
overexpressing another GR-RBP (i.e. RBP-2) led to improved germination, seedling growth and freezing tolerance at low temperatures.

Kim et al, 2005 showed that one such GR-RBP named atRZ-1a improves seed germination and seedling growth of Arabidopsis at lower temperatures and also enhances freezing tolerance of Arabidopsis plants. That is, loss of function mutants had retarded growth and germination under cold stress as compared to wild type plants. Kim et al (2007a) showed that GR-RBPs improve stress tolerance not only in plants but also when expressed in E. coli. Expression of GRPs in the Bx04 strain of E. coli led to better growth and improved viability at low temperature. As these proteins are known to be associated with improved stress tolerance of plants, they are candidates for improving stress tolerance in agronomically important plants.

**Agrobacterium-mediated sorghum transformation**

Plant transformation refers to the introduction of gene into recipient cells, its integration in the host genome followed by successful regeneration of transgenic plants (Newell, 2000). It has accelerated the breeding process by the introduction of desired genes into plants while eliminating the transfer of undesired linked genes that otherwise poses a significant challenge when introgression of desired genes is done using traditional breeding methods. Various methods for plant transformation have been developed which include agrobacterium-mediated transformation, protoplast transformation, particle bombardment, tissue electroporation, silicon carbide whiskers, injection of DNA etc. (Newell, 2000)

The concept of Agrobacterium-mediated gene transfer started with the discovery that agrobacterium is the casual agent of crown gall disease of plants (Newell, 2000).
Agrobacterium transfers a T-DNA segment from its plasmid into plant genome. This DNA segment has genes for the production of various products that are then produced by plants but are used by *agrobacterium*. After years of research, scientists were able to replace the Ti (Tumor inducing) segment of the T-DNA with other genes of interest and *agrobacterium* became a vehicle for gene transfer. Later agrobacterium mediated transformation was successfully applied in many monocot species like rice (Chan et al, 1992, 1993; Hiei et al, 1994), maize (Ishida et al, 1996), wheat (Cheng et al, 1997) and barley (Tingay et al, 1997).

The transformation progress of sorghum was initially hindered by the recalcitrance of the explants to be regenerated into viable plants. Battraw and Hall (1991) transformed DNA into protoplasts by electroporation and further selected transgenic cells. Unfortunately, they were not able to regenerate viable plants. Hagio et al (1991) transformed cell suspensions of sorghum using a particle bombardment method. They transformed hygromycin and kanamycin resistance by introducing hygromycin B phosphotransferase and neomycin phosphotransferase genes respectively. Although they were able to transform and express the gene, they were not able to regenerate transformant plants.

The first transgenic sorghum plant was regenerated after particle bombardment of immature zygotic embryos (Casas et al, 1993). Researchers successfully transformed a GUS reporter gene along with a selectable marker gene (bar) into a sorghum cultivar P898012. Towards the end of 20th century, significant progress was made in optimizing the sorghum transformation process. The first report of agrobacterium-mediated sorghum transformation accompanied by successful regeneration of transgenic plants was
published in 2000 (Zhao et al, 2000). Researchers used agrobacterium strain LBA4404 carrying a ‘Super-binary’ vector having a GUS gene expression cassette along with a bar gene expression cassette as a selectable marker gene. They used immature embryos as explants. They also showed that immature embryos from field grown crops gave better transformation efficiency than embryos from plants grown in the greenhouse. Further, several laboratories focussed their research to the improvement of sorghum transformation by modification of the transformation process itself and enhancing the regeneration frequencies (Tadesse et al, 2003; Carvalho et al, 2004; Gao et al, 2005a, b; Howe et al, 2006).

Among the first reports of sorghum improvement for an economically important trait was the enhancement of lysine content of the grain (Zhao et al, 2003). Researchers introduced a high lysine protein (HT12 protein) into sorghum that resulted in a 50% increase of lysine content in the seed. Further, Girijashankar et al (2005) improved insect resistance of sorghum by introduction of the Cry1Ac gene from *Bacillus thuringiensis* under the control of a wound inducible promoter. The resulting plants were resistant to the spotted stem borer of sorghum.

In most cases, the transformation frequencies were between 1-2%. Although Howe et al, (2006) was able to obtain 4.5% of transformation frequency in one of the experiments. However, other researchers have not been able to reproduce this high frequency. This suggests that there may be further means of improvement for recovery of transgenic plants.

A large number of selectable marker genes have been used in various studies. These include hygromycin phosphotransferase (hpt) (Hagio et al, 1991), bar gene (Cases
1993, Zhao et al, 2000), phosphomannose and xylose isomerase genes which gives metabolic advantage to transgenic cells over non transgenic ones (Penna et al, 2002). Further use of visual screenable marker genes e.g. green fluorescent protein gene (GFP) (Sheen et al, 1995) and yellow fluoroscent protein gene (YFP) (Nagai et al, 2002) are also reported.

In this study we used *agrobacterium tumefaciens* strain NTL4 (Luo et al, 2001) that harbors the disarmed Ti plasmid named chry5 (Palanichelvam, 2000) designated pKPSF2. npt-II was used as selectable marker gene. A number of transgenic plants were obtained and were further characterized at the phenotypic level.
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CHAPTER 2

Expression of the rice CDPK-7 in sorghum: Molecular and phenotypic analyses

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Abstract

Sorghum (*Sorghum bicolor* (L.) Moench) is an important source for food, feed, and possesses many agronomic attributes attractive for a biofuels feedstock. A warm season crop originating from the semi-arid tropics, sorghum is relatively susceptible to both cold and freezing stress. Enhancing the ability of sorghum to tolerate cold and freezing offers a route to expand the acreage for production, and provides a potential drought avoidance strategy during flowering, an important parameter for protection of yield. Targeted perturbation of the signal transduction pathway, that is triggered by exposure to abiotic stress in plants, has been demonstrated in model systems as an avenue to augment tolerance. Calcium-dependent protein kinases (CDPKs) are key players in a plant’s response to environmental assaults. To test the impact of modulating CDPK activity in sorghum as a means to enhance abiotic stress tolerance, we introduced a constitutively expressed rice CDPK-7 (*OsCDPK-7*) gene construct. Sorghum transformants carrying this cassette displayed lesions on their leaves and up-regulation of a number of pathogen related proteins, along with transcripts linked to photosynthesis. These results demonstrate that modulating the Ca signaling cascade *in planta* via constitutively expressed CDPK can lead to off-target effects likely due to the broadly integrated nature of these enzymes in signaling.
Introduction

Based on production sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most important cereal crop in the world following rice, maize, wheat and barley. Annual production is estimated at 60 million tons with Mexico, India, Nigeria and USA accounting for the majority of the harvest (Dicko et al., 2006). Recently grain and sweet sorghum have garnered the attention of the plant science community as a potential feedstock for biofuels due to its relatively low input requirements, C\textsubscript{4} photosynthesis, and superior water use efficiency. A wealth of genomics resources (Paterson, 2008) and recent completion of the genome sequence (Paterson et al., 2009) make sorghum an attractive species for research and facilitate translational efforts to improve agronomics and output traits of the crop.

Originating in the tropics sorghum is susceptible to cold and freezing stress during early development, which can negatively impact productivity in cooler environments (Ercoli et al., 2004; Staggenborg and Vanderlip, 1996; Yu et al., 2004). Improving sorghum’s ability to withstand both chilling and freezing conditions would not only provide a means to protect yield following exposure to these stresses, but also permit earlier planting, which would extend the growing season and avoid drought conditions during flowering in the mid-summer, a common environmental pattern in the cooler climates where sorghum is grown (Ercoli et al., 2004).

Intracellular levels of calcium ions are significantly increased following a plant’s exposure to either abiotic or biotic stresses (Ludwig et al., 2004; Ma and Berkowitz, 2007). Translation of intracellular calcium signatures leading to a plethora of signaling cascades is mediated by various calcium binding proteins including calmodulins,
calmodulin-like proteins (Snedden and Fromm, 1998), calcineurin B-like (Zhu, 2002), and calcium-dependent protein kinases (CDPKs) (Cheng et al., 2002). The latter comprise the largest family of the calcium sensors. CDPKs are monomeric proteins, ranging in size from 40 to 90 kDa, that harbor both calmodulin-like, and kinase domains (Cheng et al., 2002; Klimecka and Muszynska, 2007). Hence, CDPKs can sense modulations in cellular Ca signatures without direct interactions with calmodulins (Ludwig et al., 2004). CDPKs play a significant role in relaying information following a spike in cytosolic calcium levels triggered by abiotic stress, with previous reports demonstrating CDPKs induction at both the transcriptional and post-transcription levels (Xiong et al., 2002). The rice genome harbors 31 CDPKs, of which six that are up-regulated at the transcription level following exposure to cold (Ray et al., 2007). Among these is *OsCDPK-7* (*OsCPK13*), previously shown to be up-regulated in rice approximately 2-fold in rice following 3-hour, 4°C exposure of 7 day-old seedlings (Ray et al., 2007). Saijo et al (2000) demonstrated that ectopic expression of *OsCDPK-7* in transgenic rice imparts enhanced tolerance towards salt, drought and cold stresses, while suppression of endogenous *OsCDPK-7* transcripts led to an increased sensitivity to these stresses. Importantly, the ectopic expression produced no apparent phenotypic effects in regards to growth and development under greenhouse conditions. These data suggest that *OsCDPK-7* is a good candidate to impart tolerance towards these stresses, since it is assumed that post-translational activation is required for the signal transduction cascade to ensue (Saijo et al., 2000).

This study was designed to test whether the phenotypic benefits associated with ectopic expression of *OsCDPK-7* in rice (Saijo et al., 2000) for cold tolerance can be
translated to grain sorghum. We assembled constitutive expression cassettes carrying either a sorghum codon-optimized version of the OsCDPK-7 open reading frame (ORF), or a mutated, inactive, version of OsCDPK-7 ORF and introduced these two cassettes into sorghum via Agrobacterium-mediated transformation. We report here on the molecular and phenotypic evaluation of the derived transgenic sorghum events.

**Materials and methods**

**Plasmid Constructions**

The OsCDPK-7 ORF was isolated from rice (genotype Nipponbare) by RT-PCR using primers, Os7-F: ATGGGCAACGCATGCGGCGGTTCC and Os7-R: GTCTAGAAGTGACCAGGTGCGTCCCTC, based on the genbank accession number AB042550. Attempts to isolate the ORF from genotype Nipponbare resulted in several point mutations (Fig 2.1), even with high fidelity Taq polymerase. We used this mutant version of OsCDPK-7 as a control in subsequent experiments, and synthesized (GenScript, Piscataway, NJ) a codon-optimized OsCDPK-7 ORF based on the deposited sequence. The two derived ORFs were fused to the tobacco etch virus (TEV) translational enhancer element (Carrington and Freed, 1990), and subcloned downstream to the maize ubiquitin promoter coupled with its first intron (Christensen et al., 1992). The resultant cassettes were cloned into the binary plasmid pPZP212 (Hajdukiewicz et al., 1994), and designated pPTN751, and pPTN780, for the synthetic and mutant OsCDPK-7 versions, respectively. The final binary vectors were mobilized into A. tumefaciens strain NTL4 carrying the disarmed Ti plasmid pKPSF2 (Luo et al., 2001) via tri-parental mating. Sorghum transformations were conducted with genotypes Tx430 and C2-97 as previously described (Howe et al., 2006).
Bacterial OsCDPK7 expression vectors for protein purification

To assay in vitro kinase activity of the respective OsCDPK-7 genes harbored in the binary vectors pPTN751 and pPTN780, the ORFs were cloned into the bacterial glutathione S-transferase expression vector pGEX-5X-1 (Amersham Biosciences). The resultant plasmids, designated pPTN874 and pPTN875, for the synthetic and mutated OsCDPK-7 ORFs, respectively, were introduced into E. coli strain DH5α. Cell cultures were grown to an OD₆₀₀ 0.5 expression induced by the addition of IPTG and L-arabinose and cells allowed to grow for an additional 4 hours. Cells were harvested by centrifugation at 9,000 rpm (Beckman JLA-10.5 rotor) for 15 min. Bacterial pellets were suspended in GST binding buffer (10 mM NaH₂PO₄, 150 mM NaCl, 1 mM DTT, pH7.3). Lysozyme nuclease (25 U/ml) was added and the suspension was sonicated for 30 s. Following the sonication step the suspension was incubated at 4°C for 20 min with mild agitation and subsequently centrifuged for 10 min at 4,350 x g in a Beckman JA-20 rotor. The supernatant was loaded onto a 50 ml Superloop™ (GE Healthcare, cat # 19-7850-01) fitted on a FPLC. A one-ml GSTrap FF™ column (GE Healthcare, cat # 17-513-02) was washed with 5 ml GST binding buffer, and purified protein preparation was loaded at a flow rate of 0.2 ml per min. The column was subsequently washed with GST binding buffer to remove unbound protein. Following the wash step, the GSTrapFF™ column was removed from the FPLC and injected with 1 ml Factor Xa cleavage buffer (50 mM Tris HCL, 150 mM NaCl, 1 mM CaCl₂, pH 7.5) supplemented with 25 µg Factor Xa (New England Biolabs, cat # P8010L) to cleave the GST tag from the target protein. The column was incubated for 5 hours at room temperature, placed back onto the FPLC and washed with 5 ml GST binding buffer to elute the protein, and followed by a wash with 5
ml GST elution buffer (50 mM Tris HCl, 10 mM reduced glutathione, 1 mM DTT, pH 8.0) to elute the GST tag.

**Kinase activity assay**

Monitoring of *in vitro* kinase activity was performed using a modified protocol described by Saijo *et al.* (1997). The reaction mixture (40 µl final volume) was composed of 25 µM [γ-<sup>32</sup>P]ATP (8 µCi), 50 mM Tris HCl, 5 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 20 µg casein (Sigma cat # C8032-100) and recombinant purified proteins (0.75 µg) +/- 10 mM EGTA. The reaction was incubated overnight at room temperature and the proteins resolved by 14% SDS-PAGE.

**Molecular characterization of transgenic sorghum**

Total genomic DNA was isolated from sorghum leaves following a modification of the protocol described by Dellaporta *et al.* (1983). Southern blot analysis of transgenic events was conducted as previously reported (Howe et al., 2006). Membranes were hybridized with either dCT<sup>32</sup>P labeled synthetic OsCDPK-7 ORF or the 35S CaMV promoter derived from the npt II selectable marker cassette used for selection. For RNA gel blot analysis, total RNA was extracted from 200 mg of ground leaf tissue in 1.5 ml of TRIzol<sup>®</sup> LS reagent (Invitrogen). RNA sample separation and hybridizations were carried out as previously described (Buhr et al., 2002). Membranes were hybridized with either dCT<sup>32</sup>P labeled 882-bp region of the synthetic OsCDPK-7 ORF or 764-bp fragment from the OsCDPK-7 mutated ORF.

**Leaf protein extracts**

Leaf samples from greenhouse grown plants were ground in liquid nitrogen. Ground tissue was mixed with protein extraction buffer composed of 0.1 M Trizma base,
10 mM EDTA, 0.9 M sucrose, 0.4% (v/v) β-mercaptoethanol, pH 8.0. The mixture was subsequently extracted for 30 min at 4°C, with slight agitation, in equal volume Tris-saturated phenol. Following the 30 min extraction step, the suspension was centrifuged at 4,350 x g in a Beckman JA-20 rotor for 10 min. The upper phenol phase was collected and precipitated overnight at -20°C, using 5X volume of 0.1M ammonium acetate in 100% methanol. Precipitated proteins were pelleted at 17,400 x g in a Beckman JA-20 rotor for 10 min. The protein pellet was subsequently washed with 0.1M ammonium acetate in 100% methanol, followed by washings in 80% (v/v) acetone, and finally 70% (v/v) ethanol. The washed protein pellets were suspended in 8 M urea, 2M thiourea, 2% (w/v) CHAPS, and 2% (v/v) Triton X-100. Protein concentrations were quantified using the Bradford assay.

Two-dimensional gel electrophoresis

A total of 300 µg of protein was loaded onto each 7 cm pH 3.0-10.0 Ready Strip IPG strips (Bio-Rad, cat # 163-2000). Following focusing in the first dimension, the strips were run in the second dimension using 14% SDS-PAGE and the gel was subsequently stained with Coomaissie G-250. Differential spots observed in transgenic samples as compared to control samples, were picked and analyzed using tandem mass spectrometry.

RNA sample preparation and microarray analysis

Transgenic and control plants were grown side-by-side under greenhouse conditions. Same staged leaf samples were immediately frozen in liquid nitrogen. Each sample was composed of a pool of total RNA isolated from a single leaf across three plants. Three such RNA pools were used for transgenics and corresponding control
samples. Total RNA was isolated using TRIzol ® reagent (Invitrogen cat. # 10296-028) as described by the manufacturer. Following the TRIzol extraction step the preps were purified using RNeasy columns (Qiagen cat# 74204). cDNAs were generated from 15 µg of total RNA using Affymetrix on-cycle cDNA synthesis kit (Cat # 900493) following the manufacturer’s protocol. Hybridizations were performed with the Affymetrix GeneChip® Maize Genome Array (cat # 900614). Following the hybridization step the GeneChips were stained with streptavidin phycoerythrin conjugate on an Affymetrix Fludics Station 450. Images were acquired using GeneChip® 3000.

Microarray data analysis

GeneChip® Maize Genome Arrays (Affymetrix cat# 900614) were utilized for transcript profiling studies. The maize chip has 17,555 probe sets to integrate approximately 14,850 maize transcripts, which translate to 13,339 genes. Probes were mapped to genes using Netaffx Support Center from Affymetrix (http://www.affymetrix.com/analysis/index.affx). Only signals from perfect match probes were used in the analysis (Irizarry et al., 2003). Data from all Affymetrix GeneChips were normalized using the Robust Multichip Average (RMA) method (Bolstad et al., 2003; Irizarry et al., 2003) with Bioconductor’s (Gentleman et al., 2004) affy package (Gautier et al., 2004). The empirical Bayes moderated t-test was conducted on the background corrected, normalized and log²-transformed expression values using the limma package (Smyth, 2004). The corresponding P-values for transcripts represented on the GeneChips were converted to q-values to obtain approximate control of the false discovery rate at specified values (Storey and Tibshirani, 2003). Estimates of
fold change for each transcript was obtained by converting the mean treatment difference estimated as part of the test.

**Quantitative PCR**

A subset of three significantly upregulated genes were selected and primers designed based on their corresponding sorghum homologs identified from the sorghum genome (http://www.phytozome.net/sorghum). RNA isolations were conducted as described above. First stand cDNA synthesis was carried out with 1 µg RNA using SuperScriptII Platinum Two-Step qRT-PCR kit (Invitrogen cat # 11735). Concentration of cDNAs was ascertained spectrophotometrically. The qRT-PCR reactions were conducted using a BioRad iCycler using SYBERgreenER™ qPCR SuperMix (Invitrogen cat# 11761). Each reaction was carried out in a 10 µl total volume, with 50 ng cDNA, 200 nM of each primer and 5 µl SYBRgreen. The sorghum actin gene was used as the internal reference. Following the reaction, dissociation curves were derived to test for probability of primer dimmers and secondary products. The cycling conditions used were 50°C for 2 min, 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec and the respective annealing temperatures for 30 sec. Following the 40 cycle regime, a final 95°C for 1 min and 55°C for 13 min were run. The corresponding fold change in the expression of the respective genes was calculated as described by Dussault and Pouliot. (2006)

**Results**

**OsCDPK-7 gene isolation and kinase activity**

The working hypothesis driving this project was that expression of OsCDPK-7 in sorghum would improve abiotic (cold) stress tolerance in the crop. During isolation of the
native gene from rice the cDNA from rice genotype Nipponbare displayed a number of point mutations in the OsCDPK-7 ORF (Fig 2.1). The mutated sequence predicted a translational product with seven residue changes, including two changes in the N-terminal variable domain, A33G and D51E, three changes in the kinase domain, L143P, K147M and H204Y, and two residue changes in the C-terminal region downstream of the calmodulin-like, calcium binding domain, N538S and I542T (Fig 2.1). We therefore elected to have the OsCDPK-7 ORF synthesized, with codons optimized for sorghum. The two derived ORFs, synthetic OsCDPK-7 and mutated OsCDPK-7 were cloned into a bacterial expression vector to monitor kinase activity and designated pPTN874 and pPTN875, for the synthetic and mutated OsCDPK-7 versions, respectively. Kinase assays performed with casein as the substrate (Fig 2.2) showed the synthetic OsCDPK-7 active in autophosphorylation, as well as the ability to phosphorylate casein (Fig 2.2A). Successful inhibition of activity by the addition of EGTA or the removal of Mg$^{++}$ ions in the reaction is characteristic of CDPKs. Parallel experiments conducted with the mutant form of OsCDPK-7 showed this form of the gene product to be inactive (Fig 2.2B).

**Sorghum transformation**

Binary vectors pPTN751 and pPTN780 harbor constitutively active cassettes of the OsCDPK-7 synthetic and mutated ORFs respectively. Sorghum transformations were conducted with two genotypes, Tx430 and C2-97. Primary pPTN751 transformants in the Tx430 genetic background, established in the greenhouse, displayed lesions on mature leaves, and we were able to recover seed from only three independent events. Primary pPTN751 transformants in the C2-97 genetic background, and pPTN780 transformants in either Tx430 or C2-97, displayed normal development patterns (Fig 2.3).
Sorghum leaves displaying lesions were analyzed at the University of Nebraska’s Plant and Pest Diagnostic clinic to determine if the lesions represented pathogen infection sites. No pathogens were identified, suggesting that the induction of local lesions was a direct consequence of transgene expression. We selected two independent events of pPTN751 in both Tx430 and C2-97 genetic backgrounds, and two independent events from pPTN780 in Tx430 genetic background for further characterization.

The two pPTN751 lead events in Tx430 background were designated 133-3-5-1 and 137-3-5-1, and the pPTN751 lead events in C2-97 background were designated 24-1-1 and 49-2-5b. The pPTN780 lead events in Tx430 background are 18-4-1a and 39-1-1a. Figure 2.4 shows DNA gel blot analysis results with the respective events. Hybridizations involved total genomic DNA digested by an enzyme for which there is a single restriction enzyme recognition site within the T-DNA element to produce a single hybridization signal for each transgenic locus. Although multiple copies per insertion may be present, copy number in each insertion event was not determined. Genomic DNA hybridization results suggested that both the pPTN751 Tx430 events (133-3-5-1 and 137-3-5-1) harbored a single transgenic locus (Fig 2.4A), as did the pPTN780 event 18-4-1a (Fig 2.4C). The C2-97 pPTN751 events, 24-1-1 and 49-2-5b contained three and two loci, respectively (Fig 2.4B), while the other selected pPTN780 event, 39-1-1a contained two loci (Fig 2.4C).

Relative expression levels of OsCDPK-7 transgenes were monitored via RNA gel blot analysis with results shown in Fig 2.5. Among selected pPTN751 events, the two C2-97 events (24-1-1 and 49-2-5b) along with one of the Tx430 events, 137-5-1-1, showed relatively high expression of the synthetic OsCDPK-7 (Fig 2.5A&B). The second
Tx430 event, 133-3-5-1, displayed relatively low expression (Fig 2.5A). The two pPTN780 events in Tx430 background, 18-4-1a and 39-1-1a, displayed similar expression levels of the mutated OsCDPK-7 transgene, but also showed a smaller sized transcript, in addition to the predicted size (Fig 2.5C). Additional independent transgenic sorghum pPTN780 events analyzed showed the same signal pattern on RNA blots, which we did not investigate further.

We tested a number of parameters to monitor their impact on lesion formation in the Tx430 OsCDPK-7 transgenic events. These included light, axenic culture conditions, and wounding. To evaluate the effect of light on lesion formation under greenhouse conditions, fully expanded leaves, from both wildtype and Tx430 pPTN751 transgenic events, not displaying lesions, were covered with aluminum foil for a period of 15 days. The covered leaf sections from transgenic events still developed lesions, while those from control leaves were chlorotic, but lesion free (Fig 2.6A). To confirm that development of lesions on the Tx430 pPTN751 events was not the result of enhanced pathogen susceptibility, we grew surfaced-sterilized seed of control and transgenic events on MS-based germination medium supplemented with 1% sucrose in doubled stacked Magenta™ vessels. As shown in Fig 2.6B, lesion development occurred in the transgenic pPTN751 Tx430 events grown in an axenic environment, reflecting a physiological, rather than biotic, elicitation of the localized cell death response. We also addressed whether wounding could trigger the observed cell death response in the transgenic pPTN751 Tx430 events by subjecting fully expanded leaves to a needle prick, prior to lesion induction. The plants were subsequently allowed to grow under greenhouse conditions for two weeks following the wounding treatment, and lesion formation was
monitored. As shown in Fig 2.6C, the site of wounding was not associated with lesion formation on leaves of the transgenic event. These data suggest that OsCDPK-7, expressed constitutively in Tx430 background mimics signaling associated with apoptotic cell death response.

**Two-dimensional gel electrophoresis and Microarray analysis**

To investigate transcript and protein changes in vegetative tissues of the Tx430 pPTN751 events, both microarray analysis and two-dimensional (2-D) gel electrophoresis were conducted. With respect to the latter, protein samples from wildtype Tx430 and selected pPTN751 Tx430 transgenic events showing lesions on leaves grown under greenhouse conditions, were separated on 2-D gels and imaged. Three major protein differences were detected (Fig 2.7), and protein spots were analyzed via tandem mass spectrometry. The three differentially expressed proteins selected from pPTN751 Tx430 samples were identified as pathogen-related class 10 (PR-10) family proteins (Liu and Ekramoddoullah, 2006).

Alteration in the transcript profile of the Tx430 pPTN751 transgenics was monitored using microarray analysis. No publicly available sorghum gene chip is currently available, so we used the Maize Genome Array Chips, followed by gene confirmation of selected up-regulated transcripts by qRT-PCR. Transcripts displaying up-regulation greater than 2-fold or down-regulation greater than 50% were identified using Affymetrix Netflex Data Analysis Center and BLAST analysis. Only a portion of the responsive genes was identified due to incomplete annotation. Genes that were identified to be responsive in the transgenic event were classified as having cell maintenance, membrane transport, and biotic and abiotic stress-related functions. Table
1 shows the list of identifiable genes that were up-regulated in the Tx430 pPTN751 transgenics. From this list three transcripts were selected to confirm via qRT-PCR. The selected transcripts were Zm.2321.4.A1_a_at with the sorghum homolog, Sb01g023750.1 that is predicted to encode an alanine aminotransferase, Zm385.1A1 with the sorghum homolog Sb01g036030.1 that encodes uroporphyrinogen decarboxylase (UROD), and Zm10293.S1 with the sorghum homolog Sb10g026450.1 containing the basic-leucine zipper domain, cprf-2, that is associated with light regulated genes (Table 2). The means of the normalized expression ratio calculated from the qRT-PCR analysis of selected transcripts were consistent with their up-regulation (Table 1 & 2).

Discussion

Our working hypothesis was expression of OsCDPK-7 would enhance cold tolerance to sorghum, under the assumption that what was observed in rice (Saijo et al., 2000) would be translatable to other grains. The introduction of OsCDPK-7 in one genetic background of sorghum, Tx430, led to a lesion mimic phenotype, while in second genotype, C2-97, normal development under greenhouse conditions were observed. Moreover, an isolated OsCDPK-7 ORF with a series of mutations (Fig 2.1) was phenotypically neutral in both genotypes. Our data suggest that the induction of lesions was not likely due to an increased susceptibility to a biotic agent, given that the lesion formation was observed under axenic growth conditions (Fig 2.6C). Thus taken together the data indicates that with the Tx430 genetic background an environmental cue to alter the cellular calcium signature is not required to activate OsCDPK-7. Such post-translational regulation is important to avoid negative agronomic impacts under non-stress conditions. We have shown that indeed OsCDPK-7 requires Ca^{++} for activity, and
apparently Mg$$^{++}$$ as well (Fig 2.2). Hence, the apparent constitutive nature of the enzymatic activity in the Tx430 background under environmental conditions that should not alter the calcium signature is confounding.

Due to the dual signaling nature of CDPKs, transduction towards a biotic or abiotic response may occur. The phenotype per se, lesion mimic, would imply the induction is more likely towards a biotic response, given that a hypersensitive response is a hallmark resistance strategy employed in plant systems as a means to block pathogen ingress. Differential protein accumulation monitored in the 2-D gel analysis (Fig 2.3) revealed the transduction response triggered in the transgenic events led to the accumulation of a number of PR-10s proteins. There are over 100 proteins that fall in the PR-10 family and these are modulated in expression over development, upon challenge with pathogens or exposure to abiotic stresses (Liu and Ekramoddoullah, 2006). Hence, the identification of PR-10 proteins differentially expressed in the Tx430 transgenics with the lesion mimic phenotype was not surprising.

Due to the lack of a publicly available sorghum Chip, we relied upon the Affymetrix maize genome array to gain insight on the alteration in the transcriptome in Tx430 events carrying the pPTN751 OsCDPK-7 cassette. Of the modulated transcripts that were annotated many were associated with general cell maintenance and membrane transport, photosynthesis capacity, or stress response (Table 1). For example, transcript Zm2321.4A1_a_at is annotated as an alanine aminotransferase (AlaAT). This transcript was significantly up-regulated in the transgenic Tx430 events displaying lesion mimic phenotype (Table 1 & 2). AlaAT uses glutamate and pyruvate to form 2-oxoglutarate and alanine. Under a hypoxic environment, alanine accumulates in root tissues, although in
soybean it has been shown that AlaAT activity is maximum following the accumulation of alanine, which indicates that a role of AlaAt is to aid in the maintenance of the nitrogen and carbon status of the cell following exposure to low oxygen stress, and not accumulation of alanine *per se* (de Sousa and Sodek, 2003; Miyashita et al., 2007).

A second transcript observed to be up-regulated in the microarray and confirmed via qRT-PCR was Zm385.1.A1_at, sorghum homolog Sb01g036030.1 (Table 1 & 2), which was annotated as a UROD. UROD is involved in the metabolism of tetrapyrrole, generating the required building blocks for the light harvesting machinery of a plant cell (Tanaka and Tanaka, 2007). Down regulation of UROD in tobacco led to the induction of overall stunting, crinkling and necrosis in leaves of transgenic tobacco (Mock and Grimm, 1997). The UORD silenced transgenics were shown to have accumulated the metabolite, uroporphyrinogen III, along with a reduction in chlorophyll level in older leaves, as well as a decrease heme level over leaf development. These phenotypes correlated well with the light-dependent induction of cell death (Mock and Grimm, 1997). In maize, a number of lesion mimic mutants have been characterized, which most are inherited in a dominant fashion (Johal et al., 1995). One such mutant designated as *les22*, phenotypically develops defined necrotic lesions on the leaf blade. This mutation was found to reside in UROD (Hu et al., 1998). However, lesion development in *les22* maize and the down-regulated UROD tobacco events are light-dependent (Hu et al., 1998; Mock and Grimm, 1997). In the case of the latter, accumulation of tetrapyrroles metabolites in silenced UROD tobacco events also resulted in the build-up of phenolic compounds which in turn triggered the pathogen defense response, and this translated to an enhanced tolerance towards tobacco mosaic virus (Mock et al., 1999).
With respect to the lesion mimic phenotype that develops in the OsCDPK-7 Tx430 events, it is developmentally regulated, in that lesions appear following the two leaf stage, and only on fully expanded leaves. However, unlike lesion mimics induced upon mutations and/or down-regulation of UROD in plants, the lesion mimic observed in the transgenic Tx430 sorghum events are light-independent (Fig 2.6A), with an up-regulation of UROD (Table 1). Nonetheless, a number of lesion mimic mutants in plants have been linked to various genes associated with chlorophyll metabolism. Degradation of chlorophyll upon leaf senescence requires the action of accelerated cell death 1 gene (Acd1) which encodes for pheophorbide a oxygenase (PaO) (Pruzinská et al., 2003). The maize lesion mimic lethal leaf spot-1 (lls1) (Gray et al., 2002), was shown to be homologous to Acd1 (Pruzinská et al., 2003; Yang et al., 2004). The lls1 phenotype in maize is a progressive cell death in that once triggered, i.e. by wounding (Gray et al., 2002), necrosis expands through the leaf, reminiscent of the pattern of cell death observed here in sorghum, with the exception that lesion formation is not triggered by wounding in the sorghum events described here (Fig 2.6C). While initial reports classified the lls1 lesion mimic as light dependent, more recently, it has been shown that down-regulation of Acd1 in Arabidopsis induces local lesions in a light-independent fashion (Hirashima et al., 2009). Arabidopsis silenced Acd1 events, when placed in the dark, display significant water loss, enhanced electrolyte leakage, and plastid degradation, reflecting a light-independent nature of the cell death response in the silenced events (Hirashima et al., 2009). Hence, phenotypically the Tx430 pPTN751 events display some of the hallmarks of the classic lesion mimics, yet were induced through expression of a Ca-dependent protein kinase, OsCDPK-7, not via
insertional/point mutations or down-regulation of a target gene (Johal et al., 1995; Lorrain et al., 2003). The observed cell death response in the Tx430 was not likely due to a T-DNA insertional mutation, since the same phenotype occurred in two independent transgenic events (Fig 2.4A).

Experiments were designed to attempt to block the cell death response in planta, by stacking of an antiapoptotic gene with the OsCDPK-7 allele in the Tx430 background. For this study we chose Bcl-2, because we had the transgenic sorghum events in hand, and the gene has been previously shown to impede cell ingress of necrotrophic fungi in plants (Dickman et al., 2001) and ameliorate tolerance towards abiotic stress through slowing of the cell death response in plants (Awada et al., 2003; Awada et al., 2004). The Bcl-2 transgenic sorghum events carry a cassette under control of the maize ubiquitin promoter, with the binary vector designated as pPTN396. The pPTN396 events were characterized at the molecular level, and were found to be highly expressing Bcl-2 (data not shown). We stacked the Bcl-2 with OsCDPK-7 allele via crossing and F1 plants, which were confirmed crosses, the plants were monitored for lesion development under greenhouse conditions. The lesion mimic phenotype was not blocked by the expression of Bcl-2 (data not shown), suggesting either the signal transduction cascade triggered by OsCDPK-7 does not intersect with the action of Bcl-2, or Bcl-2 per se does not possess sufficient antiapoptotic affects to block the cell death response triggered by OsCDPK-7 in planta.

Phenotyping for cold tolerance in the Tx430 background was complicated by the onset of lesion mimics. However, we did, conduct a series of abiotic stress evaluations with the C2-97 events, 24-1-1 and 49-2-5b (Fig 2.2B), investigating cold, at both
germination and seedlings stages along with salt tolerance at the seedling stage. These studies did not indicate a significant enhanced tolerance towards these two stresses in the C2-97 OsCDPK-7 events (data not shown).

The studies outlined herein clearly demonstrate that translating phenotypic effects of a transgene across plant species is not straightforward. Case in point, ectopic expression of OsCDPK-7 in rice led to improved abiotic stress tolerance, without observed developmental abnormalities (Saijo et al., 2000; Saijo et al., 2001), while translation of this approach to grain sorghum, failed to enhance abiotic stress tolerance, and induced a lesion mimic phenotype, that was genotype specific.

**Acknowledgments** This work was partially supported through funds provided by the Nebraska Sorghum Board, and the Nebraska Research Initiative. TK was supported through a USDA-NRI graduate training grant award number USDA 2007-55100-17788. The authors wish to thank Amy Hilske for greenhouse care of plants and Yuannan Xia for assistance with the microarray study.
References


Figure 2.1: Alignment of peptide sequences of OsCDPK-7 ORFs that reside in binary vectors pPTN751 and pPTN780.

Residue changes between the two ORFs are indicated by purple font. Blue residues (shown in pPTN751) reflect the kinase domain, and purple residues represent the EF hand domain of CDPKs. Those residues highlighted in orange were previously reported to be critical for kinase activity of CDPKs.
**Figure 2.2**: Kinase activity assay on the respective OsCDPK-7 ORFs

**2.2A**: Top panel: Coomassie stained SDS PAGE gel OsCDPK-7 synthetic ORF. Lane 1 purified synthetic OsCDPK-7, lane 2 OsCDPK-7 complete reaction mix, lane 3 casein alone, lane 4 blank, lane 5 OsCDPK-7 complete reaction plus EGTA, and lane OsCDPK-7 complete reaction minus MgCl₂. Bottom panel: Autoradiographic image of corresponding kinase activity assay. **2.2B**: Top panel: Coomassie stained SDS PAGE gel OsCDPK-7 mutated ORF. Lane 1 purified mutated OsCDPK-7, lane 2 mutated OsCDPK-7 complete reaction mix, lane 3 mutated OsCDPK-7 complete reaction plus EGTA, and lane 5 casein alone. Bottom panel: Autoradiographic image of corresponding kinase activity assay.
**Figure 2.3:** Phenotype of transgenic sorghum events

**2.3A:** Progeny derived from Tx430 pPTN751 events 137-5-1-1 and 133-3-5-1, along with C2-97 event 24-1-1. Insert, close-up lesion on leaf blade from event 137-5-1-1

**2.3B:** Leaves from progeny derived events 24-1-1 (C2-97/pPTN751), C2-97 wild-type, 133-3-5-1 (Tx430/pPTN751), Tx430 wild-type, and 18-4-1a (Tx430/pPTN780).
**Figure 2.4:** Southern blot analysis on selected transgenic sorghum events

**2.4A:** Tx430 events 133-3-5-1 and 137-5-1-1. Membrane was hybridized with synthetic OsCDPK-7 probe. Lanes Tx430, and pPTN751 refer to wildtype DNA and 50 pg of plasmid, respectively.  

**2.4B:** C2-97 events 24-1-1 and 49-2-5b. Membrane was hybridized with synthetic OsCDPK-7 probe. Lanes C2-97, and pPTN751 refer to wildtype DNA and 50 pg of plasmid, respectively.  

**2.4C:** Tx430 events 18-4-1a and 39-1-1a. Membrane was hybridized with 35S CaMV promoter probe. Lanes Tx430, and pPTN780 refer to wildtype DNA and 50 pg of plasmid, respectively.
Figure 2.5: RNA hybridization analysis on selected transgenic sorghum events

2.5A: Tx430 events 133-3-5-1 and 137-5-1-1. Lane Tx430 refers to control total RNA from Tx430. Probe used in analysis was synthetic *OsCDPK*-7. Bottom panel is ethidium stained gel. 2.5B: C2-97 events 24-1-1 and 49-2-5b. Lane C2-97 refers to control total RNA from C2-97. Probe used in analysis was synthetic *OsCDPK*-7. Bottom panel is ethidium stained gel. 2.5C: Tx430 events 18-4-1a and 39-1-1a. Lane Tx430 refers to control total RNA from Tx430. Probe used in analysis was mutated *OsCDPK*-7. Bottom panel is ethidium stained gel.
Figure 2.6: Lesion formation under varying conditions

2.6A: Left, leaf blade of transgenic event 133-3-5-1, after covering with foil, showing lesion formation occurs in a light-independent fashion. Right, leaf blade of Tx430 wild-type, displaying only chlorosis due to coverage of foil. 2.6B: Leaf blade of transgenic event 133-3-5-1 displaying lesion formation under axenic growth conditions. 2.6C: Tx430 wild-type following wounding treatment, 2.6D: transgenic event 133-5-1 following wounding treatment showing formation of lesion does not emanate from wound site.
Figure 2.7: Two-dimensional gel electrophoresis

2.7A: 2-D gel of control TxB30 wild-type protein samples. 2.7B: 2-D gel of transgenic event 133-3-51 protein samples. Arrows indicate differential spots analyzed by tandem mass spectrometry, which indicated the differential proteins were members of the PR-10 family of pathogen related proteins.
Table 2.1: Transcripts with an observed greater than 2-fold increase in expression in Tx 430 pPTN751 transgenic events

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Category</th>
<th>Fold</th>
<th>q-value</th>
<th>Gene</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zm.2321.4.A1_at</td>
<td>Cell maintenance</td>
<td>5.12</td>
<td>0.00023</td>
<td>Alanine aminotransferase</td>
<td>Reversible transfer of amino group from ala to 2-oxoglutarate to form pyruvate and glutamate</td>
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<tr>
<td>Zm.4684.1.A1_at</td>
<td>Cell maintenance</td>
<td>2.3</td>
<td>0.00689</td>
<td>NADH-dependent glutamate synthase</td>
<td>Ammonium assimilation</td>
</tr>
<tr>
<td>Zm.264.1.S1_at</td>
<td>Cell maintenance</td>
<td>2.12</td>
<td>0.008</td>
<td>Acetyl-coenzyme A carboxylase ACC1B</td>
<td>Acetyl-CoA carboxylase. First step in fatty acid biosynthesis</td>
</tr>
<tr>
<td>Zm.13934.1.S1_at</td>
<td>Membrane transport</td>
<td>2.82</td>
<td>0.0078</td>
<td>Plasma membrane H⁺ ATPase</td>
<td>Generates an electrochemical gradient across plasma membrane, aids in maintaining cell turgor</td>
</tr>
<tr>
<td>Zm.17243.1.S1_at</td>
<td>Membrane transport</td>
<td>2.12</td>
<td>0.008</td>
<td>CBL-interacting protein kinase 23</td>
<td>Activates the potassium channel for K⁺ uptake</td>
</tr>
<tr>
<td>Zm.9197.1.A1_at</td>
<td>Abiotic stress</td>
<td>2.8</td>
<td>0.0194</td>
<td>Tonoplast aquaporin</td>
<td>Aquaporin expressed in zones of cell division and elongation</td>
</tr>
<tr>
<td>Zm.16907.1S1_at</td>
<td>Abiotic stress</td>
<td>2.72</td>
<td>0.0013</td>
<td>Phosphoethanolamine N-metyltransferase betaine aldehyde dehydrogenase</td>
<td>Key step in choline biosynthesis leading to the production of glycine betaine BADH1: involved glycine betaine synthesis</td>
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<tr>
<td>Zm.13775.1S1._at</td>
<td>Abiotic stress</td>
<td>2.02</td>
<td>0.0057</td>
<td>Uroporphyrinogen decarboxylase, plastid precursor</td>
<td>Involved in the porphyrin pathway</td>
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<tr>
<td>Zm.385.1.A1_at</td>
<td>photosynthesis</td>
<td>4.0</td>
<td>0.0207</td>
<td></td>
<td></td>
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<tr>
<td>Zm.7091.2.S1_at</td>
<td>photosynthesis</td>
<td>2.5</td>
<td>0.0146</td>
<td>Chlorophyll a/b binding protein</td>
<td>Chlorophyll a/b binding apoprotein precursor mRNA for adenosine kinase</td>
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<tr>
<td>Zm.247.1.A1_at</td>
<td>Signal transduction</td>
<td>2.1</td>
<td>0.0856</td>
<td>Adenosine kinase</td>
<td></td>
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<tr>
<td>Zm.4270.2.A1_at</td>
<td>Defense related</td>
<td>5.7</td>
<td>0.0708</td>
<td>Bowman-Birk serine protease inhibitor</td>
<td>Involved in pathogen defense response</td>
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<tr>
<td>Zm.10293.1.S1_at</td>
<td>Transcription factor</td>
<td>2.9</td>
<td>0.0143</td>
<td>Cprf-2</td>
<td>Phosphorylated in response to light, regulates other light responsive genes</td>
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**Table 2.2:** qRT-PCR results on selected up-regulated transcripts

<table>
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<tr>
<th>Affymetrix ID/Sorghum gene</th>
<th>Normalized expression ratio mean (±SD)</th>
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<tbody>
<tr>
<td>Zm2321.4.A1_a_at/Sb01g023750.1</td>
<td>3.74±1.03</td>
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<tr>
<td>Zm385.1.A1_at/Sb01g036030.1</td>
<td>2.65±1.4</td>
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<tr>
<td>Zm10293.S1_at/Sb10g026450.1</td>
<td>2.3±1.5</td>
</tr>
</tbody>
</table>
CHAPTER 3

Sorghum transformation: overview and utility

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Abstract

Over the past decade genomics resources available for sorghum have rapidly expanded (Paterson 2008), these resources, coupled with the recent completion of the genome sequence which is relatively small in size (730 Mb) (Paterson et al. 2009) makes sorghum a rather attractive species to study. Moreover, the USDA germplasm system maintains 42,614 accessions, of which more than 800 exotic landraces have been converted to day length-insensitive lines to facilitate their use in breeding programs. In addition, a set of EMS mutation stocks developed by the USDA Plant Stress and Germplasm Development Unit in Lubbock, TX (Xin et al. 2009) will be a valuable resource for functional genomics studies in sorghum. However, in order to be a robust system for study, a suite of functional genomics tools are necessary to complement these other resources to aid in down-stream hypothesis testing. A key functional genomics tool is the ability to modulate gene expression through the introduction of transgenic genetic elements. This is exemplified by recent work (Cook et al. 2010) in which RNAi experiments were employed to specifically reduced expression of two alkylresorcinol synthases to demonstrate their role in the synthesis of the allelopathic molecule sorgoleone. In addition to its value as a functional genomics tool, plant transformation offers a route to broaden access to novel input and output traits for sorghum breeding programs.
Sorghum Transformation

In general plant transformation can be partitioned into two components, competence of a cell for culture regeneration into a whole plant and receptiveness of that same cell for foreign DNA integration. In sorghum, like most monocotyledonous plants, *in vitro* culture regimes are primarily somatic embryogenesis based systems (Elkonin and Pakhomova 2000; Jogeswar et al. 2007; Kaeppler and Pedersen 1996; Pola et al. 2008; Pola and Mani 2006; Sato et al. 2004a). As per the second component of plant transformation, integration of genetic elements, sorghum has been successfully transformed using both direct DNA delivery methods (Battraw and Hall 1991) and *Agrobacterium*-mediated transformation protocols (Cai et al. 2002; Gao et al. 2005a; Gao et al. 2005b; Gurel et al. 2009; Howe et al. 2006; Nguyen et al. 2007; Zhao et al. 2000). While both DNA delivery systems are proven technologies for recovery of stable sorghum transformants, more laboratories are moving towards implementing the latter due to the tendency of *Agrobacterium*-mediated transformants to carry lower copy number insertions and/or have a higher frequency of co-expression of the non-selected transgenic cassette (Dai et al. 2001; Gao et al. 2008; Zhao et al. 1998).

While multiple explants have been evaluated as the starting material for sorghum transformation, clearly the primary explant reported on is immature embryos. One of the factors that have hampered transformation efficiencies of sorghum with the immature embryo explant is the rapid production of phenolic compounds. Phenolics are produced during the *in vitro* culturing of sorghum immature embryos, but the production of these secondary metabolites is enhanced upon inoculation with *A. tumefaciens*. To alleviate the negative effects of phenolics on sorghum transformation media supplements such as
polyvinylpolypyrrolidone (PVPP) (Cai et al. 1987), and elevation of potassium phosphate levels (Elkonin and Pakhomova 2000; Sato et al. 2004a), or the exposure of explants to reduced temperature (Nguyen et al. 2007) have been shown to be able to reduce, but not totally eliminate the negative impact of these compounds. Triggering of the plant’s defense response upon challenge with A. tumefaciens may lead not only to the production of secondary metabolites, but also to cell death, which can further hamper the efficiency of recovery of transgenic plants. For example in banana the triggering of apoptosis by A. tumefaciens can be effectively countered by the expression of anti-apoptotic genes (Khanna et al. 2007). While such a strategy has not been evaluated in sorghum, a heat shock pre-treatment, which was previously reported to counter apoptosis in banana embryogenic callus, leading to improved transformation efficiency (Khanna et al. 2004), was recently shown to be a translatable technique using sorghum immature embryos (Gurel et al. 2009).

Key to any transformation system is the ability to rapidly, and efficiently distinguish transgenic differentiating cells from non-transgenic cell lineages. Two means typically used to differentiate transgenic from non-transgenic cell lineages are the use of visual or selectable marker genes. In sorghum the visual marker genes green fluorescent protein (gfp) and β-glucuronidase (GUS) are each effective in monitoring for transgenic cells (Jeoung et al. 2002). Using the former visual marker Gao et al (Gao et al. 2005a) reported a 3.0% transformation efficiency as means to monitor for transgenic differentiating cell lineages from immature embryos of sorghum.
Selectable marker genes used to provide a competitive edge in culture for plant transformation systems typically rely upon providing resistance to antibiotics, such as hygromycin (Gritz and Davies 1983), and the aminoglycoside kanamycin, or various derivatives thereof (Fraley et al. 1983) or tolerance towards herbicidal agents glyphosate (Barry et al. 1992) and glufosinate (Thompson et al. 1987). In addition the positive selectable marker gene phosphomannose isomerase (PMI) (Joersbo and Okkels 1996) has been shown to be a rather robust selection system for the identification of transgenic plants (Negrotto et al. 2000), including sorghum (Gao et al. 2005b).

Outline of an *Agrobacterium*-mediated transformation of sorghum using *npt II* as a selectable marker gene

As indicated above, there have been multiple reports of successful transformation of sorghum following the communicated success in 1993 (Casas et al. 1993). Outlined below is the system reported on by Howe et al (Howe et al. 2006) that utilizes npt II as the selectable marker gene, coupled with G418, as the selection agent. While the overall transformation efficiency with this system is relatively low, typically ranging from 1% to 3%, the system is consistent, and importantly, teachable with minimal training.

This sorghum transformation system relies upon immature embryos as the starting material. One of the disadvantages of using this explant is the need for continual plantings of stock plants to ensure a constant supply of immature embryos, adding labor and cost to the system. Nonetheless, the stock plants used to supply immature embryos are maintained under greenhouse conditions. Heads are harvested when 70% of the head have embryos ranging in size from 1.2 to 2.2 mm in length. Each head is then excised from the plant and placed in a 1000 ml graduated cylinder filled with 500ml of 50%
commercial bleach plus 250 ml of Tween 20. The cylinder containing the sorghum head submerged in the bleach solution is mixed with a stir bar for 20 min. Following the 20 min surface sterilization with bleach the heads are washed three times with sterile water and allowed to air dry in laminar flow hood. Seeds are isolated and subjected to a secondary sterilization process consisting of a 1 min soak with agitation in 70% ethanol, followed by a single wash with sterile water, proceeded by a soak with agitation in 10% bleach solution, with a subsequent triple wash in sterile water.

Immature embryos are isolated from 50 sterilized seeds and placed in a 35X10mm Petri plate containing 1 ml of co-culture medium supplemented with 300 µM of acetosyringone. The co-culture medium is composed of 0.5X MS major and minor salts (Murashige and Skoog 1962), 0.5 mg/l each of nicotinic acid and pyridoxine HCl, plus 1 mg/l each of thiamine HCl and casamino acids. The carbohydrate sources are 2% sucrose and 1% glucose and growth regulator, 2,4-D, at 2 mg/l. The medium is buffered with 3 mM MES (pH 5.2). The medium is filter sterilized, with no components being autoclaved.

Once 50 immature embryos have been isolated the co-culture medium is removed and replaced with 1 ml of A. tumefaciens inoculum. The inoculum is an A. tumefaciens strain NTL4/pTiPKPSF2 (Palanichelvam et al. 2000), suspension in co-cultivation medium (OD$_{660}$ 0.3-0.5). Inoculation time is 5 min. Following the co-cultivation step the explants are placed scutellum side up on 100X20 mm Petri plates containing four sterile Whatman™ filter papers saturated with 4.2 mls of co-cultivation medium. The plates are incubated for 2 days at 24°C in the dark.
Following the co-cultivation period the explants are cultured on delay medium which is composed of Elkonin’s major salts (Elkonin and Pakhomova 2000), MS minor salts and vitamin mix, 2 g proline, and 1 g asparagine. The carbohydrate source is 3% sucrose, the medium is buffered with 3mM MES (pH 5.7) and solidified with 2% phytagel. To counter select against A. tumefcaiens the medium is supplemented with 100 mg/l carbenicillin. The growth regulator 2,4-D is used at a level of 1.5 mg/l. Cultures are incubated at 28°C in the dark for 3 days.

The selection phase is immediately implemented following the delay period. A total of 20 embryos are placed on to 100X20 mm Petri plates containing the delay medium supplemented with 20 mg/L G418. The tissue is transferred to fresh selection medium every 2-3 weeks. As coleoptiles develop they are systematically removed from the explants. As the embryogenic callus begins to form about the explants the tissue is broken up into 1-3 mm pieces, with care being taken to ensure tracking of tissue from the original explant, given the fact that most transformants derived from the same initial immature embryo tend to be clones, hence, the best way to track transformation efficiencies. The callus tissue remains in the selection phase for a period of 6 to 9 weeks.

Following the selection phase proliferating embryogenic tissue is transferred to regeneration medium composed of MS major/minor salts and vitamins, supplemented with 0.5 mg/l kinetin and 1.0 mg/l IAA. The medium is solidified with 2% phytagel, carbohydrate level was 3% sucrose, and buffered with 3 mM MES (pH 5.7). The selection pressure is reduced to 10 mg/l G418, and the carbenicillin level remains at 100 mg/l. The cultures are placed under a 16/8 light regime at 24°C. Typically after 4 weeks
on regeneration, with one subculture at the two-week period, shoots with well established roots will form, that are ready to be acclimated to soil (Fig 3.1).

Once acclimated, the primary transformants are confirmed using a commercial ELISA kit to monitor npt II expression (Agdia Corp.). Primary transformants are screened to identify a minimum of two lead events for down-stream characterizations. A lead event is selected based on simple integration pattern of the transgenic element(s), and expression of the target phenotype(s) of interest.

**Considerations in designing binary vectors for sorghum transformation**

An attribute of *Agrobacterium*-mediated transformation is that T-DNAs can be integrated, albeit relatively infrequently, at unlinked positions. This ability of *A. tumefaciens* can be exploited to derive marker-free transgenic events through the simultaneous delivery of two T-DNAs, where one of the T-DNA elements carries the marker gene, and the other carries gene(s) of interest. If integrated at unlinked positions, the T-DNAs, will segregate in the progeny. This strategy has been successfully used to derived marker-free transgenic plants in a number of systems (Daley et al. 1998; Jacob and Veluthambi 2002; Komari et al. 1996; Sato et al. 2004b; Xing et al. 2000), including sorghum (Zhao et al. 2003). The integration of unlinked T-DNA alleles in sorghum is exemplified in Fig 3.2. Transgenic sorghum events were generated that harbored a transgenic cassette with the cyanamide hydratase (cah) gene (Maier-Greiner et al. 1991), under control of the sugarcane polyubiquitin promoter ubi4 (Wei et al. 2003), housed within a single T-DNA binary vector designated pPTN181 (not shown). A Southern blot analysis is performed using a restriction enzyme wherein one recognition site resides within the T-DNA element, hence each hybridization signal will reflect a single
integration locus, on a subset of primary transformants derived from pPTN181 as shown in Fig 3.2A. As can be seen the event 168 carries one locus, while events 165 and 166 harbor two loci, and event 164 contains 3 loci. Monitoring segregation of the transgenic alleles in progeny derived from these events revealed a 15:1 pattern for events 165 and 166, and a 3:1 pattern for events 164 and 168 (Data not shown). Southern analysis on a subset of the derived progeny is in agreement with the observed segregation patterns (Fig 3.2B&C). It can be seen in the T₁ individuals derived from events 165 and 166 that some individuals display the genotype of the parent, while others only carry one of the transgenic alleles. On the other hand all T₁ derived from event 164 had the same genotype as the parent, hence all alleles appear to be linked. However the single locus event, 168, segregated as expected, with T₁ individuals genotyped the same as the parent.

When implementing the tool of plant transformation for targeted output and input traits in sorghum such as improvement in grain quality or stress tolerance, respective, it is critical to have promoter elements with the desired specificity so to limit the probability of negatively impacting agronomic performance that may arise if the phenotype is mis-expressed in non-target tissues. To this end it is prudent to verify promoter specificity if using a promoter known to be tissue specific in other species, before assembling cassettes for use in sorghum. For example interest in modifying seed components of sorghum may require specific expression in the embryo. A logical candidate promoter for desired embryo-specific expression would be the maize globulin-1 promoter (Belanger and Kriz 1991). To evaluate whether the glob-1 promoter specificity would translate to sorghum a GUSPlus™(www.cambia.org) cassette under control of the glob-1 promoter was assembled and introduced into sorghum. As a constitutive control transgenic sorghum
carrying a GUSPlus™ cassette under control of the maize polyubiquitin promoter (Christensen et al. 1992) was used for comparison. Tissue samples were assayed over development in T₁ or T₂ individuals, looking at GUS expression within root, leaf, stem, glume, scutellum and embryos. As can be seen in Fig 3.3, embryo-specificity of the maize glob-1 promoter effectively translates to sorghum. While this result is not surprising, these are data that need to be gathered to fully exploit sorghum transformation as a translational genomics tool.

**Target Input Traits for Sorghum through Transformation**

A critical trait for any breeding program is yield. Addressing yield directly through transgenic approaches is a considerable challenge. A more practical and obtainable goal in the short term is protection of yield through control of biotic and abiotic stresses. In sorghum, like most crops, key stresses that compromise yield will vary across regions. Sorghum production can be severely impacted by a number of insect pests. Not only can insects impact production directly, but in some cases they can also provide an entry for secondary pathogen attack at the site of insect feeding. The success of the Bt technology in maize (Armstrong et al. 1995; Barry et al. 2000), and cotton (Cattaneo et al. 2006), is a strong rationale for the evaluation of this technology in sorghum as a means to combat specific target insect pests. Importantly, in addition to Bt’s direct impact in impeding insect pest feeding damage, a positive secondary effect observed with the use of this technology is a significant reduction in accumulation of various mycotoxins in plant tissues (Abbas et al. 2008; Bakan et al. 2002; Hammond et al. 2004). This secondary attribute of the Bt technology may serve as a valuable mechanism to limit quality issues of sorghum related to contamination of these toxins.
that may occur under certain conditions and fungal infestation levels (Ghali et al. 2009; Reddy and Raghavender 2008; Reddy et al. 2010). However, like all disease resistance traits, the Bt technology needs to be used in conjunction with proper integrated pest management practices to maximize its durability over time (Kumar and Pandey 2008; Sharma and Ortiz 2000).

A number of viral agents have been shown to be capable of replication in sorghum (Jensen and Giorda 2002), including members of the potyvirus family including sugarcane mosaic virus, maize dwarf mosaic virus, and sorghum mosaic virus. Limited resistance towards these viral agents have been identified within sorghum germplasm, although some reports have been communicated (Henzell et al. 1982). The seminal work which demonstrated introduction of viral coat protein genes in transgenic plants to confer virus resistance (Abel et al. 1986; Nelson et al. 1987; Stark and Beachy 1989), has opened the door for the translation of this technology to other plant systems, implementing various genetic constructs that target silencing of critical gene products required for the replication of the virus of interest (Beachy et al. 2003; Prins 2003), including known pathogens of sorghum (Gilbert et al. 2005). Hence, such strategies offer great potential for the introduction of durable virus resistance for sorghum.

*Striga*, commonly referred to as witch weed, contains two species, *S. hermonthica* and *S. asiatica*, that are parasitic on sorghum and other cereals (Aly 2007). Parasitic plant species infest nearly 50 million hectares crop plants on an annual basis, and great strides have been made in developing resistance in sorghum through conventional breeding approaches (Ejeta 2007). More recently Tuinstra *et al* (Tuinstra et al. 2009) communicated a herbicide seed treatment strategy, that exploits the introgression of
acetolactate synthase (ALS) herbicide resistance from shattercane into elite sorghum genotypes (Hennigh et al. 2010). Implementing this seed-coating approach significantly reduced *Striga* emergence under both greenhouse and field tests (Tuinstra et al. 2009). While this is a very promising tool to combat this devastating parasite, given that ALS inhibiting herbicides are typically classified as high risk for development of resistance, the durability of such a strategy may be limited without proper management. Hence, other approaches are needed to ensure long-term control towards *Stiga*. To this end, there has been a report looking at targeting critical genes in parasitic plant’s life cycle by expression of hair-pin constructs in the host plant which resulted in an enhanced tolerance phenotype in the *Orobanche aegyptiaca*/tomato host parasite interaction (Aly et al. 2009). However, this approach was not successfully translated as a means to control to the *Striga*/maize parasite interaction (Yoder and Scholes 2010). Clearly additional research is required to further our understanding of the underlying biology involved during the early stages of parasitism by these plants. More efforts around the assembly of ‘omics’ databases that carry this information (Torres et al. 2005) are needed to facilitate the development of alternative control strategies towards *Striga*, that may serve as a complement to the herbicide seed coating approach (Tuinstra et al. 2009).

Addressing a plant’s response to stresses that are governed in a multigenic fashion is more challenging than single gene traits. In order to investigate multigenic abiotic stress response traits such as drought and heat, researchers are evaluating a coordinated expression of a suite of genes triggered by exposure to the targeted stress by the introduction of a single transcription factor (Karaba et al. 2007; Nelson et al. 2007; Suzuki et al. 2005). Theses transcription-factor based technologies hold great promise as
a means to reduce multigenic expressed phenotypes to a single transgene fashion (Century et al. 2008), however, the transcription factor based strategy undoubtedly will require tight regulation, necessitating the need for tissue-specific and/or inducible promoter systems.

With respect to adaptation to low nitrogen environments, Yanagisawa et al (Yanagisawa et al. 2004) demonstrated that expression of the maize *Dof1* transcription factor improved nitrogen assimilation in transgenic plants. However, it is feasible to directly perturb nitrogen flux in plants. Nitrogen assimilation and metabolism in plants occurs through coordinated action of a variety of enzymes acting upon a variety of substrates. Two key enzymes involved in nitrogen metabolism in plants are glutamine synthetase (GS) and glutamate synthase (GOGAT). Previous studies have shown that enhancing GS or GOGAT activities can impact nitrogen metabolism in plant species (Cai et al. 2009; Good et al. 2004). Enhancing activity of another enzyme that impacts nitrogen flux in plants, alanine aminotransferase (Ala-AT), that catalyzes the production of alanine and 2-oxoglutarate from pyruvate and glutamate, has been shown to augment nitrogen use efficiency in both rape seed and rice (Good et al. 2007; Shrawat et al. 2008).

A caveat to these studies communicating enhancing nitrogen use efficiency through transgenic approaches is that most reports used data sets gathered from greenhouse or growth chamber studies, with minimal information on the impact of the respective transgenes on yield under field conditions (Brauer and Shelp 2010). Moreover, no data sets have been communicated to date on the impact of gene stacking strategies on nitrogen use efficiencies with these selected genes.
Target Output Traits for Sorghum through Transformation

Digestibility of sorghum limits protein availability, and ultimately impacts the nutritional quality of the grain (Duodu et al. 2003). The major sorghum proteins, prolamins, found in sorghum reside in the endosperm. The prolamin storage proteins found in sorghum and maize are designated kafirins and zeins, respectively. The prolamins are assembled into protein bodies, with a very defined pattern, where the $\alpha$ class reside in the core along with the $\delta$ class, albeit to a lower extent, while the $\beta$ and $\gamma$ classes decorate the periphery of the protein body (Wu and Messing 2010). A number of parameters can influence digestibility of sorghum protein, including structure and shape of the protein body (Duodu et al. 2003). Reduction of the zein proteins found in the maize mutants *opaque*-2 (Hartings et al. 1989) and *floury*-2 (Coleman et al. 1995) leads to a concomitant increase in lysine and tryptophan due to a compensation mechanism in seeds resulting in an increase in non-zein proteins (Coleman and Larkins 1999). Deliberate reduction in the 19 kDa $\alpha$-zeins in maize manifests the opaque kernel phenotype, and enhances levels of lysine and tryptophan in the grain (Huang et al. 2004). Similarly, reduction in the level of both the $\beta$- and $\gamma$-zeins resulted in drastic changes protein bodies, and triggered the opaque kernel phenotype (Wu and Messing 2010). Hence, modulation of the prolamins is a target that could be pursued in sorghum as a means to simultaneously address digestibility, and nutritional quality.

Oria et al (Oria et al. 2000) described a highly digestible, enhanced lysine sorghum mutant. The protein bodies observed within this mutant are highly folded, with a redistribution of the $\gamma$-kafirin about the body. These factors lead to increased exposure
of the core α-kafirins, which translates to the increased digestibility phenotype (Duodu et al. 2003).

Like the maize floury-2 and opaque-2 mutants, the highly digestible, enhanced lysine mutant of sorghum has value in both food and feed applications. However, translation of these traits to application has yet to be realized, undoubtedly due to the tendency of these altered prolamin grains to have reduced agronomic properties, and negative post harvest issues (Huang et al. 2004). However, these drawbacks may not be insurmountable. Breeding efforts are making progress in addressing the issues blocking deployment of the high digestible, enhanced lysine mutant of sorghum (Tesso et al. 2006; Tesso et al. 2008a). Further understanding of the underlying biology governing protein deposition in these mutants and the influence of the various genetic modifiers, coupled with better breeding strategies, will help to utilize this mutant, without negatively altering the endosperm characteristics. Hence, in the end, the successful deployment of a high quality grain sorghum will require an interdisciplinary approach that brings together the expertise of plant breeding, biotechnology, molecular biology/genetics and food science.

**Potential of outcrossing to weedy relatives**

One of the concerns raised about release of transgenic sorghum is the potential for outcrossing to its weedy relatives Johnsongrass and Shattercane. This can result in transfer of transgene into these weedy species and can improve their adaptive capabilities. Hence it can lead to altered balance in the ecosystem, changes in the plant community structure, and persistence of weeds in agricultural lands (Tesso et al. 2008b).

A number of parameters must be met for a successful sorghum outcross event to occur. First, the crop and weed species must be in close proximity, and flowering times
synchronized. Hybrids derived from outcrosses between grain sorghum and shattercane (*S. bicolor* subsp. *drummondii*) do not appear to be compromised in fitness (Sahoo et al. 2010). This lack of fitness drag in sorghum X shattercane hybrids has a benefit when introgressing desirable alleles from the shattercane into the cultivated genotypes, for example ALS resistance gene (Hennigh et al. 2010). However, this attribute that benefits conventional breeding strategies used to broaden diversity of cultivated sorghum, negatively impacts the use of transformation as a tool for introduction of novel traits into the crop, given there will undoubtedly be a call for more extensive regulatory testing addressing the potential ecological impact of a given transgenic sorghum event, which in turn will lead to higher costs and delay in release, with getting a transgenic sorghum event on the market.

One approach that may limit the concern of transgenic sorghum impacting the ecosystem above what is already occurring with production of conventional cultivars is the use of a male sterility system that may effectively limit pollen flow of transgenic sorghum under field conditions (Pedersen et al. 2003). However, such containment systems in many cases may not be required. The current regulatory system has a ‘one size fit all approach’, in that regardless of the trait developed through transgenic approaches, a series of laborious and costly studies must be conducted. While it is very reasonable to assume that deployment of a transgenic sorghum event will eventually be followed by outcross to a wild relative, this must not be the only deciding factor to block production. Rather than a ‘one size fits all’ model, perhaps a more adaptable, scientific regulatory process, in which decisions are made on a case-by-case model is more appropriate. Recently, Hokanson et al (Hokanson et al. 2010) communicated a
straightforward and scientific-fact based risk assessment process that hopefully will open the door for more dialog in this area, and ultimately will allow for advances in transgenic technologies to enter the marketplace expeditiously in a safe and effective manner.
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Figure 3.1: Overview of sorghum transformation steps

3.1A: Sterilization step of immature seeds,
3.1B: Inoculation step of immature embryos
3.1C: Somatic embryogeneic tissue
3.1D: partitioning of somatic embryogenic tissue to ensure lineage tracking
3.1E&F: Regeneration steps
3.1G: Acclimation of plants
3.1H: Transgenic sorghum in greenhouse
**Figure 3.2:** Southern blot analysis of transgenic sorghum events carrying cyanamide hydratase (cah) gene

3.2A: Primary transformation events designated 164, 165, 166, and 168 probed with cah ORF. + lane indicates 50 pg linear binary vector pPTN181. Tx430 lane is 10 µg of wild type DNA.

3.2B: Southern blot analysis of T₁ progeny derived from events 164 and 165, highlighting segregating transgenic alleles in event 165, and linked alleles in 164. WT lane indicates 10 µg genomic DNA from Tx430. + lane is 50 pg of linear binary vector pPTN181.

3.2C: Southern blot analysis of T₁ progeny derived from events 166 and 168, highlighting segregating transgenic alleles in event 166, and linked locus in 168. WT lane indicates 10 µg genomic DNA from Tx430. + lane is 50 pg of linear binary vector pPTN181.
Figure 3.3: GUS expression profile observed in transgenic sorghum

3A-3F: Transgenic sorghum event carrying the glob-1-GUS cassette showing embryo specific expression. 3A: stem section, 3B root section, 3C: glume, 3D leaf, 3E root, and 3F seed, endosperm and embryo (blue).
3G-3L: Transgenic sorghum event carrying the ubiquitin-1-GUS cassette showing constitutive GUS expression. 3A: stem section, 3B root section, 3C: glume, 3D leaf, 3E root, and 3F seed, endosperm and embryo.
CHAPTER 4

Modulation of kernel storage proteins in grain sorghum (*Sorghum bicolor* (L.) Moench)
Summary

Grain sorghum (*Sorghum bicolor* (L.) Moench) ranks fifth among the cereals world-wide with respect to its importance for food and feed applications. The grain is approximately 13% protein, of which the kafirins comprise over 80% of the protein component of the grain endosperm. The kafirins are categorized into subgroups α-, β-, and γ. The kafirins are co-translationally translocated to the ER where they are assembled into discrete protein bodies that tend to be poorly digestible with low functionality in baking applications. As a means to address these two issues in sorghum we employed a biotechnology approach that is designed to simultaneously alter the protein body structure, along with synthesis of a co-protein in the endosperm fraction of the grain. To this end, we report here on the molecular and phenotypic characterizations of transgenic sorghum events that are down-regulated in γ- and the 24 kDa α-kafirins, and the expression of a high molecular weight glutenin protein from wheat, and the creation of various gene stacks with the derived transgenic alleles.
Introduction

Grain sorghum (Sorghum bicolor (L.) Moench) is a staple food for a large proportion of world population. Grain harvested from sorghum and the millets provides an important source for dietary calories and protein for approximately one billion people in the semi-arid regions of the world (Belton and Taylor, 2004). However, grain sorghum products are known to have relatively poor digestibility, only approximately 46%, in comparison other grains such as wheat and maize, which tend to have digestibility percentages over 80 and 70, respectively (MacLean et al., 1981). Protein with high digestibility is by definition nutritionally superior due to the increased availability of amino acids. Digestibility can be impacted by both protein-protein and/or protein-non-protein interactions (Duodu et al., 2003; Taylor et al., 2007). However, with respect to grain sorghum it is expected that the major factor influencing digestibility is the protein-protein interactions leading to high protein cross-linking around the protein body (Duodu et al., 2003).

Protein content of grain sorghum is approximately 13% (Beta et al., 1995), of which the kafirins comprise over 80% of the protein component of the grain endosperm (Hamaker et al., 1995). The kafirins are categorized into α-, β- and γ- groupings, with α1 and α2 approximately 24 kDa and 26 kDa, respectively, and β- and γ- having molecular masses of 18.7 kDa and 20 kDa, respectively (Belton et al., 2006). The kafirins are assembled into discrete protein bodies (PB) in the ER, whereby the α-kafirins compose the core and the β- and γ- decorate the periphery of the PB. It is thought that the organizational structure of the PB has a major impact on protein digestibility of sorghum food and feed products (Hicks et al., 2001). As mentioned above, cooking leads to the
increased cross-linking of the β- and γ-kafirins that blocks the access to the more digestible α-kafirin core (Hamaker et al., 1994; Oria et al., 1995; Oria et al., 2000). This model is supported by the observation that the addition of reducing agents during cooking improves the in vitro digestibility of sorghum (Hamaker et al., 1987; Arbab and El Tinay, 1997). Moreover, studies evaluating highly digestible sorghum mutants further support the structural role of the PB on this parameter (Weaver et al., 1998). Furthermore in the sorghum mutant with high digestible high lysine (HDHL) phenotype, the PB are highly folded, with reduction of the γ-kafirin about the periphery (Oria et al., 2000).

In addition to digestibility, the bread making properties of sorghum flour are also relatively poor. As with digestibility, functionality is also influenced by protein/protein and protein/non-protein interactions. Therefore, to mirror the viscoelastic properties of wheat dough, it is necessary to functionalize the kafirins in such a fashion to allow them to mimic polymeric structures formed during processing between high molecular glutenins and gliadins. Recently it has been reported that blending of co-proteins, such as casein, with sorghum flour in a baking process can stabilize the β-sheet over time, thereby mirroring the functionality of wheat gluten (de Mesa-Stonestreet et al., 2010).

We describe here the generation and characterization of novel sorghum genotypes in which deliberate perturbation of both γ-kafirin, and the 24 kDa α-kafirin have been achieved, in addition to sorghum grain in which high levels of the wheat high molecular weight glutenin protein is produced.
Experimental procedures

Plasmid Constructions

To down-regulate the alpha kafirin, an RNAi approach was used. The alpha kafirin ORF was isolated from the sorghum genotype Tx-430 by RT-PCR on mRNA from immature seed using primers, Kaf-5: ATGGCTACCAAGATATTTGTCCTCCTTGGG and Kaf-3: AATCTAGAAGATGGCACTTCCAACGATGGG, based on the genbank accession number EU424175. Further a 500bp region from 5’ end of this ORF was amplified and restriction sites were designed at its ends using primers GTTAACGGGTTGGATGCAAGTAGCTGTTGT to add HpaI at 5’ end and TCTAGAGCCCTTATGGCCTACCAAGATATTGT to add XbaI at 3’ end. Again the same region was amplified to add different restriction sites at its end using primers GAGCTCGGGTTGGATGCAAGTAGCTGTTGT to add sst1 at 5’ end and CTCGAGGCGCTTATGGGCTACCAAGATATTGT to add XhoI at 3’ end. These amplified fragments were cloned in opposite orientation and put under the control of alpha kafirin promoter. Alpha kafirin promoter was cloned using primers palphaKaf-1: AGACCTCCCAACCCATGCTCGCCACGTTG and palphaKaf-2: TTGGAAGGACGTTGCTAGTTCGTTCCAC to amplify 832bp upstream sequence from translation start site based on gene bank accession number X16104. The gene expression cassette was terminated with 35s terminator. The resultant cassettes were cloned into the binary plasmid pPZP212 (Hajdukiewicz et al., 1994), and designated pPTN1017.

To down-regulate gamma kafirin, a ribozyme mediated RNAi approach was used. To amplify the gamma kafirin promoter, primers were designed as per sequence from gene bank accession number X62480. Primers gKaf-5:
CCGTGTACAACGAAGTGGTGAGTCATGAG and gKaf2-3:
GGTGTCGAGTTTCTGTCTGCTCTTG were used to amplify the 493bp region upstream to translation start site of this gene by PCR on sorghum (Genotype Tx430) genomic DNA. The gamma kafirin ORF was amplified using primers gKaf5:
ATGAAAGGTGTGCTCGTTGCCCTCGCTC and gKaf3:
TCTTTAATAGTGACACCACCGGCAAAAGG by RT-PCR on sorghum (Genotype Tx430) mRNA from immature seed based on gene bank accession number X62480.

Further the gamma kafirin gene was cloned under the control of gamma kafirin promoter and terminated by ribozyme (Buhr et al., 2002). Ribozyme is a self cleaving RNA molecule that cleaves itself and leads to the production of aberrant RNA. Further this RNA along with the targeting endogenous gene are silenced through RNAi mechanism. (Buhr et al., 2002; Haseloff et al., 1988). The resultant cassettes were cloned into the binary plasmid pPZP212 (Hajdukiewicz et al., 1994), and designated pPTN915. The binary vector designated pPTN883 was prepared as described by Blechl et al. (1996). The final binary vectors were mobilized into A. tumefaciens strain NTL4 carrying the disarmed Ti plasmid pKPSF2 (Luo et al., 2001) via tri-parental mating. Sorghum transformations were conducted with genotypes Tx430 as previously described (Howe et al., 2006).

**Molecular characterization of transgenic sorghum**

Total genomic DNA was isolated from sorghum leaves following a modification of the protocol described by Dellaporta et al. (1983). Southern blot analysis of transgenic events was conducted as previously reported (Howe et al., 2006). Membranes were hybridized with either dCT\(^{32}\)P labeled HMW-GS ORF or gamma kafirin ORF or the
spacer fragment separating the two RNAi arms of the alpha kafirin down-regulation cassette. For RNA gel blot analysis, total RNA was extracted from ground individual seeds (20 days after anthesis) in 1 ml of TRIzol® LS reagent (Invitrogen). RNA sample separation and hybridizations were carried out as previously described (Buhr et al., 2002). Membranes were hybridized with either dCT\(^{32}\)P labeled 2288-bp region of the HMW-GS ORF or 502-bp fragment from the alpha kafirin ORF or 819-bp fragment from the gamma kafirin ORF.

**Seed protein extracts**

Individual seeds were ground in liquid nitrogen. Ground tissue was mixed with protein extraction buffer composed of 0.1 M Trizma base, 10 mM EDTA, 0.9 M sucrose, 0.4% (v/v) β-mercaptotethanol, pH 8.0. The mixture was subsequently extracted for 30 min at 4°C, with slight agitation, in equal volume Tris-saturated phenol. Following the 30 min extraction step, the suspension was centrifuged at 4,350 x g in a Beckman JA-20 rotor for 10 min. The upper phenol phase was collected and precipitated overnight at -20°C, using 5X volume of 0.1M ammonium acetate in 100% methanol. Precipitated proteins were pelleted at 17,400 x g in a Beckman JA-20 rotor for 10 min. The protein pellet was subsequently washed with 0.1M ammonium acetate in 100% methanol, followed by washings in 80% (v/v) acetone, and finally with 70% (v/v) ethanol. The washed protein pellets were suspended in 8 M urea, 2M thiourea, 2% (w/v) CHAPS, and 2% (v/v) Triton X-100. Protein concentrations were quantified using the Bradford assay.

**Two-dimensional gel electrophoresis**

A total of 300 µg of protein was loaded onto each 7 cm pH 3.0-10.0 gradient Ready Strip IPG (Bio-Rad, cat # 163-2000). Following the run in the first dimension, the
protein was run in the second dimension using 14% SDS-PAGE and the gel was subsequently stained with Coomaissie G-250. Differential spots observed in transgenic samples as compared to control samples, were picked and analyzed using tandem mass spectrometry.

**Microscopy**

Immature seeds 12 days after anthesis were sliced transversely into 1–2-mm pieces with a razor blade and fixed in 4% (vol/vol) paraformaldehyde and 1% (vol/vol) glutaraldehyde in 0.05M potassium phosphate buffer (pH 6.8) at 4°C for 16 h. For microstructural analysis, the specimens were stained with 2% osmium tetroxide in 0.05 M potassium phosphate buffer (pH 6.8). After washing with the same buffer, the specimens were dehydrated in a graded ethanol series (15 min each in 10, 30, 50, 70, 90, and 95% aqueous ethanol, and in 100% ethanol). After gradual dehydration in the ethanol series, samples were infiltrated for 2 h each in 20, 40, 60, and 80% LR White resin in ethanol, and then for overnight in 100% LR White resin. The infiltration with 100% LR White resin continued for 2 more days with several changes of fresh resin. The samples were placed in plastic molds and polymerized for 2 days in an oven at 55°C. Prepared specimens were sectioned with a diamond knife on an ultramicrotome (LKB Ultrotome III, Stockholm, Sweden). Copper grids coated with formvar membrane and carbon were used. Sections were post stained for 5 min with 2.5% (wt/vol) uranyl acetate and for 3 min with 0.1% (wt/vol) lead citrate and examined with a Hitachi H7500 transmission electron microscope (Hitachi, Tokyo, Japan) at 80 kV at the University of Nebraska-Lincoln Microscopy Core Facility.
Digestibility and amino acid analysis

*In vitro* pepsin digestibility was carried out on seed derived from a subset of the transgenic sorghum events expressing the HMW-GS. Seed samples were uniformly ground using a Udy mill equipped with a 0.5 mm screen. The *in vitro* digestibility was ascertained following the procedure described by Mertz et al (Mertz et al, 1984). For amino acid analysis the seed samples were sent to Eurofins, Iowa.

**Results**

**Molecular analysis of transgenics**

Binary vector designated pPTN883 harbors gene expression cassette for the expression of high molecular weight glutenin subunits (HMW-GS) under the control of seed specific promoter. Binary vector designated pPTN915 and pPTN1017 contained gene expression cassettes for the down-regulation of gamma (Fig 4.1a) and alpha kafirin (Fig 4.1b) genes under the control of gamma and alpha gene promoters respectively. More than ten independent events were obtained with each binary vector. Southern blot analyses were conducted on all the transgenic events to check the integration of the transgene in the host genome (Fig 4.2a, 4.2b, 4.2c). Total genomic DNA was digested with an enzyme for which there is a single recognition site within the T-DNA element to produce a single hybridization signal for each transgenic locus. Although multiple copies per insertion may be present, copy number in each insertion event was not determined. Genomic DNA hybridization suggested the presence of varying number of inserts in different events. Northern blot analyses were conducted to evaluate the expression of these genes at the mRNA level. The Dx5 promoter from wheat resulted in very high expression of the HMW-GS in the sorghum seed (Fig 4.3a). Furthermore, the expression
of the genes at the protein level was evaluated by two dimensional SDS-PAGE (Fig 4.4a, 4.4b, 4.4c). Mass spectrometry was used to confirm the expression of HMW-GS or down-regulation of alpha or gamma kafirin proteins. Lead events showing high expression of HMW-GS and efficient down-regulation of the endogenous alpha or gamma kafirin genes were selected for further assays. The lead events i.e. 127-1-1-1 (pPTN883) is a single insert event while 128-2-1-1 (pPTN883) has three inserts in it (Fig 4.2a). These showed high expression levels of HMW-GS as indicated by Northern analysis (Fig 4.3a) and the two dimensional gels (Fig 4.4a). Events 133-3-1-1 9 (pPTN915), 288-1-1-2 (pPTN1017) and 285-1-2-1 (pPTN1017) have single insert in them (Fig 4.2b, 4.2c) and showed efficient down-regulation of the endogenous genes (Fig 4.3b, 4.3c).

**Digestibility assay**

Digestibility of the transgenic seeds was evaluated as mentioned in the material and methods section. For HMW-GS, five independent events were used for the evaluation of the digestibility assay and all of them showed an improvement of digestibility of the transgenic flour over the wild type flour (Fig 4.5a). These five independent events were evaluated further using RP-HPLC to determine the relative amount of HMW-GS in these events (Fig 4.5b). A novel peak was observed at 3.8 minute of retention time which is the area where HMW-GS are known to elute using RP-HPLC separations. This peak appeared in all of the five transgenic events and was absent in the wild type seed. The area of this peak indicates the relative amount of HMW-GS in the respective events. A regression analysis showed a strong correlation between the area of the peak and digestibility (Fig 4.5c). Cooked flour from the events expressing HMW-GS
and cooked/uncooked flour from events showing down-regulation of gamma kafirin did not show any improvement in flour digestibility.

**Amino acid analysis**

Amino acid content of various lead events was evaluated. The down-regulation of gamma kafirin protein resulted in decreased amounts of proline, glutamate and leucine (Fig 4.6). The amino acid content of all other amino acids was largely unchanged. Down-regulation of alpha kafirin protein led to a large increase in the amounts of lysine, arginine and aspartate. In addition a slight decrease in glutamate and leucine content was observed. Expression of HMW-GS in the sorghum seed did not lead to any significant change in the amino acid profile of the transgenic seed.

**Microscopy assay**

Microscopic studies were conducted at the microscopy facility at University of Nebraska-Lincoln on the developing seed 12 days after anthesis. The protein bodies present in the selected events expressing HMW-GS and showing down-regulation of gamma kafirin protein did not show any significant changes as compared to protein bodies from wild type seed (Fig 4.7a). All the wild type and transgenic seeds showed protein bodies of varying sizes indicating the presence of protein bodies at different stages of development or it could be the result of dissection blade cutting through protein bodies at different positions. Though they were of varying sizes, all of them were surrounded by a smooth surface and has a clear boundary. The clear appearance of ER membranes and other organelles e.g. mitochondria indicates that the sample was well processed. The events showing down-regulation of alpha kafirin showed the presence of distorted protein bodies that were absent in the wild type seed (Fig 4.7b). These protein
bodies in the transgenic events were small in size and showed invaginations on the surface going deep into the protein bodies. This indicates that proper expression of alpha kafirin protein was required for the formation of wild type protein bodies while exclusive down-regulation of gamma kafirin was not able to show significant change in the appearance of protein bodies (Fig 4.7c).

**Seed phenotype**

Seed from all the genotypes were evaluated for any change in their phenotype. Transgenic events showing the down-regulation of gamma and alpha kafirins showed an opaque phenotype (Fig 4.8a, 4.8b). Seeds from the segregating head were easy to separate on the light box on the basis of opacity. The cut sections of the seed also showed the change in the opaque region. Cut sections of the vitreous seed showed a small opaque region present at the center of the seed while this region was extended well towards the margins of the seed in opaque seeds. While the seed from down-regulation of gamma kafirin did not show any significant change in seed weight, down-regulation of alpha kafirin led to a 22% decrease in seed weight. Seed with the expression of HMW-GS did not show any change in the phenotype.

**Discussion**

Sorghum is a staple food and is a source of energy and proteins for many people living in developing countries. Despite this importance, sorghum suffers from limitations of low digestibility and poor bread making properties. In an attempt to improve the digestibility, we followed a transgenic approach to down-regulate various endogenous proteins and to express the high molecular weight glutenin subunits (HMW-GS) in sorghum seed. The poor digestibility of sorghum flour is attributed to the presence of
cross-linked proteins in the form of protein bodies that persist through most food preparations. Cooking sorghum in the presence of water further reduces the digestibility. It is proposed that cooking may lead to the formation of polymeric structures that are less susceptible to digestion and also result in reduced accessibility of digestive enzymes to proteins (Hamaker et al., 1987; Oria et al., 1995). It is known that the beta and gamma kafirins have extensive cysteine residues which is 5% in β kafirs and 7% in γ kafirins (Hamaker and Bugusu, 2003). These result in formation of strong disulfide bonds upon cooking and in reduced accessibility of digestive enzymes to centrally located alpha kafirins. The digestion of protein bodies initiates at the surface where gamma and beta kafirins are present and then proceeds towards the center (Oria et al., 1995). In order to improve the digestibility of the sorghum flour we down-regulated the gamma kafirin protein that is present in abundance on the surface of the protein bodies. This was expected to improve the accessibility of the digestive enzymes to centrally located alpha kafirin proteins. In addition, down-regulation of cysteine rich gamma kafirin proteins was expected to result in reduced polymeric structure formation that is also expected to lead to improved digestibility. Contrary to what was expected, the elimination of gamma kafirin did not lead to improved digestibility of sorghum flour. Expression of HMW-GS in the sorghum seed did result in improvement of digestibility of uncooked sorghum flour (Fig 4.5a, 4.5c). We speculated that change in the interaction between various components of the seed could be the reason of this improvement. When the flour containing the endogenously expressed HMW-GS was cooked, it did not show any change in digestibility as compared to wild type flour. We speculated that upon cooking, gelatinization of the starch could have formed a strong network. This could have
prevented various protein interactions to take place in the flour and prevented any improvement in the digestibility. Earlier studies conducted by Duodu et al. (2002) have shown that treatment of cooked sorghum with alpha–amylase leads to improved digestibility of proteins. This indicates that not only the protein structure, but also the interactions of protein with sorghum starch could determine the digestibility of sorghum proteins.

The evaluation of amino acid content of the transgenic seed with down-regulation of the gamma kafirin gene showed that there was a decrease in the amount of proline, glutamate and leucine amino acids (Fig 4.6). Gamma kafirin protein has the highest amount of proline and leucine and hence down-regulation of this protein is expected to result in the decreased amount of these amino acids. Reduction in the amount of glutamate could have resulted from the non-specific silencing of some other glutamate rich protein. Down-regulation of alpha kafirin protein led to large increase in the amount of lysine, arginine and aspartate (Fig 4.6). Similar observation is known to result in maize with the down-regulation of zein proteins. o2 is a transcription factor that controls the expression of zein genes (Schmidt et al., 1992). A mutation in this gene results in the decreased synthesis of zein proteins. This nearly doubles the lysine content of the corn seed (Mertz et al., 1964). Reduction in zein synthesis led to increased accumulation of various other non zein proteins that have higher lysine content (Habben et al., 1993) that resulted in the improved nutritional quality of the corn. Further Huang et al. (2006) transgenically silenced the 19- and 22-kD zein proteins using RNAi approach which led to increased expression of other proteins that maintained the total protein content while changing the amino acid composition of the seeds. In our down-regulated alpha kafirin
mutants, although there was an increase in the amount of lysine, arginine and aspartate amino acids, the seed weight was reduced. Transgenic seeds showed about 22% reduction in the seed weight. Like other major cereals, sorghum seed contain low amounts of essential amino acids such as lysine, tryptophan and threonine (Badi et al., 1990). An increase in the amount of the essential amino acid lysine in the transgenic sorghum would have a great significance as sorghum is a protein source for a large population worldwide. Improvement in the nutritional components of the seed would help to meet the nutritional needs of these people. While down-regulation of endogenous kafirin proteins showed a change in the amino acid profile of the seed, expression of HMW-GS in the sorghum seed did not lead to any significant change in the amino acid profile of the transgenic seed (Fig 4.6).

The seeds showing down-regulation of alpha and gamma kafirin proteins showed an opaque phenotype. This opaque phenotype is known to be associated with maize mutants with down-regulated zein proteins (Hunter et al., 2002). Although the exact mechanism for the opaque phenotype is not known, disturbance of correct arrangement of proteins and other components of the seed are hypothesized to be the reasons for the appearance of the opaque phenotype (Wu et al., 2010).

Sorghum proteins are present in the seed in the form of protein bodies that have very organized structures. The alpha kafirin is present at the center and is surrounded by beta and gamma kafirin proteins. It is expected that all these proteins are required for the proper assembly of the protein bodies and elimination of any one fraction of these could lead to change in the structure of the protein bodies. To evaluate the effect of down-regulation of specific protein fraction on the formation of protein bodies, microscopy
studies were conducted. In the mutants showing the down-regulation of gamma kafirin (Fig 4.7c) or expression of high-molecular weight glutenin subunit (Fig 4.7a), no specific difference was observed in the protein bodies of transgenic and wild type events. Immunolocalization studies on these mutants are needed to determine any changes in the distribution of the endogenous proteins. The down-regulated alpha kafirin protein mutants (Fig 4.7b) showed the presence of distorted protein bodies. These protein bodies have deep invaginations on the surface and were smaller in size as compared to the wild type protein bodies. These protein bodies are similar to mutant protein bodies reported earlier by Oria et al. (2000). They reported that the kafirins were redistributed in the mutant protein bodies. Gamma kafirins were found to be present on the base of the lobes and alpha kafirin was more exposed to the outside. We speculated that similar observation in our studies could be the result of redistribution of various kafirin proteins in the protein bodies. In other study conducted in maize, Wu et al. (2010) down-regulated different zein proteins and analyzed the changes in the protein body structure. They found that when alpha zein is down-regulated, it leads to the formation of small bulges at the surface of the protein bodies. However in our studies shrinken protein bodies with invaginations of various degrees were seen as a result of the down-regulation of alpha kafirin proteins. These observations indicate that although alpha kafirin is present in the center of the protein bodies, it is very important component of the protein bodies for their proper assembly.

The down-regulation of alpha kafirin in the sorghum seed, showed a simultaneous increased expression of various other proteins as shown by the two dimensional SDS-PAGE gels (Fig 4.4c). *In silico* analysis of the possible function of these proteins was
performed. Spot numbers 21 and 22 gave the highest scoring hit for S-like RNAse (Table 1). It has been reported that the increase in the amount of this protein is associated with leaf senescence (Lers et al., 1998), phosphate starvation (Dodds et al., 1996) and pathogen infection (Wilson, 1975). Hence these S-like RNAse are expected to be associated with phosphate remobilization, plant defense and mRNA decay (Green, 1994). RNA is a rich source of organic phosphate. Hence increasing the rate of turnover of this molecule could increase the availability of P₃ in the plant (Dodds et al., 1996). In our study we down-regulated the alpha kafirin in the seed using RNAi technique. As RNAi leads to breakdown of dsRNA into 21-23 bp fragments, we speculated that the increase in the amount of S-like RNAse could be the result of accumulation of these small RNA fragments and is required for their turnover. Hence while these small RNAs would be eliminated, the remobilized phosphate could be used for other cellular purposes.

The second high-scoring hit for spot number 21 is 2-cys peroxiredoxin. It catalyzes the conversion of hydroperoxides to their corresponding alcohols (Baier et al., 1999). Hence it protects DNA and other organelle from oxidative damage (Dietz et al., 2006). Its increased expression in our study indicates a probable increase in the production of reactive oxygen species in the seed. Hence its role could be to protect macromolecules present in seed at various stages of development. Peroxiredoxins are also known to be activated upon pathogen infection (Dietz et al., 2006). The highest scoring hit for spot numbers 23 and 24 showed sequence homology to PCC 13-62. This protein is induced upon dehydration and salt stress in *Craterostigma plantagineum* (Bernacchia et al., 1996; Smith-Espinoza et al., 2003). Similarly spot number 25 gave highest scoring hit for isoflavone reductase like protein. This protein is known to be induced in response to
UV light, wounding and pathogen infection (Lers et al., 1998). Although in this study, no stress was applied to the plants, the accumulation of these proteins indicated that plant is sensing some kind of stress. Spot number 26 gave the highest scoring hit for Glyoxalase 1. This gene is known to be up-regulated by salt stress (Espartero et al., 1995, Veena et al., 1999). Transgenic tobacco plants with increased expression of this gene led to improved salt tolerance (Singla-Pareek et al., 2003). As alpha kafirin is a major fraction of the storage proteins in the sorghum seed, its down-regulation would result in major change in the metabolism in the seed that could result in stressful conditions and activation of stress related proteins.

The highest scoring hit for spot numbers 27, 28, 29 and 30 was identified to be xylanase inhibitor protein (XIP). Fungal and bacterial pathogens produce cell wall degrading enzymes in order to enter the host cells. The inhibitory activity of XIP against such enzymes protects the grain from pathogen attack (Bellincampi et al., 2004). Hence increased expression of this protein indicates its role in defense mechanism. This hypothesis is further supported by the fact that XIP in wheat does not inhibit endogenous xylanase activity but inhibits activity of xylanases from fungal sources (Juge et al., 2004).

The events obtained in this study are being crossed in all possible combinations to obtain double and triple cross events. Seed will be carried to homozygosity and will be evaluated for baking applications. Improvement in the digestibility and functionally of the sorghum flour should lead to its improved usages in various food applications. This will not only have bearing on the nutritional utility of this crop but will also have significant effect on the economic growth of the countries where sorghum is the primary grown crop.
References:

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Dodds P N, Clarke A E and Newbigin E (1996) Molecular characterization of an S-like RNAse of Nicotiana alata that is induced by phosphate starvation. Plant Mol. Biol. 31 : 227-238


**Figure 4.1a:** Binary plasmid carrying gene cassette for the down-regulation of gamma kafirin gene under the control of gamma kafirin promoter and is terminated by ribozyme. The construct also consists of nptII gene cassette as a selectable marker.

**Figure 4.1b:** Binary plasmid carrying gene cassette for the down-regulation of alpha kafirin gene under the control of alpha kafirin promoter and is terminated by 35S terminator. The construct also consists of nptII gene cassette as a selectable marker.
Figure 4.2a: Southern blot analysis of sorghum (Tx-430) events with HMW-GS gene. Lane 1-2, transgenic events; Lane 3, negative control (Tx-430); Lane 4 is a positive control (pPTN883).

Figure 4.2b: Southern blot analysis of sorghum (Tx-430) events with gamma kafirin down-regulation. Lane 1-3, transgenic events; lane 4, negative control (Tx-430); lane 5 is a positive control (pPTN908).
Figure 4.2c: Southern blot analysis of sorghum (Tx-430) events with alpha kafrin down-regulation. Lane 1-3, transgenic events; lane 4, negative control (Tx-430); lane 5 is a positive control (pPTN-1017).

Figure 4.3a: Northern blot analysis of sorghum (Tx-430) events with high molecular weight glutenin subunits. Lane 1-12, transgenic events; lane 13 is a negative control (Tx-430).
**Figure 4.3b:** Northern blot analysis of sorghum (Tx-430) events with gamma kafirin down-regulation. Lane 2-8, transgenic event; lane 1,9,10 is a negative control (Tx-430).

**Figure 4.3c:** Northern blot analysis of sorghum (Tx-430) events with alpha kafirin down-regulation. Lanes 1-8, 12-19, transgenic events; lanes 9-11 are negative control (Tx-430).
Figure 4.4a: Two dimensional gel analysis of transgenic event with high molecular weight glutenin subunits (left panel) and control event Tx-430 (right).

Figure 4.4b: Two dimensional gel analysis of control event Tx-430 (left panel) and transgenic event with gamma kafirin down-regulation (right).

Figure 4.4c: Two dimensional gel analysis of control event Tx-430 (left panel) and transgenic event with Alpha kafirin down-regulation (right).
**Figure 4.5a:** Pepsin digestibility of transgenic sorghum events expressing a hybrid HMW-GS

**Figure 4.5b:** RP-HPLC chromatogram of transgenic events (High molecular weight glutenin subunits) and control (Tx-430). A novel peak at 3.8 min (arrow) determined to be HMW-GS.
Figure 4.5c: Regression line between amount of HMW-GS and digestibility

Figure 4.6: Amino acid profile of different transgenic events and wild type sorghum seed.
**Figure 4.7a**: Microscopic pictures at 15X magnification. Top panels are of Tx-430 and lower panels are of transgenic events expressing HMW-GS

**Figure 4.7b**: Microscopic pictures at 30X magnification. Top panels are of Tx-430 and lower panels are of transgenic events with down-regulation of alpha kafirin gene
Figure 4.7c: Microscopic pictures at 15X magnification; Top panels are of Tx-430 and lower panels are of transgenic events with down-regulation of gamma kafirin gene.

Figure 4.8a: Opaque phenotype observed in transgenic events with down-regulation of gamma kafirin gene (left) Vs. wild type seed (right). Top panel: Cut sections of the seed; Lower panel: whole seed.
Figure 4.8b: Opaque phenotype observed in transgenic events with down-regulation of alpha kafirin gene (left) Vs. wild type seed (right).
**Table 4.1**: The upregulated proteins as the result of down-regulation of alpha kafirin protein (Corresponding Fig-4c)

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<th>Gel-Spot Number</th>
<th>Protein ID and function</th>
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<th>Score</th>
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