SORGHUM TRANSFORMATION AND

EXPRESSION OF GENES RELATED

TO PLANT DEFENSE

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TABLE OF CONTENTS

Chapter .... Page
Chapter 1 Introduction 1
1.1 Sorghum transformation 1
1.1.1 Sorghum transformation protocol improvements 1
1.1.2 Antimicrobial transgenic sorghum 3
1.1.3 Antifungal transgenic sorghum 5
1.1.4 Gene expression under phloem specific promoters in transgenic sorghums and development of greenbug resistant sorghum 6
1.2 Tobacco transformation 8
1.3 Thaumatin-like (TL) protein expression in plants encounters resistance 9
1.4 Rationale 11
1.5 Objectives 12
1.6 References 13
Chapter 2 Sorghum Transformation 18
2.1 Abstract 18
2.2 Introduction 20
2.2.1 Transformation method development 20
2.2.2 Development of antimicrobial sorghum for bacterial diseases 23
2.2.3 Antifungal transgenic sorghums 24
2.2.4 Gene expression under phloem-specific promoters in transgenic sorghum and development of greenbug resistant sorghum 25
2.3 Materials and Methods 27
2.3.1 Plant materials 27
2.3.2 Bacterial strains and plasmids 27
2.3.2 Tobacco transformation 28
2.3.3 Sorghum transformation 28
2.3.4 GUS assay 33
2.3.5 PCR analysis 33
2.4 Results 34
2.4.1 Tobacco transformation 34
2.4.2 Sorghum transformation 37

2.5 Discussion 47
2.5.1 Development of a method for sorghum transformation using immature inflorescences 47
2.5.2 Development of *Agrobacterium*-mediated sorghum transformation ..51
2.5.3 Development of antimicrobial transgenic sorghum cultivars 54
2.5.4 Development of other transgenic sorghum cultivars 55
2.6 References 56
Chapter 3 Differential expression of thaumatin-like protein in sorghum infested with greenbug 61
3.1 Abstract 61
3.2 Introduction 62
3.3 Materials and methods 64
3.3.1 Plant material and growth conditions...64
3.3.2 Greenbug infestation 64
3.3.3 RT-PCR and Real-time RT-PCR 65
3.4 Results and Discussion 67
3.5 Discussion 72
3.6 References 75

LIST OF TABLES

Table 2-1. Sorghum transformation and regeneration media 31
Table 2-2. Media for *Agrobacterium*-mediated sorghum transformation 53
Table 3-1. Real-time PCR threshold cycle analysis of three different sorghum lines when infested with greenbugs...69

LIST OF FIGURES

Fig. 2-1. Tobacco leaves on shoot-induction medium with shoot buds..............35
Fig. 2-2. Tobacco plants on root-induction medium with well developed shoots and roots..35
Fig. 2-3. GUS staining of a leaf from the AMP transgenic tobacco plant...........36
Fig. 2-4. GUS staining of a leaf from a 121 transgenic tobacco plants...............36
Fig. 2-5. PCR for the GUS gene of genomic DNA from transgenic AMP and 121 tobacco plants..37
Fig. 2-6a. Regenerated sorghum calli of Tx430 (immature inflorescence) with developed shoot buds…………………………………..............................39
Fig. 2-6b. Sorghum calli of Tx430 (immature inflorescence) with well-developed shoots on shoot regeneration medium…………………… 39
Fig. 2-6c. Transformed explants of Tx430 (immature inflorescence) with well developed shoots and roots of sorghum in magenta boxes on root regeneration medium. 40
Fig. 2-7a. GUS staining of transformed callus. 44
Fig. 2-7b. GUS staining of leaves of a regenerated transformed plant................44
Fig. 2-8a. The gel results of PCR for the GUS gene...45
Fig. 2-8b. PCR results for target genes CO1 and Ct in transformed regenerated plant genomic DNAs...46
Fig. 3-1. RT-PCR DNA gel showing the transcriptional induction of thaumatin-like protein (TLP) of three sorghum lines when infested with greenbug at different treatment times…..68
Fig. 3-2. Threshold cycle fold changes of thaumatin-like protein (TLP) at the transcriptional level in three sorghum lines when infested with greenbugs.71

Chapter 1

Introduction

1.1 Sorghum transformation

1.1.1 Sorghum transformation protocol improvements

Plant biotechnology challenges the age-old agricultural practices of plant genetic improvement, especially in the field of genetic transformation. Gene transfer techniques allow the incorporation of beneficial genes for specific agronomic traits into diverse crop species (Vaeck *et al.* 1987). Genetic transformation allows plant breeders to get new improved varieties by installing desired foreign genes such as insect resistance and pathogen resistance genes into the commercial lines in a shorter period of time than traditional breeding methods (Amoah *et al*. 2001; Gao *et al*. 2000; Hilder *et al*. 1987). As previously reported, many of the world's most important crops like wheat (Triticum aestivum) (Cheng *et al.* 1997), maize (Zea Mays L.) (Ishida *et al.* 1996) and rice (Oryza sativa) (Hiei *et al.* 1994) have already been engineered with increased resistance to insects and diseases.

There are only a few reports on sorghum (*Sorghum bicolor* L. Moench) transformation and almost all of these experiments were conducted to deliver foreign marker genes into plant cells (Jeoung *et al.* 2002). Although sorghum regeneration (Cai *et al.* 1987) and transformation has been reported (Casas *et al*. 1993a), no agronomically useful transgenic sorghum cultivar has been developed. The reason that sorghum transformation is lagging

behind other major crops is partly due to difficulties associated with its tissue culture and the lack of efficient protocols for transformation (Huang 2005).

Development of an efficient plant regeneration system is the first critical step for a successful sorghum transformation system. Microprojectiles, *Agrobacterium* and pollen-mediated transformation have all been studied for sorghum transformation. Although *Agrobacterium*, a natural engineer, may be a better system for DNA delivery in higher plants, including graminaceous monocots (Ishida *et al*. 1996; Schlappi and Hohn 1992), microprojectile bombardment may be a better mechanism for grass species including sorghum. Microprojectile bombardment has been developed for sorghum transformation (Casas *et al*. 1993b), which apparently has some advantages, considering it has no specification in plant genotypes or target tissues.

A commercial sorghum line Tx430 was used in several transformation studies, (Casas *et al*. 1993a; Casas *et al*. 1997; Howe *et al*. 2006), and proved to be amenable to genetic transformation. Thus, Tx430 should be an ideal plant genotype for the study of sorghum transformation. Tx430 is a widely used greenbug-susceptible fertility restorer sorghum line (Miller 1984), while Tx2737 is a greenbug biotype C-resistant fertility restorer line (Johnson *et al*. 1982.), so both genotypes were chosen for the development of a successful transformation system.

Transgenic fertile sorghum plants were obtained by microprojectile bombardment of immature embryos and shoot tips (Casas *et al.* 1993a; Girijashankar *et al*. 2005). Immature tissues, including inflorescences and immature embryos, have proved to be the

most suitable sources of morphogenic structures in cereals (Maddock *et al.* 1983; Rout and Lucas 1996). Meanwhile, inflorescence cultures have many advantages over immature embryos (Amoah *et al.* 2001). Plant cells may lose their competence at an early stage. If the immature inflorescences are collected as soon as the flag-leaf comes out, they will probably have more ability to regenerate.

Selectable marker genes are normally used to select the transformed explants. Case *et al*. (1993) used bialaphos as the selection agent. A novel mannose sorghum transformation selection system has also been reported recently (Gao *et al.* 2005). Antibiotics, like geneticin and paromomycin, were used as the selectable markers in sorghum transformation in a recent report (Howe *et al.* 2006). Based on Howe's study, hygromycin and kanamycin might also be used as selectable markers in sorghum transformation.

While some sorghum transformations have been reported, the transformation rate has been low, and few of the gene constructs used for sorghum transformation carried additional agronomical value. In this study, we decided to develop a highly efficient sorghum transformation and regeneration system by using a commercial sorghum line and microprojectile bombardment with kanamycin as the selectable marker.

1.1.2 Antimicrobial transgenic sorghum

Sorghum is the fifth most important cereal crop in the world. Sorghum is not only used to produce food, but can also be an importance source of animal feed and fodder,

especially in dry areas (Sharma 1993). Sorghum is now also considered an important feed-stock for ethanol production (Rains *et al.* 1993). Development of new sorghum cultivars has to date been relying on traditional breeding methods (Able *et al.* 2001). Most of the world's important crops have already been engineered with increased resistance to insects and diseases (Cheng *et al.* 1997; Hiei *et al.* 1994; Ishida *et al.* 1996), while sorghum is lagging behind. To date, there are no agronomically useful transgenic sorghum cultivars.

Pathogenic bacteria and fungi can cause important diseases in crop plants. For example, bacterial stripe (*Pseudomonas andropogoni*) and bacterial spot (*Pseudomonas syringe*) are two important sorghum diseases caused by bacteria (Frederiksen and Odvody 2000). Disease resistant plants have their own mechanisms for defending against pathogens, but through genetic transformation, the pathogen resistance of crops can be greatly improved. Plants have many defense mechanisms to protect themselves from pathogenic organisms, like the ability to produce many kinds of antimicrobial proteins (Turrini *et al.* 2004). One group of plant antimicrobial proteins is the cysteine-rich peptides group, which includes thionins (Bohlmann and Apel 1991), lipid-transfer proteins (LTPs) and defensins (Broekaert *et al.* 1995). Defensins, found in mammals, insect, and plant systems, are cysteine-rich cationic proteins active against bacteria, fungi and enveloped viruses (Broekaert *et al.* 1995). Antimicrobial peptides, like plant defensins, have been used for effective and sustained control of fungal and microbial pathogens in modern agriculture (Gao *et al.* 2000). Though there are many reports about

using plant antimicrobial peptides to develop transgenic crops, there has been no previous demonstration using a gymnosperm peptide to develop disease resistant crops.

When studying the genetic mechanism of host resistance in loblolly pine, antimicrobial properties were found in the tissue and a novel type of antimicrobial peptide was characterized based on the sequence; this peptide was named PtAMP. This AMP has conserved domains and multiple disulfide bridges that are believed to be the corresponding part of cysteine-rich AMPs from animals and plants. PtAMP showed the ability to inhibit the growth of a number of pathogenic organisms as described by Huang et al in 2003. Developing transgenic Pt-AMP tobacco plants is the first step to study the antimicrobial function of PtAMP in *vivo*. PtAMP-transgenic tobacco plants might have enhanced pathogen resistance. Like other plant antimicrobial peptides, PtAMP could be used to develop disease resistant crop plants (Topping 1998). In this report, the *PtAMP* gene, which was isolated from the tissue of loblolly pine, was used to genetically engineer pathogen resistant sorghum. Expression of the *PtAMP* gene under the control of a promoter will be analyzed. The efficiency of PtAMP sorghum against bacterial diseases will be evaluated.

1.1.3 Antifungal transgenic sorghum

Sorghum is an important cereal crop in the world. Several fungal pathogens cause some important diseases in sorghum. Previously improvement of disease resistance in sorghum mainly relied on traditional breeding (Able *et al.* 2004). Plant biotechnology,

especially plant transformation, has been widely used in crop development for disease resistance. Some plant species produce a wide range of proteins that can inhibit the growth of pathogenic microorganisms. Plant chitinase plays an important role in host defense against fungi because the chitin hydrolysis enzyme can degrade the cell walls of fungi (Cramer *et al.* 1985; Lamb *et al.* 1989; Dixion and Lamb 1990; (Schlumbaum *et al.* 1986). Thus, the plant chitinase gene is a promising tool for crop disease improvement (Grison *et al.* 1996). There are three classes of chitinase genes: basic chitinase; acidic chitinase; lysozyme homogenous chitinase (Metraux *et al.* 1998).

The rice (*Oryza sativa* L.) RCH10 gene could also be induced by a fungal cell wall elicitor in suspension cultured cells. The rice RCH10 not only encodes a basic chitinase gene, but also might encode an acidic chitinase gene (Zhu and Lamb 1991).

The RCH10 chitinases accumulate mostly in roots, and a few accumulated in leaf and stem tissues (Zhu and Lamb 1991). The plasmid Ct contains a rice chitinase RCH10 gene. This gene could be used to develop fungus resistant sorghum.

1.1.4 Gene expression under phloem specific promoters in transgenic sorghums and development of greenbug resistant sorghum

Sorghum ranks fifth in importance among cultivated cereal crops. The aphid greenbug (*Schizaphis graminum*) has been reported as one of the major pests of sorghum since 1968 (Porter *et al.* 1997). It causes damage that costs tens of millions of dollars annually in the USA (Park *et al.* 2005). Until present, most farmers used insecticides to

kill greenbugs, but insecticides can cause harmful contamination to the environment and many are costly. An alternative aphid control is to enhance sorghum's resistance to greenbugs without using chemicals.

Greenbug management on sorghum has relied on conventional breeding methods, which have become an important component of the sorghum improvement program. It is important to develop more effective ways to improve sorghum resistance (Park *et al.* 2005) as the traditional methods cannot overcome the difficulty of natural barriers (i.e. sexual incompatibility) and the narrow genetic variability (limited gene pool) of sorghum (Huang 2005). Currently, plant biotechnology utilizing plant transformation is a promising tool as the most effective way to overcome these barriers. Greenbugs always feed on phloem (Porter *et al.* 1997). So, it is better if the foreign greenbug resistant genes are specifically expressed in the phloem. The plasmid of SUC_2 contains an *A. thaliana* $AtSUC_2$ gene promoter -GUS fusion which can direct the gene expression specifically in the phloem. The plasmid SUC_2 contains both the reporter gene *uidA*, encoding beta-glucuronidase (GUS) and the selectable marker Kanamycin gene (Truernit and Sauer 1994; Zhao *et al.* 2004). Plasmid CO1 contains a CoYMV (*Commelina yellow mottle* virus) GUS gene fusion. This CoYMV promoter can also direct gene expression specifically to the phloem (Medberry *et al.* 1992).

The objective of our project is to study gene expression in sorghum using phloem specific expression promoters (both SUC_2 and CO1 vectors contain the phloem specific promoters). For this purpose, some transgenic SUC_2 and CO1 sorghum plants, which

could be used to study and compare gene expression in sorghum under these kinds of promoters by using GUS staining, were obtained (Thomma *et al.* 2002). Since greenbugs feed on the phloem of sorghum, these kinds of promoters can be used to develop anti-greenbug transgenic sorghum plants in the future. Snowdrop lectin (*Galanthus nivalis agglutinin*; GNA) is toxic to lepidopteran, while safe to human and animals. It has been cloned into the pholem-specific promoter and used for the development of transgenic crops with improved host defense against other insects (Gatehouse *et al.* 1997). Recent work in Dr. Huang's lab demonstrated novel greenbug resistance genes in resistant sorghum. Some of the host defense genes have been identified in greenbug resistant sorghum lines using microarray methods (Park *et al.* 2005).

Further, novel greenbug resistant loci have also been found by an SSR QTL mapping method (Wu *et al.* 2007). Once the newly identified greenbug resistance genes are cloned, they can be used with the phloem specific promoters to develop greenbug resistant transgenic sorghum plants in the future. This may be the first study to utilize sorghum greenbug resistance genes to develop transgenic sorghum cultivars for greenbug management.

1.2 Tobacco transformation

Plants can produce many types of antimicrobial proteins to protect themselves from pathogenic organisms, such as cysteine-rich peptides (Turrini *et al.* 2004). A novel type of antimicrobial peptide was found in loblolly pine and characterized based on its sequence. This peptide was named PtAMP (Huang *et al.* 2003). The AMP conserved

domains and multiple disulfide bridges of PtAMP can be thought of as the corresponding part of cysteine-rich AMPs found in animals and plants. The PtAMP protein exhibited inhibition of the growth of a wide range of plant pathogenic bacteria and fungi in *vitro* (Huang *et al.* 2003).

As an ongoing study of the PtAMP functions in *vivo*, transgenic tobacco plants will be used in this study of PtAMP function. Tobacco (*Nicotiana spp*., L.) has been a model plant for tissue culture and transformation (Nester *et al.* 2005). Dicotyledons like tobacco are widely used in *Agrobacterium*-mediated transformation. In this study, plasmids of AMP and 121 (negative control) were used to develop two different kinds of transformed tobacco plants. Constructing the transgenic tobacco plants is the first step to study the antimicrobial function of PtAMP in *vivo*. PtAMP transgenic tobacco plants may have enhanced pathogen resistance. Like other plant antimicrobial peptides, PtAMP can be used to develop disease resistant crop plants like sorghum in the future (Topping 1998).

1.3 Thaumatin-like (TL) protein expression in plants encounters resistance

Plants have their inherent mechanisms for resistance to pathogens and many factors are involved in those mechanisms: some are preformed, others are inducible (Hammerschmidt 1999). Pathogen-related (PR) proteins such as chitinase, osmotins, and β-1,3-glucanase, are defined as proteins that are encoded by the plant genome and induced specifically in response to infections by pathogens such as fungi, bacteria, or viruses, or by adverse environmental factors (Breiteneder 2004). PR proteins are divided

into several families. The PR-5 family has amino acid sequence similarities to thaumatin proteins, which are sweet and were first found as a mixture of proteins isolated from the katemefe fruits (Van der Wel and Loeve 1972). Thaumatin-like proteins (TLP) that belong to the PR-5 family are involved in the plant resistance mechanisms. There are three classes of thaumatin-like proteins: proteins that are produced in response to pathogen infection; osmotic proteins; and plant antifungal proteins (AFPs) which are constitutive in plants, especially in seeds (Breiteneder 2004).

Thaumatin-like proteins, which are involved in pathogen resistance, can be induced by a large spectrum of pests (not only insects, but also virus, bacteria and fungi) and stimuli-like chemicals, wounding, cold stress, etc (Bol *et al.* 1990; Lotan and Fluhr 1990; Trudel *et al.* 1998). For example, *Rhizoctonia solani*, the sheath blight fungi, can cause the induction of TLPs in rice based on molecular analysis; and two different TLPs involved in this response were revealed by western blotting (Velazhahan *et al.* 1998). A recent study showed that salicylic acid (SA) and jasmonic acid (JA) could induce TLPs and *β*-1,3-glucanase production in wheat plants at the protein level, and caused system acquired resistance (SAR) leading to enhanced resistance to bacterial diseases. The induction level was time-dependent (Jayaraj *et al.* 2004).

An antifungal thaumatin-like protein was isolated from sorghum leaves in 2002 (Velazhahan *et al.* 2002). The aphid greenbug (*Schizaphis graminum*) might also be an inducer of TLPs in sorghum (Hammerschmidt 1999). Although many studies showed the induction of TLPs when a plant encounters a stimulus or pathogen, no study has

quantatively compared the time-dependent expression of TLPs in different cultivars especially between resistant and susceptible cultivars.

A recent report showed that pathogen related proteins were differentially expressed among different cultivars when barley plants interacted with a bacterial pathogen (Geddes *et al.* 2008). There may also be differential expression of TLP in sorghum at the cultivar level. Real-time PCR is a quantatively and time-dependent technique that is widely used in the molecular world that has proven to be a useful tool in the in-depth study of pathogen-related TLPs (Klein 2002). In this study, the real-time PCR technique was used to quantatively analyze the expression of TLP at the transcription level of two greenbug resistant lines (PI550607 and PI550610), and one greenbug susceptible line (Tx7000), when they were infested with greenbug.

1.4 Rationale

Plant tissue culture is a prerequisite of plant transformation systems. Tobacco is a model plant for tissue culture and transformation, which can be conveniently used to validate gene function. The plasmid AMP contains the antimicrobial gene which was isolated from the loblolly pine in our lab. The *PtAMP* gene function *in vivo* will be tested in tobacco first, which should demonstrate the potential of the *PtAMP* gene for crop improvements.

Sorghum transformation is much more difficult than tobacco transformation, so genetic improvement of sorghum has depended on conventional plant breeding methods. As a result, sorghum insect management has mainly relied on the development of pest

resistant varieties through traditional breeding and improved cultural management practices.

Thaumatin-like protein (TLP) is reported as a defense related response in plants. Real-time PCR is a method to quantitatively analyze the levels of gene expression during different treatment time points. These methods will be used to study sorghum's TLP gene transcription changes when infested by greenbugs.

1.5 Objectives

This research project focused on three objectives. The first was to develop transgenic plants to test the gene constructs in the model plant tobacco. The second objective, as the core project, was to develop disease and greenbug resistant sorghum plants through the transgenic approach. To reach the goal, sorghum regeneration and transformation methods must be developed first; thus both particle bombardment and *Agrobacterium* infection methods were used in the development of a sorghum transformation system. Once a sorghum transformation system was developed, target genes such as disease resistance and aphid resistance genes were to be used to produce transgenic sorghum plants with improved resistance to sorghum diseases and pests.

The last research objective was to examine the expression of a defense-related protein in sorghum plants. The aphid greenbug is a notorious pest of important crops, including wheat and sorghum. According to our preliminary data, greenbug feeding induced the expression of thaumatin-like protein (TLP) expression, which is reported as a defense-related protein. Thus this experiment was designed to analyze the expression of TLP in

sorghum plants in response to greenbug attack and to compare its expression in resistant and susceptible lines.

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Chapter 2

Sorghum Transformation

2.1 Abstract

The objective of the study was to develop a protocol for sorghum transformation and regeneration, leading to development of transgenic sorghum plants with enhanced greenbug resistance. Immature inflorescences were collected as soon as the flag leaves appeared and cultivated on a co-cultivation medium for 5 to 14 days prior to microprojectile bombardment. Various levels of antibiotic selection were imposed to the shoot and root regeneration media to select transformants while shoots and/or roots were developing. The GUS gene vector's selectable marker expression was stably expressed not only in propagation calli, but also in regenerated sorghum plantlets. The efficiency of regeneration and transformation varied and was affected by the following factors: the amount of plasmid DNA that was used for bombardment, the vector that was used to deliver genes into the plant genome, the stage of which the immature inflorescences were collected, the cultivation length before the explants were bombarded, the ingredients of the cultivation medium, especially shoot and root regeneration media, and the selection antibiotic selection pressures. Other factors might also affect the transformation efficiency, such as the bombardment pressure, the plasmid-coated particles, and the

bombardment distance from the rupture disk to the stopping plate. At an optimized condition, the sorghum transformation rate could be significantly improved.

The investigation of critical factors for sorghum regeneration and transformation is an important first step for the development of improved sorghum cultivars. During this study, the sorghum regeneration and bombardment transformation protocol was well-developed and established with sorghum line Tx430.

From the GUS staining and PCR results, some transgenic sorghum plants were obtained. Microprojectile bombardment of immature inflorescences of sorghum Tx430, resulted in transgenic sorghum plants carrying the *PtAMP* (containing the antimicrobial gene isolated from loblolly pine) and the rice chitinase gene. In addition, the DNA sequences of two phloem-specific promoters (one from *Commelina yellow mottle* virus and the other from the *A. thaliana* $AtSUC_2$ gene) were also transferred into the sorghum genome. These transgenic sorghum plants might express the target genes and could be developed into disease resistant cultivars in the future.

2.2 Introduction

2.2.1 Transformation method development

Plant biotechnology challenges the age-old agricultural practices of plant genetic improvement, especially in the field of genetic transformation. Gene transfer techniques allow the incorporation of beneficial genes for specific agronomic traits into diverse crop species (Vaeck *et al.* 1987). Genetic transformation allows plant breeders to get new improved varieties by installing desired foreign genes, such as insect and disease resistant genes into the commercial lines in a shorter period of time than traditional breeding methods (Amoah *et al*. 2001; Gao *et al*. 2000; Hilder *et al*. 1987). As previously reported, many of the world's most important crops like wheat (Cheng *et al.* 1997), maize (Ishida

et al. 1996) and rice (Hiei *et al.* 1994) have already been engineered with increased resistance to insects and diseases.

There are only a few reports on the trials of sorghum transformation and almost all of these experiments were conducted to deliver foreign marker genes into plant cells (Jeoung *et al.* 2002). Although sorghum regeneration (Cai *et al.* 1987) and transformation have been reported (Casas *et al*. 1993a), to my knowledge no transgenic sorghum plant with agronomically traits has been developed to date. The reason that sorghum transformation is lagging behind other major crops is partly due to difficulties associated with its tissue culture and partly due to the lack of efficient protocols for transformation (Huang 2005).

Development of efficient plant regeneration protocols is the first critical step for a successful sorghum transformation system. Microprojectiles, *Agrobacterium*, and pollen-mediated transformation have all been successfully used in sorghum transformation. Microprojectile bombardment has been used more in sorghum transformation (Casas *et al*. 1993b). Microprojectile bombardment has some advantages, for example, it has no limitation to plant genotypes or target tissues. However, *Agrobacterium*, a natural engineer, may be a better system for higher frequency of DNA delivery in higher plants, including graminaceous monocots (Ishida *et al*. 1996; Schlappi and Hohn 1992).

There are a few reports of successful transformation using the commercial sorghum line Tx430 (Casas *et al*. 1993a; Howe *et al*. 2006), so it might be an ideal plant genotype for the study of sorghum transformation. Tx430 is a widely used greenbug-susceptible fertility restorer sorghum line (Miller 1984), while Tx2737 is a greenbug biotype C-

resistant fertility restorer line (Johnson *et al*. 1982.); thus both genotypes were chosen to develop a successful transformation system for sorghum.

Transgenic fertile sorghum plants (*Sorghum bicolor* L. Moench) were reportedly obtained by microprojectile bombardment of immature embryos and shoot tips (Casas *et al*. 1993a; Girijashankar *et al*. 2005). Immature tissues, including inflorescences and immature embryos, have proven to be the most suitable sources of morphogenic structures in cereals (Maddock *et al.* 1983; Rout and Lucas 1996). Meanwhile, inflorescence cultures have many advantages over immature embryos (Amoah *et al.* 2001). Since inflorescences cells lose their competence at an early stage, the immature inflorescences were collected as soon as the flag-leaf appeared to achieve better regeneration.

During transformation experiments, selectable markers are usually used to select transformed explants. Case *et al*. (1993) first used bialaphos as the selection agent for sorghum. Later, a novel mannose transformation selection system was also reported in sorghum (Gao *et al.* 2005). Other antibiotics, like geneticin and paromomycin have also been used as the selectable markers in sorghum transformation (Howe *et al.* 2006). Based on Howe's study, hygromycin and kanamycin might be the effective selectable markers for sorghum transformation.

While some sorghum transformations have been reported, the transformation rate is relatively low, and none of the transgenic sorghum had agronomic value. In this study, our goal was to develop a highly efficient sorghum regeneration and transformation system by using a commercial sorghum line and microprojectile bombardment. We used the antibiotic kanamycin as the selection agent. Based on our transformation system, an

agronomically useful sorghum cultivar with improved disease resistance could be developed.

2.2.2 Development of antimicrobial sorghum for bacterial diseases

Sorghum, *Sorghum bicolor* (*L.*) Moench, is the fifth most important cereal crop in the world. Sorghum cannot only be used to produce food, but can also be an importance source of animal feed and fodder, especially in dry areas (Sharma 1993). Sorghum is now also considered an important biomass source for ethanol production (Rains *et al.* 1993). The development of a new sorghum cultivar mainly relies on traditional breeding methods (Able *et al.* 2001). To date, most of the world's important crops have already been engineered with increased resistance to insects and diseases (Cheng *et al.* 1997; Hiei *et al.* 1994; Ishida *et al.* 1996), while sorghum is lagging behind. Yet there has been no successful production of agronomically useful transgenic sorghum cultivars.

Pathogenic bacteria and fungi can cause some important diseases in crop plants. For example, bacterial stripe (*Pseudomonas andropogoni*) and bacterial spot (*Pseudomonas syringe*) are two important sorghum diseases caused by bacteria (Frederiksen and Odvody 2000). Disease resistant plants have their own mechanisms for defending against pathogens, but through genetic transformation, the pathogen resistance of crops can be greatly improved. Plants have many defense mechanisms to protect themselves from pathogenic organisms including the ability to produce many kinds of antimicrobial proteins (Turrini *et al.* 2004). One group of the plant antimicrobial proteins is cysteine-rich peptides, which includes thionins (Bohlmann and Apel 1991), lipid-transfer proteins (LTPs) and defensins (Broekaert *et al.* 1995). Defensins, found in mammal, insect, and plant systems, are cysteine-rich cationic proteins active against bacteria, fungi and

enveloped viruses (Broekaert *et al.* 1995). Antimicrobial peptides, like plant defensins, are used for effective and sustained control of fungal and microbial pathogens in modern agriculture (Gao *et al.* 2000). Though there are many reports of using plant antimicrobial peptides to develop resistant transgenic crops, there has been no previous demonstration of using a gymnosperm peptide to develop resistant crops.

When studying the genetic mechanism of host resistance in loblolly pine, antimicrobial properties were found in the tissue and a novel type of antimicrobial peptide was characterized based on sequence; this peptide was named PtAMP. The AMP conserved domains and multiple disulfide bridges of PtAMP can be thought of as the corresponding part of cysteine-rich AMPs from animals and plants. Therefore, the PtAMP may have the potential ability to inhibit the growth of pathogenic organisms (Huang *et al.* 2003). Developing transgenic tobacco plants is the first step to study the antimicrobial function of PtAMP in *vivo*. PtAMP transgenic tobacco plants might have enhanced pathogen resistance. Like other plant antimicrobial peptides, PtAMP could be used to develop disease resistant crop plants like sorghum (Topping 1998). In this report, PtAMP, which was isolated from the tissue of loblolly pine, was used to genetically engineer pathogen resistant sorghum. The expression of the *PtAMP* gene under the control of a promoter was analyzed. The efficiency of PtAMP sorghum against bacterial diseases was evaluated.

2.2.3 Antifungal transgenic sorghums

Plant biotechnology, especially plant transformation, is widely used in crop development. Plant chitinase, which is involved in host defense, is a promising tool for the crop disease improvement (Grison *et al.* 1996). Plants can produce a wide range of

proteins to inhibit the growth of pathogenic microorganisms; chitinase, a hydrolyzing enzyme of chitin, is one example in the defense against pathogen attack (Cramer *et al.* 1985; Lamb *et al.* 1989; Dixion and Lamb 1990). Like plant antimicrobial pepetides, plant chitinase could potentially inhibit the fungi growth by attacking and digesting the cell wall of fungi (Schlumbaum *et al.* 1986). There are three classes of chitinase genes: basic chitinase; acidic chitinase; lysozyme homogenous chitinase (Metraux *et al.* 1998).

The rice (*Oryza sativa* L.) RCH10 gene may also be induced by a fungal cell wall elicitor in suspension cultured cells. The rice RCH10 gene not only encodes a basic chitinase gene, but may also encode an acidic chitinase gene (Zhu and Lamb 1991). The RCH10 chitinase accumulates mostly in plant roots, and with limited accumulation in leaves and stem tissues (Zhu and Lamb 1991). The plasmid Ct contains a rice chitinase RCH10 gene.

This plasmid was used to develop fungal resistant crops, like sorghum, in the future.

2.2.4 Gene expression under phloem-specific promoters in transgenic sorghum and development of greenbug resistant sorghum

Greenbug management on sorghum has relied on conventional breeding methods and has become an important component of future sorghum improvement. It is important to develop more effective ways to improve sorghum resistance (Park *et al.* 2005). But traditional methods cannot overcome the difficulty of natural barriers (i.e. sexual incompatibility between species) and the narrow genetic variability (limited gene pool) of sorghum (Huang 2005). Currently, plant biotechnology utilizing plant transformation is a promising tool and the most effective way to overcome these limitations. Greenbugs always feed on phloem (Porter *et al.* 1997), so it is necessary to express foreign greenbug

resistant genes in the phloem. The plasmid of SUC_2 contains an *A. thaliana* $AtSUC_2$ gene (2137bp) with a GUS fusion promoter which directs gene expression to the phloem. Plasmid SUC_2 contains the reporter gene *uidA*, encoding beta-glucuronidase (GUS) and the selectable marker Kanamycin gene (Truernit and Sauer 1994; Zhao *et al.* 2004). Plasmid CO1 contains a CoYMV (*Commelina yellow mottle* virus) GUS gene fusion promoter. This promoter can direct gene expression specifically to the phloem (Medberry *et al.* 1992). This project is to study gene expression using phloem-specific expression promoters (plasmids of SUC_2 and CO1 contain the phloem-specific promoters) in transgenic sorghum. First, I need make some transgenic SUC_2 and CO1 sorghum plants, and then compare gene expression in sorghum with these promoters by using GUS staining (Thomma *et al.* 2002). Since greenbugs feed on the phloem of sorghum, these phloem-specific promoter can be used to develop greenbug resistant transgenic sorghum plants.

Snowdrop lectin (*Galanthus nivalis agglutinin*; GNA) is toxic to lepidopterans, while safe to human and animals. It is used for the development of transgenic greenbug resistant crops (Gatehouse *et al.* 1997). Recent work in Dr. Huang's lab demonstrated novel greenbug resistance genes in resistant sorghum. Greenbug resistant genes have been identified using microarray methods (Park *et al.* 2005).

Further, a novel greenbug resistant locus was also found by SSR and AFLP methods (Wu *et al.* 2007). The newly identified greenbug resistance genes can be used with the phloem specific promoters to develop transgenic sorghum plants. This may be the first study utilizing of sorghum greenbug resistance genes to develop transgenic sorghum cultivars for greenbug management in field crops.

2.3 Materials and Methods

2.3.1 Plant materials

Two different grain sorghum lines with different genetic backgrounds were used in this research. Tx430 is a sorghum line widely used for transformation, while Tx2737 was used as a control in this study. Sorghum plants were grown in a greenhouse at 25 □ for two to three months after seeding. Immature inflorescences were collected as soon as the flag leaf appeared; the head of plant was cut, all the sheaths were removed and the immature inflorescences were cut into small pieces and placed onto co-cultivation medium.

2.3.2 Bacterial strains and plasmids

Four gene constructs were used in this study.

Plasmid A was AMP (pBI121-AMP) that contains the reporter gene *uidA* encoding beta-glucuronidase (GUS). It also contains an antimicrobial protein gene (the *PtAMP* gene) that was isolated from loblolly pine (Huang *et al.* 2003). Plasmid pBI121 containing no AMP gene was used as a negative control.

Plasmid B was Ct that contains the GUS gene, a kanamycin resistance gene, and an antifungal (chitinase) gene (Zhu and Lamb 1991).

Plasmid C was CO1 which contains the GUS gene and a kanamycin resistance gene; both genes are driven by a phloem-specific expression promoter (i.e., the Commelina yellow mottle virus, CoYMV promoter) (Medberry *et al.* 1992).

Plasmid D was SUC_2 that contains the GUS reporter gene and a marker kanamycin resistance gene. Both genes are driven by the promoter of the $AtSUC_2$ gene which directs phloem-specific expression (Zhao *et al.* 2004).

Agrobacterium strain 4404 was used in this study for developing transgenic tobacco plants. This disarmed *Agrobacterium* strain was employed to deliver the binary vectors, (i.e., one containing the 121-AMP gene fusion, the other containing the plasmid 121 only), into plant cells separately.

2.3.2 Tobacco transformation

According to the method of Svab *et al* (1975), *Agrobacterium* was inoculated on YEM medium with 50 mg/ml kanamycin for 1-2 days at 28 °C. *Agorbacterium* cultures (5 ml) were then centrifuged and the upper liquid phase was removed. The *Agrobacterium* pellet was resuspended with 25 mL callus induction medium (CIM) (dilution rate is 1:5). Young leaves of tobacco plants were collected and cut into small pieces, and the small pieces were immersed in the *Agorbacterium* solution for 10 minutes at room temperature. Filter paper was used to remove the excess *Agrobacterium*-CIM liquid from the surface of tobacco leaves. The tobacco leaves were transferred onto CIM medium for co-cultivation with *Agrobacterium* for. 3-5 days in the dark, then transferred to shoot induction medium (SIM) (containing timetin 200 mg/L) and subcultured every 2 weeks for 2-4 weeks. When shoots formed, the regenerated plantlets were transferred onto root induction medium (RIM) (containing timentin 200 mg/L and kanamycin 50 mg/L), after 4-6 weeks they were transferred to soil and grown in greenhouse.

2.3.3 Sorghum transformation

2.3.3.1 Preparation of Microprojectiles

The microprojectiles were prepared using the method developed by Sanford *et al*. (1993). Sixty milligrams of gold particles (Bio-Rad, 1.0-1.6 µm in diameter) were washed once in 70% ethanol, then three times with sterilized water, and then the pellet

was resuspended in 1000 μl of 50% glycerol (for deagglomeration of the particles). While vortexing, 50 μl aliquots of the mixture were put into 20 sterile screwcaped microtubes and stored at -20 □ until use.

For coating DNA onto the microprojectile, the 50 μl of mixture stock were placed on wet ice and used within 4 hours. While vortexing, the following components were added in order: 10 μl plasmid DNA （0.1 to 1 μg/μl, Amp, CT, SUC_2 or CO1), 50 μl $CaCl_2$ (2.5 M) and 50 μl ice-cold spermidine (0.1 μM, tissue culture grade, base free). The mixture was then vortexed for another 2-3 minutes, allowed to settle down for 2-5 minutes, then centrifuged at high speed for 2-5 seconds. The supernatant was discarded and the pellet was washed with 250 μl absolute ethanol, and then resuspended in 75 μl absolute ethanol. The final concentrations of all ingredients in the suspension were: golden particle 40 μg/μl, plasmid DNA 0.013~0.13 μg/μl, $CaCl_2$ 1.5 to 1.6 M and spermidine 63 mM. Ten milliliters of the suspension (0.13~1.3 μg of the plasmid DNA and 400 μg of the gold particles) was used per bombardment.

2.3.3.2 Microprojectile bombardment

Transformation experiments were conducted with the Biolistics PDS 1000/He system. Immature inflorescences, which were cultivated on co-cultivation medium for 10-14 days, were then bombarded with microprojectiles (four kinds of microprojectiles, each made from different plasmid). Sterilized filter paper was used to absorb the water from the surface of the inflorescences prior to bombardment. The bombardment pressure was 900 to 1300 psi and the distance from rapture disc to the launch plate was 10 cm to 13 cm. Ten microliter of coated micoprojectiles were used per bombardment. The biolistic

sample chamber and acceleration tube of the PDS- 1000 were cleaned with 95% ethanol. During bombardment gas pressure was held at 23-25 psi.

Following bombardment, immature inflorescences were transferred onto the sorghum propagation medium incubated at 25 °C in dark. To develop shoots, the immature inflorescences were transferred onto shoot regeneration medium with a period of 16 hours of light and eight hours of dark everyday and sub-cultivated every two weeks until shoots had developed well. Under these conditions, some shoots even developed roots after grown on the shoot regeneration medium for four to eight weeks. The regenerated plants were then transferred into soil and grown in the greenhouse. Poorly developed or unrooted plants were transferred onto root regeneration medium. After the roots were well-developed, they were transferred into soil and placed in the greenhouse. Table 2-1 shows the basic ingredients of each medium.

Table 2-1. Sorghum transformation and regeneration medium

Medium	Auto-claved	Filter-sterilized	Time
Co-cultivation	Sucrose 20 g/L Glucose 10 g/L Ms salt 4.4 g/L Phytagel 2.2 g/L pH 5.8	Vitamin B5 I ml Myo-insitol 0.1 g/L L-proline 0.7 g/L ABA 10 mg/L MES 0.5 g/L 2,4-D 2 mg/L	20 to 30 days Subculture every 5-7 days
Propagation	Sucrose 20 g/L Glucose 10 g/L Ms salt 4.4 g/L Phytagel 2.2 g/L pH 5.8	Vitamin B5 I ml Myo-insitol 0.1 g/L L-proline 0.7 g/L ABA 10 mg/L MES 0.5 g/L 2,4-D 2 mg/L Kinetin 0.5 mg/L	4 to 8 weeks Subculture every 5-7 days
Shoot regeneration	Sucrose 20 g/L Glucose 10 g/L Ms salt 4.4 g/L Phytagel 2.2 g/L pH 5.8	Vitamin B5 I ml Myo-insitol 0.1 g/L L-proline 0.7 g/L MES 0.5 g/L 2,4-D 2 mg/L Kinetin 0.5 mg/L IAA 1 mg/L	4 to 6 weeks Subculture every 7-10 days
Root regeneration	Sucrose 20 g/L Glucose 10 g/L Ms salt 4.4 g/L Phytagel 2.2 g/L pH 5.8	Vitamin B5 I ml Myo-insitol 0.1 g/L L-proline 0.7 g/L MES 0.5 g/L 2,4-D 2 mg/L Kinetin 0.5 mg/L NAA, IBA 1mg/L	2 to 4 weeks Subculture every 7-10 days

In order to increase the frequency of shoot and root regeneration, some critical ingredients of the media were tested. For shoot regeneration, 0.35 g/L L-proline was added to the shoot regeneration medium. Then the concentration of L-proline was increased to 0.7 g/L. Additional MES was also added to the medium at a final concentration of 0.5 g/L.

2.3.3.3 Selection agents of transformation (antibiotic selection conditions)

Kanamycin, ampicillin and hygromycin were used as the selection agents in these experiments. Antibiotics were dissolved in water (50 mg/mL) and sterilized by filtration and stored at -20 □ prior to use. To test the antibiotic's effect on shoot regeneration, kanamycin (50 mg/L) and hygromycin (1.5 mg/L) were first added to the shoot regeneration medium (without L-proline and MES, did not have enough time to test the one with L-proline and MES).

To find the best selection condition for development of the transgenic sorghum plants, a different group of explants were used. Fifty mg/L of kanamycin was imposed to the propagation medium (as selection medium) for two to three weeks, and then decreased to 25 mg/L for another two weeks. The calli were transferred onto shoot regeneration medium with or without selection pressure at a final concentration of 50mg/L. In addition, the shoot regeneration medium was supplemented with L-proline and MES at a series of concentrations of 0 mg/L, 15 mg/L, and 50 mg/L.

For the selection of Amp-transformed explants, kanamycin and hygromycin were all used. For the selection of CT-transformed explants, CO1-transformed and SUC_2-transformed explants, kanamycin was used for the selection of the transformed calli.

2.3.4 GUS assay

GUS assay was performed to determine the gene delivery into immature inflorescences, calli, shoots and leaves (Jefferson *et al.* 1987). Immature inflorescences, calli, shoots or leaves of sorghum and leaves of tobacco were transferred into small tubes containing GUS-staining buffer (1 mM 5-bromo-4-chloro-3-indolyl-D-glucuronide [X-Gluc], 100 mM sodium phosphate buffer pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 0.1% Triton X-100). The tubes were incubated overnight at 25 °C, and at room temperature a further 24 h. Explants, especially leaves and shoots, were then washed once with sterile distilled water and steeped in 70% ethanol overnight to extract any chlorophyll that may be present in the tissues. Explants were then examined under a microscope and the ratio of explants producing blue spots per treatment and the number of spots per explant was counted and statistically analyzed.

2.3.5 PCR analysis

Genomic DNA was isolated from T_0 plant (the regenerated plant) leaf samples of transformed and control plants of sorghum and tobacco using the CTAB method (Sambrook *et al.* 1989). PCR amplification of a 500 bp DNA fragment of the *GUS* gene was carried out using a pair of gene-specific primers; 412 5'-CCCTTACGCTGAAGAGATGC-3' and 413 5'-GGCACAGCACATCAAAGAGA-3'. The PCR reaction mixture contained 100 ng of genomic DNA in a final volume of 25 µl containing 1× PCR buffer, 10 m*M* dNTPs, 10 pmol of each primer, and *Taq* DNA Polymerase (2.5 U). Amplification was carried out by the program containing denaturation at 94 □ for 1 min, annealing at 58.5 □ for 1 min, and extension at 72 □ for 1.5 min for 40 cycles. A pre-denaturation step of 2 min and a final elongation and pre-denaturation step of 2 min, and a final elongation step of 5 min were included.

In the case of the gene construct AMP, a 0.3 kb fragment was amplified using the forward primer 6936 5'ATGGAAACCAAGCGCTTG-3' and reverse primer 6937 5'-TTAGCACTGGATGAAAAAAC-3'. Denaturation was carried out at 94 ℃ for 1 min, annealing at 50 ℃ for 1 min, and extension at 72 ℃ for 1.5 min.

In the case of the gene construct CT**,** a 0.5 kb and a 1 kb fragments were amplified using the forward primer RCH105 5'-AATCAGTCAATCTGTATAC-3' and the reverse primer RCH10MR 5'-TCTGCTGGCAGTAGTCC-3'.

In the case of the construct Co1**,** a 0.4 kb fragment was amplified using the forward primer 5'-CAGGATATCGGCAAATTGGT-3' and reverse primer 5'-TCTTTCGGTGCTTCTTGGAT-3'.

In the case of the gene construct SUC2, a 0.4 kb fragment was amplified using the forward primer 5'-CACGTGTCACGAAGATACCC-3' and reverse primer 5'-AGGGTTTTTGGTGGTTGTTG-3'. Denaturation was carried out at 94 ℃ for 1 min, annealing at 50 ℃ for 1 min, and extension at 72 ℃ for 1.5 min.

2.4 Results

2.4.1 Tobacco transformation

2.4.1.1 Plant transformation and regeneration of tobacco

The tobacco plants were transformed and regenerated on callus induction medium (CIM), shoot induction medium (SIM) and root induction medium (RIM) media (Fig. 2-1, and Fig. 2-2). Tobacco leaves were cut into small pieces and co-cultivated on the CIM medium with *Agrobacterium* for three to five days. Shoot buds developed from the leaf pieces following incubation on the SIM medium for one to two weeks (Fig. 2-1). When

the shoots were well-developed, the regenerated plants were then transferred onto RIM medium to induce roots (Fig. 2-2).

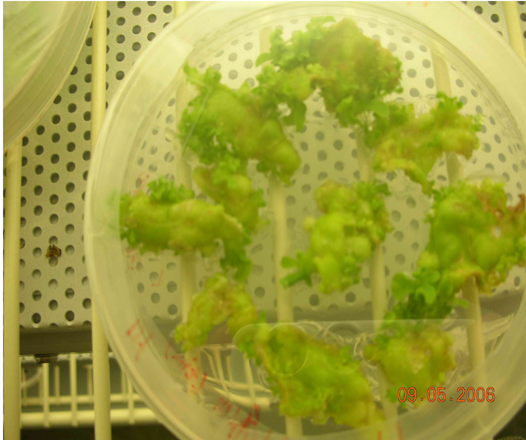


Fig. 2-1. Tobacco leaves on shoot-induction medium with shoot buds. Shoot buds from the explants came out after co-cultivated on the SIM medium for one to two weeks.

Fig. 2-2. Tobacco plants on root-induction medium with well developed shoots and roots.

2.4.1.2 GUS staining

The regenerated tobacco leaves were bleached and stained with GUS solution. The leaves turned blue after being stained with GUS overnight (Fig. 2-3 and Fig. 2-4), so these tobacco plants which regenerated on the kanamycin selection medium and stained

blue were transformed; from the antibiotic selection and GUS staining results, about 30% percent of the regenerated tobacco plants were transformed. In the case of AMP-transformed plants, the GUS staining provides the first evidence for the nature of genetic transformation in these plants.

Fig. 2-3. GUS staining of a leaf from the AMP-transgenic tobacco plant.

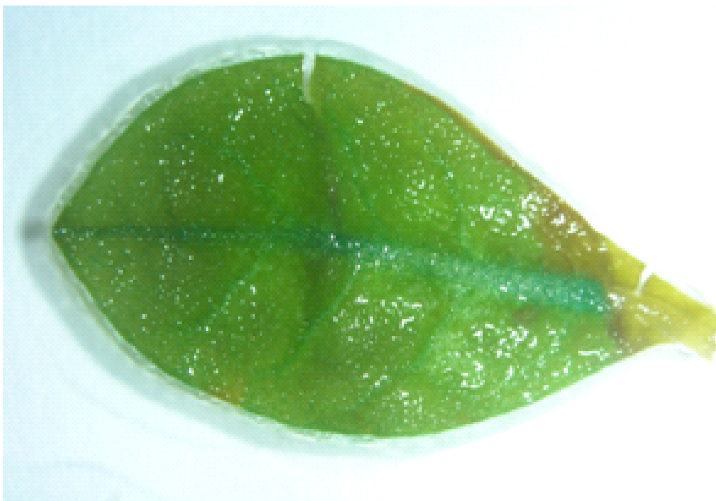


Fig. 2-4. GUS staining of a leaf from a plasmid-121 transgenic tobacco plants.

2.4.1.3 PCR for transgenic tobacco plants

PCR analyses were carried out to determine the transfer and the presence of the target gene in the GUS positive T_0 plants. The expected 500 bp GUS gene amplification band was detected (Fig. 2-5). From the PCR results, these regenerated plants proved to be transgenic tobacco plants carrying the GUS gene.

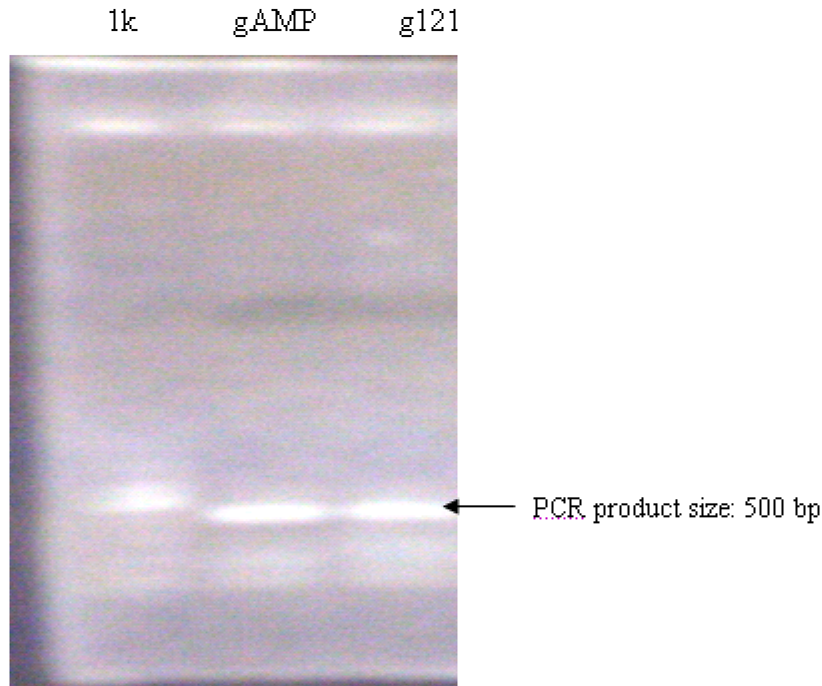


Fig. 2-5. PCR product for the GUS gene from genomic DNA of transgenic AMP and 121 tobacco plants. The product was close to the 500 bp line of 1 k marker. The gAMP was the genomic DNA from a transgenic AMP tobacco plant; while the g121 was the genomic DNA from a transgenic 121 tobacco plant. 1k was the PCR marker.

2.4.2 Sorghum transformation

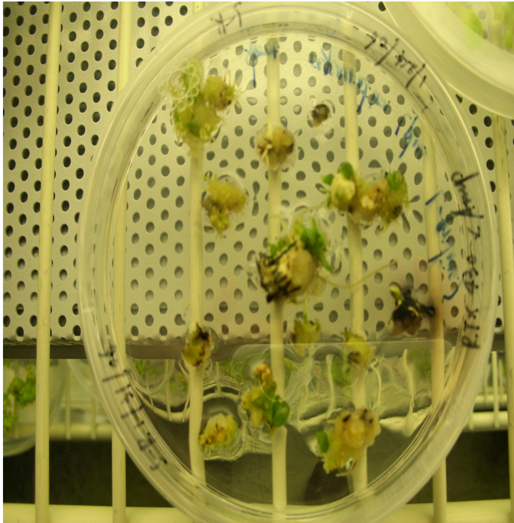
2.4.2.1 Method development

In this study, the sorghum regeneration system was considerably improved by using immature inflorescences of an elite sorghum line, Tx430. The microprojectile

bombardment transformation rate of the target gene was increased from the evidence of permanent expression of the GUS gene in the transformed tissues. The current studies suggest that transformation frequency can be increased further at optimized conditions.

2.4.2.2 Plant regeneration and transformation

Calli derived from the immature inflorescences of Tx430 were bombarded with DNA coated microprojectiles and grown on co-cultivation medium for one month (Fig. 2-6a), and then propagated on propagation medium for another month. The calli of Tx430 were then transferred to shoot regeneration medium and shoots were regenerated on shoot regeneration medium (with 0.7 g/L L-proline and 0.5 g/L MES) without selection pressure (Fig. 2-1b,Fig. 2-6c and Fig. 2-6d). Under the culture conditions, some unique calli (featured as white and compressed in structure) were developed (Fig. 2-6a), which had the ability to regenerate plants. The Fig. 2-6b to Fig. 2-6d shows morphogenetic development on the shoot regeneration medium at different development stages: shoot initiation, well-developed shoots, and plantlets with fully-developed shoots with roots, respectively. Sometimes multiple shoots developed from the base of compact callus clusters (Fig. 2-6c, Fig. 2-6d). After roots were induced, the fully-developed plantlets were separated and cultured *in vitro* for further growth. Finally the fully regenerated plants were transplanted in sterilized soil in plastic pots (Fig. 2-6d) and grown in the greenhouse at 25 □. For the AMP transgenic sorghum plants, five were regenerated from the same callus; the sixth one was regenerated from another callus. The putatively transformed T_0 sorghum plants produced seeds.



Compress parts of the calli

Shoot buds

Fig. 2-6a. Callus induced from Tx430 immature inflorescence in vitro and shoots buds developed at the base of the compact callus clusters on the shoot regeneration medium.

Multiple shoots were developed

Fig. 2-6b. Shoots development from callus (Tx430 immature inflorescence).

Fig. 2-6c. Regenerated transgenic sorghum plantlets with well-developed shoot and root systems in a magenta box on root the regeneration medium.

Fig. 2-6d. Transgenic *PtAMP* sorghum plants regenerated from immature inflorescences of Tx430. Each of the individual shoots developed into a mature sorghum plant.

2.4.2.3 The effects of plant genotype and explants type on transformation and plant regeneration

Protocols used in this study showed that the sorghum lineTx430 was amenable to in vitro propagation. The calli developed from Tx430 explants were able to grow bigger clusters and amplified much quickly than Tx2737 on propagation medium (picture not taken). These calli exhibited greater potential for transformation and regeneration under the regeneration conditions as shown in Table 2-1.

Two types of explants, immature embryos and immature inflorescences of Tx430, were compared for their capacity for regeneration. It was noted that under the defined cultivation conditions (given in Table 2-1), immature inflorescences were easier to propagate and to regenerate than immature embryos. Thus, immature inflorescences of Tx430 proved very useful for genetic transformation in sorghum.

Immature inflorescences pieces of Tx430 from the lower part of the panicle were more responsive to shoot induction under the tissue culture conditions and were able to develop into light-colored compact calli. Successful regeneration was limited to these light-colored parts of the calli (Fig. 2-6b).

2.4.2.4 The effects of different ingredients on regeneration and genetic transformation

In order to optimize the plant tissue culture and regeneration protocol for sorghum, several chemicals that are used as the supplements of tissue culture medium were tested for their effects on plant regeneration. From our results, it appeared that L-proline and MES can increase shoot and root regeneration of Tx430 immature inflorescences. Without L-proline and MES, only small “shoot buds” (picture not taken) developed after cultivation on shoot regeneration medium for 3 to 7 days; then the “shoot buds” became withered and no further regeneration occurred. These “shoot buds” were unlike the

regeneratable shoot buds shown in Fig. 2-6b; they seemed like a morphological alteration which was resulted from the expansion of immature inflorescences in vitro that did but not involve tissue redifferentiation.

Further, effects of media on shoot regeneration were observed when the calli of Tx430 immature inflorescences were incubated on different shoot regeneration media. Shoot regeneration was much better on media without L-proline (picture not taken); shoot regeneration appeared almost normal but morphogenesis was not normal (picture not taken). When incubated on medium with both L-proline and MES, normal shoots regenerated and some of the calli even developed into multiple shoots, and roots also developed (Fig. 2-6c).

2.4.2.5 The effects of antibiotics on cell division, morphogenesis, and plant regeneration

The calli of Tx430 immature inflorescences were cultured on propagation medium for one month before being exposed to any selection pressure. Under these conditions, the calli multiplied with normal appearance. Following this culture period, the calli were transferred onto shoot regeneration medium with kanamycin (50 mg/L) and hygromycin (1.5 mg/L) as well as without selection pressure to assess the effect of antibiotics on callus development and shoot bud differentiation. Interestingly, the cultures exposed to kanamycin (50 mg/L) grew even better than those without selection pressure (picture not taken), but the morphogenesis of the shoot buds appeared different in morphology; these shoot buds were longer and thicker than normal shoot buds and turned white after more than two weeks on the medium containing antibiotics, resulting in no regenerated shoots. Shoots exposed to hygromycin (0.5, 1.5 mg/L) grew almost the same as shoots without selection pressure.

Based on the trial, we developed a strategy for selection of transformed tissues with antibiotics. Calli were propagated on the propagation medium with kanamycin (50 mg/L) for two weeks (picture not taken); then kanamycin was reduced to 25 mg/L for another two to three weeks. Cultures that survived the selection appeared almost normal. The only difference from shoots without selection pressure was that those calli which grew with selection pressure did not have compressed parts which have the ability to regenerate. After 4 to 8 weeks on the propagation medium with selection pressure, those calli propagated with selection pressure were transferred onto shoot regeneration medium (with L-proline and MES) with the selection pressure of kanamycin (0 mg/L, 30 mg/L and 50 mg/L), but no shoot buds or shoots developed.

2.4.2.6 GUS staining

Freshly bombarded immature inflorescences, calli and leaves of regenerated sorghum plants were collected for GUS staining to evaluate the transfer and expression of the reporter gene (i.e. the GUS gene) in putatively transformed sorghum tissues. It is believed that some target samples are transformed based upon the expression of the reporter gene in plant cells as shown in Fig. 2-7a and Fig. 2-7b. The same experiments were done for all bombardment-treated calli with different gene constructs: Amp, CT, CO1, and SUC_2. The GUS staining results were permanent blue, which provided the evidence that these were indeed transgenic sorghum tissue (Fig 2-7b). In Fig. 2-7a, almost the entire callus was permanently stained blue, suggesting that the transformation rate was high.

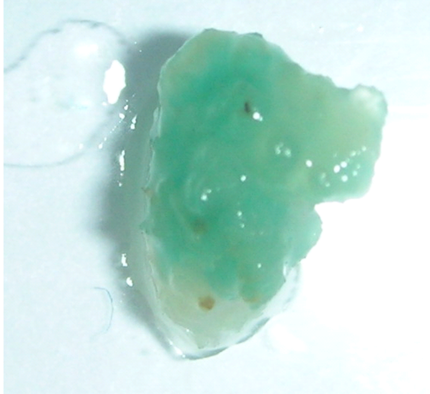


Fig. 2-7a. GUS staining of transformed callus. The photograph shows the calli of Tx430 immature inflorescences that were bombarded with the AMP construct, stained in GUS buffer overnight.

Fig. 2-7b. GUS staining of leaves from a regenerated transformed plant from Tx430 immature inflorescences that were bombarded with the microprojectile prepared with AMP DNA. A segment of leaf was stained in GUS buffer over night.

2.4.2.7 Confirmation of the GUS gene in plant cells by PCR

To confirm the GUS staining results, PCR was done before the regenerated T_0 plants were transferred to the greenhouse. Two grams of leaf tissues were collected from each regenerated plant. Genomic DNA was isolated from each sample, and PCR was performed using the specific primers for the GUS gene. There were 10 regenerated plants, including six PtAMP-transformed sorghum plants, three transformed with the Ct gene and one transformed with the CO1 construct. The results from the gene specific

PCR amplification showed that all of them contained the GUS gene. Fig. 2-8a shows the PCR results from four transgenic sorghum T_0 plants. As mentioned above, five individual PtAMP-transformed sorghum plants (Fig. 2-6d) were regenerated from the same callus cluster, and each of them were analyzed by PCR and all of them contained the GUS gene. The last regenerated PtAMP sorghum plant, which was regenerated from another calli, was also positive for the PCR amplification.

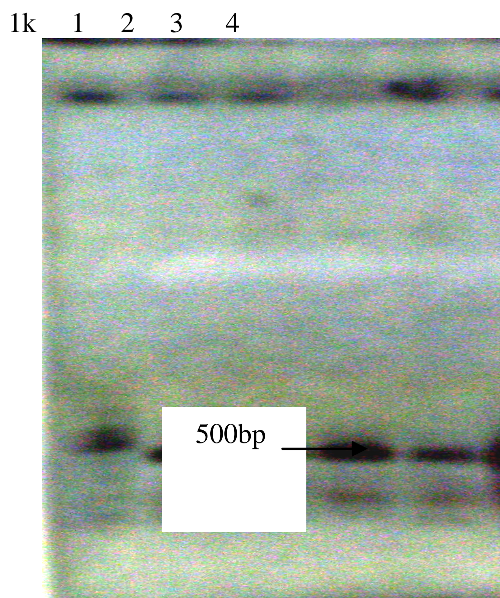


Fig. 2-8a. The gel results of PCR for the GUS gene. Lane 1k is 1 kb marker as a molecular standard, lanes 1, 2, 3, and 4 are the PCR products for the GUS gene from the DNA of four individual transgenic plants. The PCR product for the GUS gene is about 500bp in size.

2.4.2.8 Confirmation of other target genes in transformed sorghum plants

To further confirm the above PCR results, PCR amplification was done for the target genes using the genomic DNA of the four kinds of transformed plants (AMP, Ct, Co1, SUC2). As each of the gene constructs carry one target gene and the GUS reporter gene,

the two foreign genes are closely-linked, residing within the same construct. In general they can be co-transformed into the same target plant cell. Fig. 2-8b shows that the Ct gene has been introduced with the GUS gene into the same transformed sorghum plants. Similarly, the CO1 gene showed the positive in the GUS-transformed plants.

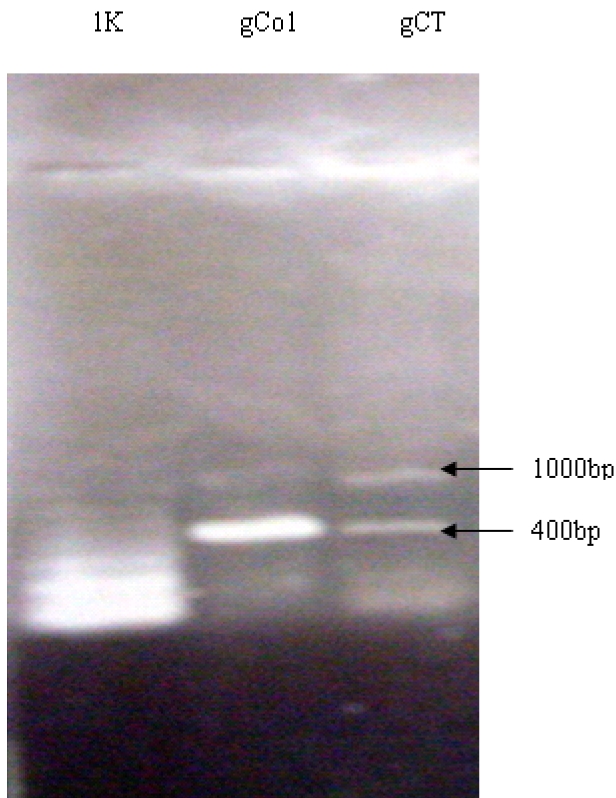


Fig. 2-8b. PCR results for the target genes CO1 and Ct-AMP in transformed regenerated plant genomic DNAs. Lane gCo1 is the PCR result for Co1-transformed sorghum plant using the primers designed from the CO1 sequence, the PCR product size is about 400 bp. Lane gCt are the PCR products for the CT-transformed plant using Ct-AMP gene specific primers, the PCR products sizes are about 400 bp and 1000 bp.

2.5 Discussion

2.5.1 Development of a method for sorghum transformation using immature inflorescences

In order to develop new transgenic sorghum cultivars, the first critical step was to develop and establish a highly efficient transformation system. During this study, an immature inflorescence-based transformation was successfully developed for sorghum using microprojectile bombardment. From the results of the GUS staining and PCR analysis, the transformation rate in immature inflorescence is more than 50%. If the transformation rate was from 25% to 75%, the antibiotic selection pressure and the antibiotic selectable marker, which might result in a negative impact on the environment, might not be needed in the future. Once the putatively transformed plants are produced, the GUS staining or PCR analysis for the regenerated plants could be used to determine whether the regenerated plants are transformed before planting them into the greenhouse.

The inflorescence-based method proved a useful tool for sorghum transformation. However, many factors may affect the frequency of transformation and regeneration which lead to the final production of transgenic plants. Those factors that need to be further investigated in the future in order to optimize the method are briefly discussed here.

2.5.1.1 Plant genotype and target tissue

The first step for a successful transformation in sorghum is to choose an ideal plant genotype and target tissue for gene delivery. The sorghum line Tx430 was chosen as one of the lines to test in this study because it showed amenability for transformation in an earlier report (Casas *et al*. 1993a). In this study, Tx430 was easier to regenerate than the cultivar Tx2737. Various types of tissues were tested for transformation in sorghum

during early efforts. The most widely used target tissue has been immature embryos. In cereal crops, like rice and wheat, cells seem to be competent at an early developmental stage. Immature tissues like immature inflorescences were proven to be the most suitable sources for morphogenesis (Maddock *et al.* 1983; Rout and Lucas 1996). Rasoco-Gaunt and Barcelo's study in 1999 also showed that immature inflorescences are advantageous over immature embryos (Rasco-Gaunt and Barcelo 1999). From the results of this study, immature inflorescences were easier in plant regeneration than immature embryos. When immature inflorescences were collected immediately before the flag leaves came out, transformation frequency was high.

2.5.1.2 Tissue culture conditions

The in vitro culture conditions affect frequency of regeneration and transformation. Phytohormones are the most critical regulators for plant tissue culture. Abscisic acid (ABA) was reported to improve the quality of calli in rice (Jiang *et al.* 2006), and promoted the calli to produce compressed parts. In this study with sorghum, ABA seemed to direct the development of callus type, producing calli with a compact structure. It was observed that only the light colored compact calli had the ability to regenerate a plant.

Mannitol and sorbitol, as osmotic regulators are often used in the pre-cultivation medium, as they were reported to increase GUS gene transit and stable expression (Girijashankar *et al.* 2005). Like mannitol and sorbitol, L-proline was supplemented in the co-cultivation and propagation medium in this study. Whether L-proline has the same function of mannitol and sorbitol still needs to be investigated. L-proline is reported to promote plant somatic embryogenesis (Armstrong and Green 1985). Rout *et al.*(1995)

also reported that the addition of L-proline to the culture medium promoted development of high-frequency somatic embryogenesis and secondary somatic embryogenesis in rice (Rout *et al.* 1995). The L-proline was also believed to promote adventitious shoot regeneration from immature embryo in sorghum (in the line Tx430) (Hagio 2002). Based on our experiment, an additional 0.7 g/L of L-proline resulted in a high frequency of shoot and root regeneration from immature inflorescences in sorghum cultivar Tx430 (Fig. 2-6c). It is believed that L-proline plays a role in the regulation the osmotic pressure of the medium as it absorbs free hormones such as IAA, IBA or NAA from the medium.

The pH of tissue culture medium affects plant growth and is also critical for plant regeneration. The pH of the medium may change under some situations, for example after autoclaving, while the explants grew on the medium, etc. MES (Morpholine ethane sulphonic acid) is often used as a buffering agent, and it can adjust the Pka value of 6.15 at 20 °C (Good *et al.* 1966). The initial pH value of the sorghum transformation and regeneration medium was 5.8. From this study, it was determined that an additional MES (0.5 g/L) benefited the shoot and root regeneration (Fig. 2-6c). MES and L-proline proved useful for shoot and root regeneration in sorghum.

2.5.1.3 Parameters of the microprojectile bombardment process

The parameters of the microprojectile bombardment determine a successful transformation system. Micoprojectile bombardment has been used for gene delivery in sorghum but the frequency of transformation varied (Casas *et al*. 1993a; Girijashankar *et al*. 2005). Many factors can affect the success of DNA delivery. The bombardment pressure, the distance from the rupture disk to the stopping plate, the metal particle, the purity and the amount of DNA, the vacuum, and the pellets of the DNA will all affect the

transformation rate (Able *et al.* 2001; Tadessem *et al.* 2003). In this study, we manipulated some parameters, leading to higher transformation rates in some groups; some parameters still need to be investigated further.

From this study, the amount of plasmid DNA might be a critical component for the success of gene transfer using bombardment. Varying amounts of plasmid DNA/golden particle per bombardment, ranging from 0.2 μg/400 μg to 2 μg/400 μg, were evaluated. Lowering the amount of plasmid DNA might increase the transformation rate. Decreasing the amount of plasmid might increase the stable delivery of the target gene into the sorghum genome. To investigate it in the future, different plasmid amounts could be used for bombardment and then place the treated explants on propagation medium for two to four weeks, and then assess the gene transfer by GUS staining method.

For bombardment, we adopted a chamber pressure of 900 psi to 1300 psi, which was commonly used in previous studies. But the distance from the rapture disc to the launch plate was modified to 10 cm to 13 cm, which was a greater distance than used in previous reports. This modification might be one of the factors that improved our transformation rate in sorghum.

2.5.1.4 The selection conditions

The selection media conditions also affect successful sorghum transformation. There are no successful reports of sorghum transformation when using antibiotic selection pressure. Kanamycin (15-50 mg/ml), when added to the shoot regeneration medium alone (i.e., without L-proline and MES), seemed to benefit plant transformation rates in this study (Fig. 2-6). In the future, further studies are needed to confirm the effect of kanamycin on shoot regeneration.

Hygromycin (0.5-1.5 mg/ml), which was added to the shoot regeneration medium (without L-proline and MES) at a low concentration, seemed to not affect the propagation of the calli. The reason might be the low concentration of hygromycin, but it did effect the regeneration of sorghum explants. The concentration could be increased to 15 mg/L to 25 mg/L and L-proline and MES could be added in the future.

In our selection experiments, Kanamycin (50 mg/L) was added to the propagation medium. At this level, the calli developed almost normally but lost regeneration ability completely. There may be several reasons for this loss: the selection pressure was too high, thus it might be decreased in the future; the materials were too old and did not have the potential to regenerate; the quality of the ABA was not good, which was reported to promote the calli to produce the compressed and light-colored parts of the calli; the antibiotic did affect the future regeneration ability of callus. To solve these problems, good quality immature inflorescences, which have the potential to regenerate should be used, and several selection pressures should be tried (for example 25, 50 mg/L for kanamycin and hygromycin), and a good quality ABA should be used.

2.5.2 Development of *Agrobacterium*-mediated sorghum transformation

Since the regeneration of sorghum Tx430 from immature inflorescences was well established, we could also use this regeneration system to develop the methods for *Agrobacterium*-mediated sorghum transformation.

Agrobacterium can cause the release of phenolics from explants into the medium which is harmful for the regeneration of sorghum from calli (Zhao *et al.* 2000). To solve the problem, researchers usually shorten sub-cultivation duration to 5~7 days, and add PVPP to co-cultivation and propagation media to prevent the production of phenolics

(Gao *et al.* 2005; Howe *et al.* 2006). According to Hagio's report (2002), 1000 mg/L PVPP, in combination with 1000 mg/L proline, increased adventitious shoot regeneration. Since 100 μM to 200 μM acetosyringone was reported as a critical factor to induce transfer of T-DNA into the plant genome (Amoah *et al.* 2001), we may add acetosyringone to the *Agrobacterium* medium when preparing *Agrobacterium* for co-cultivation with plant tissues. Carbencillin is widely used to kill *Agrobacterium* during the propagation of plant callus. It is usually imposed for less than four weeks and does not affect the growth of the calli, but as the concentration of carbencillin increases, it can decrease potential regeneration ability (Zhang 2004). So, the ideal concentration of carbencillin should be 100-150 mg/L.

Table 2-2. Media for *Agrobacterium*-mediated sorghum transformation

Medium	Auto-claved	Filter-sterilized	Time
Precultivation	Sucrose 20 g/L Glucose 10 g/L Ms salt 4.4 g/L Phytagel 2.2 g/L pH 5.8	Vitamin B5 1 ml Myo-insitol 0.1 g/L L-proline 0.7 g/L ABA 10 mg/L MES 0.5 g/L 2,4-D 2 mg/L	10 to 14 days Subculture every 5-7 days
Inoculation	Sucrose 68.5 g/L Glucose 36 g/L Ms salt 4.4 g/L pH 5.2	Vitamin B5 1 ml Myo-insitol 0.1 g/L L-proline 0.7 g/L; ABA 10 mg/L MES 0.5 g/L; 2,4-D 2 mg/L PVPP 1 % Acetosyringone 100-200 µM	10-20 minutes
Co-cultivation	Sucrose 20 g/L Glucose 10 g/L Ms salt 4.4 g/L Phytagel 2.2 g/L pH 5.8	Vitamin B5 1 ml Myo-insitol 0.1 g/L L-proline 0.7 g/L; ABA 10 mg/L MES 0.5 g/L; 2,4-D 2 mg/L PVPP 1 % Acetosyringone 100-200 µM	Rest for 2-3 days Then imposed carbencilline (100 mg/L)
Propagation	Sucrose 20 g/L Glucose 10 g/L Ms salt 4.4 g/L Phytagel 2.2 g/L pH 5.8	Vitamin B5 1 ml Myo-insitol 0.1 g/L L-proline 0.7 g/L; ABA 10 mg/L MES 0.5 g/L; 2,4-D 2 mg/L Kinetin 0.5 mg/L PVPP 1%	6-8 weeks Subculture every 7-10 days
Shoot regeneration	Sucrose 20 g/L Glucose 10 g/L Ms salt 4.4 g/L Phytagel 2.2 g/L pH 5.8	Vitamin B5 1 ml Myo-insitol 0.1 g/L L-proline 0.7 g/L; MES 0.5 g/L Kinetin 0.5 mg/L; IAA 1 mg/L PVPP 1%	4 to 8 weeks Subculture every 7-10 days
Root regeneration	Sucrose 20 g/L Glucose 10 g/L Ms salt 4.4 g/L Phytagel 2.2 g/L pH 5.8	Vitamin B5 1 ml Myo-insitol 0.1 g/L L-proline 0.7 g/L; MES 0.5 g/L NAA, IBA 1mg/L PVPP 1 %	2 to 4 weeks Subculture every 7-10 days

Agrobacterium strain 4404 is widely used and highly efficient in rice transformation (Ignacimuthu *et al.* 2000), and is also used with sorghum cultivars

(Gao *et al*. 2005; Zhao *et al*. 2000). A recent report showed a rapid and reproducible transformation method by using the *Agrobacterium* strain NTL_4 (Howe *et al.* 2006). Both 4404 and NTL_4 can be used in the future. Bombarding the calli with the microprojectile prior to *Agrobacterium*–plant co-cultivation may also increase the T-DNA delivery of the target gene into plant genomes (Bidney *et al.* 1992).

Based on a previous report and this study, the suggested protocol of *Agrobacterium*-mediated cultivation is set up in Table 2-2. But, we still need to investigate the best selection conditions using antibiotics. Also, during autoclaving, sucrose can hydrolyze into glucose and fructose and fructose can further hydrolyze and produce harmful products in the medium (Hsiao *et al.* 1991). In order to optimize the regeneration and selection conditions, it may be better to filter-sterilized the sucrose in the future.

2.5.3 Development of antimicrobial transgenic sorghum cultivars

Plant antimicrobial genes were used to develop transgenic species which have enhanced pathogen resistance (Shah 1997). The novel antimicrobial protein isolated from loblolly pine has the ability to inhibit the growth of pathogen microorganisms in *vivo* (Huang *et al.* 2003). As an ongoing study of the PtAMP peptide, transgenic tobacco plants containing the *PtAMP* gene were developed in this study (Fig. 2-1 to Fig. 2-5). Also, putatively transgenic AMP sorghum plants were developed using immature

inflorescences (Fig. 2-6 to Fig. 2-8), although characterization of the transgenic plants needs to be performed.

The tobacco transformation work was designed to develop transgenic tobacco in order to check the in *vivo* function of the PtAMP protein. The tobacco plants were regenerated (Fig. 2-1 to Fig. 2-2) and were either AMP-transformed tobacco or 121-transformed tobacco plants (Fig. 2-3 to Fig. 2-5). To further confirm the nature of integrated transformation, Southern blot analysis and functional analysis of the PtAMP protein *in vivo* and *in vitro* are needed.

Developing transgenic antimicrobial sorghum was one of the research aims of this project. The transformed sorghum plants have been regenerated (Fig. 2-6). The results from PCR analysis indicate these plants are genetically transformed (Fig. 2-7 to Fig. 2-8). Testing the gene function of the AMP peptide first in tobacco will help us developed transgenic sorghum. If the PtAMP peptide has antimicrobial ability in tobacco, it might be also used as a source for sorghum disease development. The target bacterial pathogens would be *Pseudomonas syringe* and *Pseudomonas andropogoni,* which can cause important sorghum diseases (Frederiksen and Odvody 2000).

2.5.4 Development of other transgenic sorghum cultivars

Based on the developed biolistic transformation method, we can develop transgenic sorghum cultivars.

Plant antimicrobial peptides are powerful tools to enhance plant pathogen disease resistance by genetic transformation. One focus of the current project is the AMP construct that contains an antimicrobial gene (*PtAMP* gene) which has been isolated from loblolly pine. In addition, the rice disease resistance chitinase gene (Zhu and Lamb 1991)

is being used in sorghum transformation research. It is anticipated that these two genes are promising for developing disease resistant sorghum plants.

It is important to direct expression of the defense genes to the correct destination. For example, greenbugs attack host plants by feeding on phloem tissues of a plant. Thus for the best result, aphid resistance genes should be directed by a phloem-specific promoter. In this study we were testing two phloem-specific promoters, the promoter of the *A. thaliana* $AtSUC_2$ gene (Zhao *et al.* 2004) and the Commelina yellow mottle virus, CoYMV promoter (Medberry *et al.* 1992). The Galanthus Nivalis Agglutinin (GNA) gene has been widely used in crop development for insect-resistance. The GNA gene may be toxic to aphid greenbugs but not harmful to animals and humans (McCaffertya *et al*. 2008) . It is worthwhile to develop gene constructs, having the GNA directed by the phloem-specific promoter, to achieve development of greenbug resistance sorghum cultivars.

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Chapter 3

Differential expression of thaumatin-like protein in sorghum infested with greenbug

3.1 Abstract

This study was designed to quantatively analyze the expression of thaumatin-like protein (TLP) at the transcriptional levels in different sorghum lines when they were infested with greenbug. Three sorghum lines, Tx7000, PI550607 and PI550610, were used. RNAs from the different sorghum lines which were infested with greenbugs at different infestation times were isolated; RNA was reverse transcribed into cDNA and the RT-PCR products were separated by agrose gel. Then, real-time PCR data were analyzed by using the $2^{-\Delta\Delta Ct}$ method, which relies on comparison of the TLP gene expression to the β-actin reference gene and the expression of TLP gene in target samples against reference samples. The results show that the transcriptional levels of the TLP were increased and the increased levels were time-dependent. For the susceptible line, the threshold cycle changes ($2^{-\Delta\Delta Ctn}$) of the TLP's transcripts increased several thousand fold at 120 hpi（hours post infestation）, while for the two resistant sorghum lines, the $2^{-\Delta\Delta Ctn}$ value increased but less than one hundred fold.

3.2 Introduction

Plants have their inherent mechanisms for resistance to pathogens and many factors are involved in those mechanisms: some are preformed and some are inducible (Hammerschmidt 1999). Pathogen-related proteins (PRs) like chitinase, osmotins, and *β*-1,3-glucanase are defined as proteins that are encoded by the plant genome and induced specifically in response to infections by pathogens such as fungi, bacteria, or viruses, or by adverse environmental factors (Breiteneder 2004). PR proteins are divided into several families. The PR-5 family has amino acid sequence similarities to thaumatin proteins, which are sweet and were first found as a mixture of proteins isolated from the katemefe fruits (Van der Wel and Loeve 1972). These thaumatin-like (TL) proteins belong to the PR-5 family and some are involved in plant resistant mechanisms. There are three classes of thaumatin-like proteins: proteins produced in response to pathogen infection, osmotic proteins and plant antifungal proteins (AFPs) which are constitutive in plants, especially seeds (Breiteneder 2004).

Thaumatin-like proteins, which are involved in pathogen resistance, can be induced by a large spectrum of pests (not only insects, but also virus, bacteria and fungi) and stimuli--like chemicals, wounding, cold stress, etc (Bol *et al.* 1990; Lotan and Fluhr 1990; Trudel *et al.* 1998). For example, *Rhizoctonia solani* , the sheath blight fungal, caused the induction of TLPs in rice, based on molecular analysis, and two different TLPs involved in this mechanism were revealed by western bloting (Velazhahan *et al.* 1998). A recent

study showed that, salicylic acid (SA) and jasmonic acid (JA) could induce TLPs and *β*-1,3-glucanases in wheat plants, and cause system acquired resistance (SAR) leading to the enhanced resistance to bacterial diseases. The induction level was time-dependent (Jayaraj *et al.* 2004).

An antifungal thaumatin-like protein was isolated from sorghum leaves in 2002 (Velazhahan *et al.* 2002). The aphid greenbug (*Schizaphis graminum*), one of the major pests of sorghum (*Sorghum bicolor* L. Moench), might be an inducer of this TLP in sorghum (Hammerschmidt 1999; Porter *et al.* 1997). Although many studies are about TLP's production when plants encounter outside stimulus, no study has quantatively compared the time-dependent expression of TLPs in different sorghum cultivars, especially between resistant cultivars and susceptible ones to greenbugs.

A recent report showed that pathogen related proteins were differentially expressed among different cultivars when barley plants interacted with their bacteria pathogen (Geddes *et al.* 2008). It is reasonable to expect similar differential expression of TLP in sorghum at the cultivar level. Real-time PCR is a quantative and time-dependent technique that is widely used in the molecular world. It is a promising tool for the in-depth study of TLP-related pathogen response (Klein 2002). For this study, RT-PCR and relative real-time PCR technique were used to quantatively analyze the expression of TLP at the transcription level of greenbug in resistant lines PI550607 and PI550610, and one greenbug susceptible line Tx7000, when they were infested with greenbug biotypes I.

3.3 Materials and methods

3.3.1 Plant material and growth conditions

Three sorghum (*Sorghum bicolor* (L.) Moench) lines were used in this study: Tx7000, PI550607 and PI550610. Tx7000 is a greenbug-susceptible line, while PI550607 and PI550610 are greenbug-resistant lines. Each sorghum line was grown in five individual pots, which were designed for the time-dependent study. For each pot, fifteen to twenty seeds were added into the soil and covered with a thin layer of soil. Sorghum plants were grown in pots at 20-25 ℃ for a week in a greenhouse.

3.3.2 Greenbug infestation

Aphid greenbugs (*Schizaphis graminum)* (type I) were used in this study. Each pot of plants was infested with fifteen to twenty greenbugs at the same age and at the same time except the control plants. Each infested plant was covered with a plastic cage. Control plants which were not infested with greenbugs (0 hpi; hpi means hours post-infestation) were collected as soon as the other sorghum seedlings were infested with greenbug. The collected leaves were covered with foil paper and put into liquid nitrogen as soon as possible, then stored at -80 ℃. The infested plants were collected at different post-infestation times: 12, 24, 72, and 120 hpi (hours post-infestation). The greenbugs were brushed off the leaves before storage.

3.3.3 RT-PCR and Real-time RT-PCR

3.3.3.1 Extraction of mRNA

RNAs of the different sorghum lines from the different infestation durations were isolated. Tissue was homogenized with Trizol reagent (Invitrogen) and RNAs were separated by chloroform, precipitated, washed, and dissolved in DEPC water.

3.3.2.2 RT-PCR (reverse transcription-polymerase chain reaction)

Five micrograms of the total cellular RNA of each sample was heat-denatured at 65 °C for 5 min with 10x buffer (Invitrogen) and used as template for reverse transcription (RT). RT reactions were performed using 50 U Superscript II reverse transcriptase (Invitrogen) at 42 °C for 60 min in the presence of 5x first strand buffer (Invitrogen), 0.5 mM dNTP, 10 mM DTT, 40 U RNaseOut (Invitrogen) and 12.5 ng random primers (Invitrogen). A 1:5 dilution of the RT reaction product was used for quantitative RT-PCR (Q-RT-PCR) analysis.

The 1:5 diluted cDNA products were amplified by PCR. The PCR running mix was 18 µl dH_2O, 2.5 µl 10X PCR buffer, 1.5 µl 25 mM $MgCl_2$, 1.0 µl dNTP (2.5 mM), 0.5 µl forward primer, 0.5 µl reverse primer, 0.2 µl *Taq* enzyme, 1.0 µl cDNA. PCR was run with the following program: 95 °C, 3 min; 94 °C, 5 min; 58 °C, 0.5 min; 72 °C, 1 min; 72 °C, 5 min for 30 cycles. The PCR fragments were fractionated on a 0.3 % agarose gel.

3.3.2.3 Quantative real-time PCR and threshold cycle analysis

Quantative real-time PCR was carried out with the instruction of Takara Co. at a final volume of 15 μl. cDNA was diluted to the ratio of 1:10, TLPs and ß-actin forward and reverse primers were used to further quantatively analyze the transcriptional levels of the TLP gene. The reaction mix was as follows: 7.5 μl Takara mix, 3.0 μl cDNA（cDNA dilution 1:10）, 1.5 μl 25 μM forward primer, 1.5 μl 25 μM reverse primer. Annealing temperature was 58 □ for both the actin gene and the TLP gene. The running cycle was 40 times and set up as: 95 □ 10 sec, 55 □ or appropriate temperature, 58 □ 30 sec, 72□ 30 sec, repeat for 40 times, 95 □ 1 min, 55 □ 1min, 55 □ 10 sec, 4 □ hold. The threshold cycle data were collected and analyzed by using the $2^{-\Delta\Delta CTn}$ method (Livak and Schmittgen 2001).

The fold change in expression of the TLP gene was calculated using the ΔΔCt method with the levels of the β-actin gene RNA as an internal control. The parameter CT (threshold cycle) was defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Threshold cycle change: $\Delta Ct_n = \Delta Ct_{nTLP} - \Delta Ct_n actin$, (at the time point of 0hpi, the threshold cycle of the TLP gene is $T_{nTLP,}$ the threshold cycle of the actin gene is $T_n$actin, the threshold cycle difference of the TLP gene and the actin gene at the time point of n hpi is ΔCt_n). Threshold cycle changes compared to the control sample is -$\Delta\Delta Ct_{n-0} = \Delta Ct_n - \Delta Ct_0$ (at the time point n, the changes of the threshold cycle difference of TLP gene and actin gene at n time point compared to 0 time point). The threshold cycle fold change is $2^{-\Delta\Delta Ctn}$, which can describe the multiplication number of each sample compared to the control.

$Whereas\ \Delta Ct_{\mathrm{target}} = Ct_{control} - Ct_{treatment}$ and $\Delta Ct_{reference} = Ct_{control} - Ct_{treatment}$

$Ratio = 2^{-\Delta\Delta Ct}$

$Whereas\ \Delta\Delta Ct = \Delta Ct_{reference} - \Delta Ct_{\mathrm{target}}$

3.4 Results and Discussion

Three sorghum lines were infested with greenbugs and examined for the transcription level of TLP and ß-actin by using the RT-PCR techniques. DNA agrose gel analysis of the real-time products revealed that the transcripts of TLPs increased and ß-actin decreased for all three sorghum lines. The content TLP of the susceptible line Tx7000 elevated much more markedly than the resistant lines PI550607 and PI550610; while the substance of ß-actin of Tx7000 declined more when compared to the other two lines (Fig.3-1).

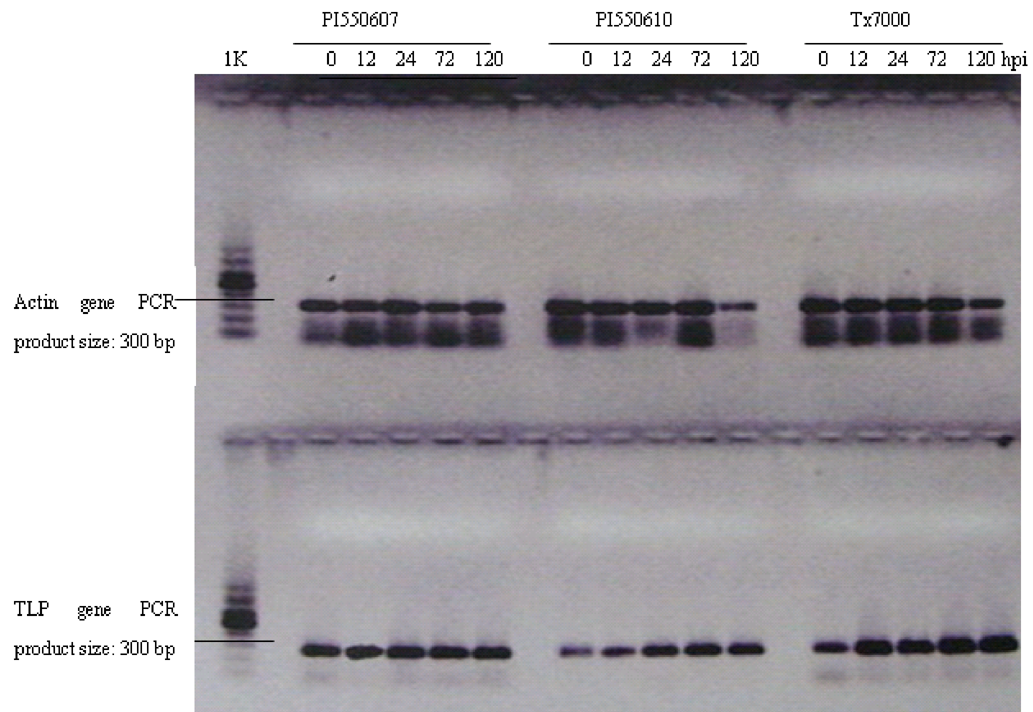

Fig. 3-1. RT-PCR DNA gel showing the transcriptional induction of thaumatin-like protein (TLP) of three sorghum lines when infested with greenbug at different treatment times. The infestation times were 0, 12, 24, 72, 120 hpi; hpi means hours post-infestation. Tx7000 was a greenbug- susceptible line; PI550607 and PI550610 were greenbug resistant lines.

Table.3-1. Real-time PCR threshold cycle analysis of three different sorghum lines when infested with greenbugs. The times of collection since infestation are indicated on the top; Tx7000 is a greenbug-susceptible line, while PI550607 and PI550610 are greenbug-resistant. Ct is the threshold of the fractional cycle number at which the fluorescence passes the fixed threshold. Thaumatin-like proteins and ß-actin gene primers were used in this study. Ctn is the threshold cycle value of each sample. ΔCtn is the threshold cycle difference of the TLPs gene and the ß-actin gene of each sample. $-\Delta\Delta Ct_{n-0}=\Delta Ct_n-\Delta Ct_0$ means the decreased amount of threshold cycle of each sample compared to the control plant (0 hpi).

		0 hpi	12 hpi	24 hpi	72 hpi	120 hpi
Tx7000	Ct_{nTLP}	28.26	20.85	21.25	19.27	18.94
	Ct_nactin	23.98	24.51	25.70	26.94	27.12
	$\Delta Ct_n=Ct_{nTLP}-Ct_n actin$	4.28	-3.66	-4.45	-7.67	-8.18
	$-\Delta\Delta Ct_{n-0}=\Delta Ct_n-\Delta Ct_0$	0	7.94	8.73	11.95	12.46
	$2^{-\Delta\Delta Ctn}$	1	245.51	424.61	3956.48	5634.22
PI550607	Ct_{nTLP}	25.07	24.48	22.26	23.65	20.88
	Ct_nactin	22.72	25.14	21.79	27.60	25.18
	$\Delta Ct_n=Ct_{nTLP}-Ct_n actin$	2.35	-0.66	0.47	-3.95	-4.30
	$-\Delta\Delta Ct_{n-0}=\Delta Ct_n-\Delta Ct_0$	0	3.01	1.88	6.30	6.65
	$2^{-\Delta\Delta Ctn}$	1	8.055	3.68	78.79	100.43
PI550610	Ct_{nTLP}	26.96	23.19	23.87	22.98	24.69
	Ct_nactin	23.32	22.55	22.64	24.23	27.26
	$\Delta Ct_n=Ct_{nTLP}-Ct_n actin$	3.64	0.64	1.23	-1.25	-2.57
	$-\Delta\Delta Ct_{n-0}=\Delta Ct_n-\Delta Ct_0$	0	3.00	2.41	4.89	6.21
	$2^{-\Delta\Delta Ctn}$	1	8	4.73	29.65	74.03

The threshold cycle analysis using real-time PCR further confirmed the PCR gel results. Upon analysis of the real-time PCR threshold cycle (CT) data, the induction of the TLP's transcriptional levels of three sorghum lines increased, which gave further confirmation of the relative real-time PCR gel results. The induction of TLP for the three sorghum lines was time-dependent; as the infestation time increased, the amount of TLP increased. At the 120 hpi, the threshold cycle fold changes of Tx7000 was several thousand compared to the 0 hpi, while the threshold cycle fold change of PI550607 and PI550610 was around one hundred. The preformed amount (ΔCt_{nTLP}) of TLPs of Tx7000 was lower than PI550607 and PI550610, while the inducted amount was much higher than the other sorghum lines. From the $-\Delta\Delta Ct_{n-0}$ value, PI550607 was almost the same but slightly higher than PI550610. The induction level of PI550607 is a slightly more than PI550610. For the susceptible line, the threshold cycle fold change ($2^{-\Delta\Delta Ctn}$) of TLP's transcripts increased several thousand fold at 120 hpi, while for the two resistant sorghum lines, the $2^{-\Delta\Delta Ctn}$ value increased, but only around one hundred fold (Table3-1).

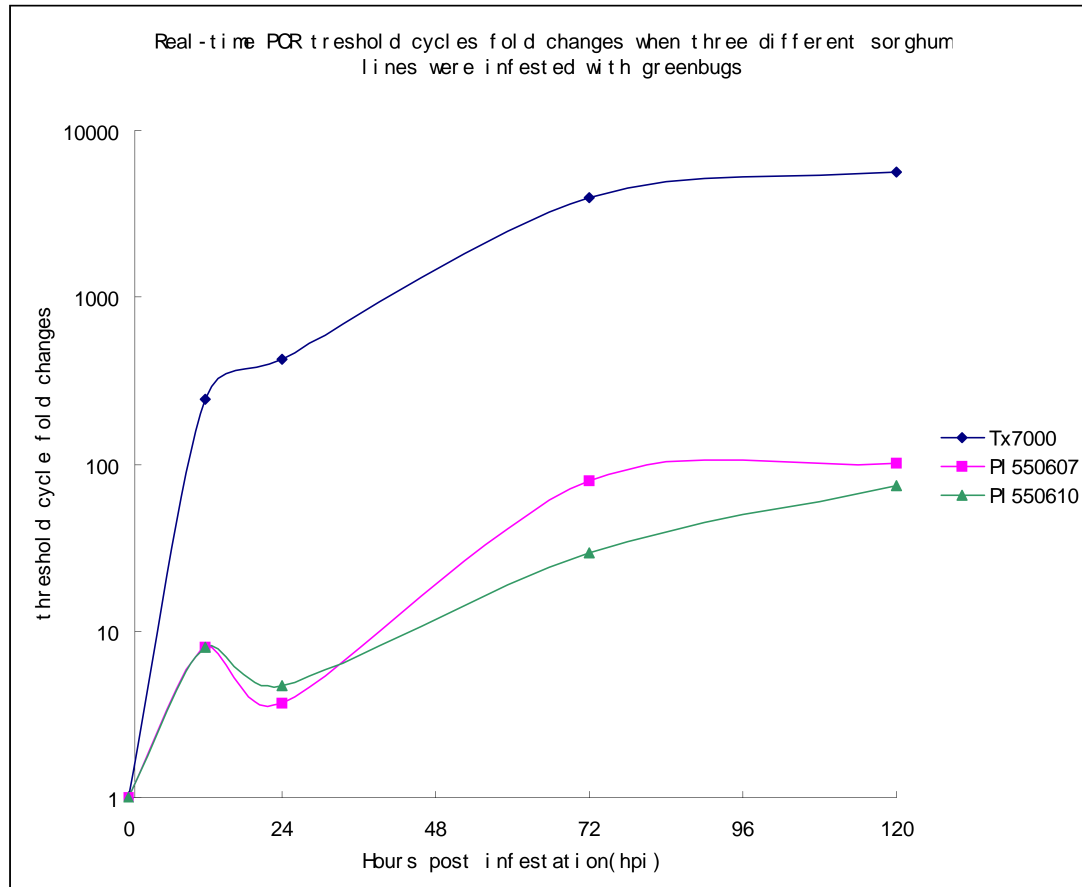

Fig. 3-2. Threshold cycle fold changes of thaumatin-like protein (TLP) at the transcriptional level in three sorghum lines when infested with greenbugs. Tx7000 is greenbug-susceptible, while PI550607 and PI550610 are greenbug-resistant lines. The Y-axis values are the calculation amounts of $2^{-\Delta\Delta Ctn}$ of each sample.

The threshold cycle fold changes were most in the sorghum line Tx7000 which was a greenbug-susceptible line. The curves of PI550610 and PI550607, which are greenbug-resistant lines, were more reduced than the Tx7000, and almost the same (Fig 3.2). Infestation of sorghum plants with greenbugs increased the transcriptional level of TLPs in three sorghum lines when ß-actin was used as the reference gene; as the infestation time increased, the induction level increased.

3.5 Discussion

Thaumatin-like proteins are reported to be associated with plant defense systems. When plants encounter chemical, wounding, pathogens, or other kinds of challenges, the production level of TLP will increase (Velazhahan *et al.* 1999). Our study shows that TLP could also be induced when sorghum plants are infested with greenbug; and as the infestation duration increased, more TLP transcripts were produced (Fig. 3-2).

From this study, the insects acted as an inducer of the resistant system and the induction levels varied based on cultivars. It is well known that insect viruses, bacteria and fungi are inducers of various resistant systems (Bol *et al.* 1990; Lotan and Fluhr 1990). The aphid greenbug (*Schizaphis graminum*), which is one of the major pests of sorghum (*Sorghum bicolor* L. Moench) since 1868 (Porter *et al.* 1997), is shown to also be an inducer of TLP in sorghum.

The induction levels of TLP have cultivar differences（Fig. 3-1, Table 3-1）. PI550607 possesses a high level of resistance to greenbug biotypes C and E (Katsa *et al.* 2002) While PI550610 has shown a little higher level of antibiosis, its TLP induction

level just a little different from that of PI550607 (Bowling and Wilde 1996). Our results indicated that with a more susceptible sorghum line, Tx7000, more TLP transcripts were produced to protect itself against greenbug damage compared to a more resistant line. There was a little difference between the resistant lines of PI550607 and PI550610. Even though there still was a slight difference between them; the induction level of PI550610 was higher than PI550607, which was consistent with the previous hypothesis that the more resistant a line was, the less TLP was induced.

TLPs are not only constitutive or preformed (seed permatins and fruit proteins), but also stress-induced (PR-5 proteins and osmotins) (Trudel *et al.* 1998). Our data indicated that the preformed amounts of thaumatin-like protein are different among three sorghum lines (Table. 3-1). The resistant sorghum line's ΔCt_0 values are lower than the susceptible line, which indicated that the preformed amount of thaumatin proteins of a resistant sorghum line was higher than a susceptible line. At the 12 hpi, the ΔCt_{12} value indicated that the TLP amount of Tx7000 surpassed PI550607 and PI550610. For the more susceptible sorghum line Tx7000, more TLP transcripts were induced to protect itself against greenbug damage compared to a more resistant line (Fig. 3-1, Table. 3-2).

Plants have many natural mechanisms to protect themselves from pathogens (Hammerschmidt 1999). Our results showed TLP apparently does play a role in attenuating the insect-pathogen response, but did not overcome the natural weak resistance and defense system of a susceptible sorghum line. There were more TLP

transcripts produced in the susceptible line and the TLP transcription level went higher as the infestation duration went longer.

When insects bite plant leaves, they cause wounding damage to the plants; metal files can mimic the insects' damage to the plants which can help us study the insect-induced pathogen system. Other pathogens or stimulus could be used in the future, like bacteria inoculation (*Pseudomonas andropogoni*), SA or JA stimulus, and wounding damage to study the effect of TLP levels.

Based on differences in the induction levels of different sorghum cultivars, we could investigate species difference in the future. For example, since the wheat plant has been reported to acquire system resistance (SAR) (Jayaraj *et al.* 2004), we could explore the differences of TLP's insect-induction between sorghum and wheat.

Real-time PCR is used in the comparison of different genes involved in the same or different mechanisms. Many factors are involved in the plant's natural resistant system (Hammerschmidt 1999); TLP is just one factor involved in the induced-resistant mechanism. Our results indicate that greenbugs induce TLP production and levels were negatively associated with the plant's natural resistant level. In the future, we could study other protein factors combined with TLP （like chitinase or glucanase） as well.

To summarize, from the one-time results, TLP was related to the sorghum-greenbug defense system, and the more susceptible a sorghum line was, the more TLP transcripts were produced. However TLP still may not overcome the weak resistance of the susceptible lines.

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VITA

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Pages in Study: 77 Candidate for the Degree of Master of Science

Major Field: Forest resources

Scope and Method of Study:

This study was attempted to develop transgenic sorghum plants and to analyze expression of thaumatin-like (TLP) gene in sorghum plants in response to greenbug feeding. Many molecular genetics techniques used in the study included extraction of plasmid DNA, PCR, RT-PCR, real-time PCR, southern blot, RNA isolation, reverse transcription and *Agrobacterium*-mediated transformation.

Findings and Conclusions:

Putatively transformed plants have been produced although they need to be confirmed. One of them was transformed with an antifungal gene, and the other was done with an antimicrobial gene. In addition, the other two lines were transformed with plasmids containing phloem-specific promoters. All of the putatively transformed plants should contain the GUS gene. Further experiments are needed to confirm that they are transgenic plants containing the genes of interest. In addition, the expression of the TLP gene in three different sorghum lines infested with greenbugs was analyzed using the real-time PCR method. The results indicated that the TLP expression was elevated in those plants in response to greenbug infestation, which may play a role in plant defense against the greenbug pest.

ADVISER'S APPROVAL: Yinghua Huang