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## Characterization of a Novel RING-type Ubiquitin E3 Ligase GhRING2 involved in Cotton Fiber Development

Shiva Theja Reddy Soma

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Characterization of a novel RING-type ubiquitin E3 ligase GhRING2 involved in cotton  
fiber development

By

Shiva Theja Reddy Soma

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Characterization of a novel RING-type ubiquitin E3 ligase GhRING2 involved in cotton  
fiber development

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Candidate for Degree of Master of Science

The ubiquitin-proteasome proteolysis pathway is responsible for the degradation of abnormal and short-lived proteins to regulate many important biochemical activities in eukaryotes. By employing affymetrix microarray analysis, we have identified a novel ubiquitin ligase E3 gene *GhRING2* that is expressed differentially between two *G. hirsutum* lines - Texas Marker-1 and Chromosome Substitution Line CS-B25. The complete *GhRING2* gene sequence was obtained by genomic and cDNA walking. The expression of *GhRING2* in cotton fiber is developmentally regulated, suggesting that the ubiquitin-proteasome pathway may regulate cotton fiber growth and development. Using a yeast two-hybrid assay GhRING2 was found to interact with a PROTODERMAL FACTOR1 (GhPDF1) protein. GhPDF1 is expressed preferentially in immature ovules and fiber initials and the gene has been suggested to play a role in cell fate determination and fiber development. Pull down and plasmid swap assays were employed to confirm this interaction.

## DEDICATION

I would like to dedicate this research to my parents, Mr. Narsi Reddy Soma and Ms. Vimala Thatikonda, and my brother Mr. Ravi Thej Reddy Soma.

## ACKNOWLEDGEMENTS

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## LIST OF ABBREVIATIONS

a.a	amino acid(s)
AMP	adenosine 5'-monophosphate
APS	ammonium persulfate
ARF	auxin response factor(s)
At	<i>Arabidopsis thaliana</i>
3-AT	3-amino-1, 2, 4-triazole
ATP	adenosine 5'-triphosphate
bp	base pair(s)
BSA	bovine serum albumin
C	cysteine
CaMV 35S	cauliflower mosaic virus promoter
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
Ct	threshold cycle
Cys	cysteine
2-D	two-dimensional
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ddH <sub>2</sub> O	double distilled water

DEPC	diethyl pyrocarbonate
dGTP	2'-deoxyguanosine 5'-triphosphate
DMSO	dimethyl sulfoxide
DNA	2'-deoxyribonucleic acid
dNTPs	2'-deoxynucleoside 5'-triphosphates
DPA	dayspost anthesis
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin ligase
EDTA	ethylenediaminetetraacetic acid
Ga	<i>Gossypium arboretum</i>
GA	gibberellic acid
Gh	<i>Gossypium hirsutum</i>
Gr	<i>Gossypium raimondii</i>
GST	glutathione S-transferase
GUS	beta-glucuronidase
H	histidine
HECT	homologous to E6-AP C-terminus
His	histidine
IAA	indole-3-acetic acid
IPTG	isopropyl-beta-D-thiogalactopyranoside

JA	Jasmonate
Kb	kilobase(s)
kDa	kilodalton
LB	Luria-Bertani
LTP	lipid transfer protein
M	molar concentration
mRNA	messenger RNA
Ni-NTA	nickel-nitrilotriacetic acid
NP-40	Nonidet P-40
OD	optical density
Os	<i>Oryza sativa</i>
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonyl fluoride
PVP	polyvinylpyrrolidone
<i>RING</i>	really interesting new gene
RING	really interesting new gene protein
RNA	ribonucleic acid
RT	reverse transcription
SCF	Skp-Cullin-F-box
SD media	synthetic dropout media
SDS	sodium dodecyl sulfate

TAE	Tris-acetate/EDTA
TEMED	N, N, N', N'-Tetramethylethylenediamine
Tris	Tris (hydroxymethyl)-aminomethane
UBC	ubiquitin conjugating enzyme
<i>UBC</i>	ubiquitin conjugating enzyme gene
UEV	ubiquitin-conjugating E2 enzyme variant
V	volt
X-gal	5-Bromo-4-chloro-3-indoxyl-beta-D-galactopyranoside
X-Gluc	5-Bromo-4-chloro-3-indoxyl-beta-D-glucuronide
YPDA	yeast peptone dextrose adenine

## CHAPTER I

### INTRODUCTION

Cotton is one of the most important economic crops for textile industries in the world and is the fourth leading crop in the US (Johnson et al. 2012). It has been estimated that cotton industry generates US \$15-20 billion in revenue worldwide (Chamandoosti 2013). Over 90% of the cotton produced in the US is from Upland cotton (*Gossypium hirsutum*) which accounts for most of the textile and other needs. Upland cotton has high percentage of lint fiber and high yield. Pima cotton (*Gossypium barbadense*) produces fiber with better qualities (longer and strong fibers) but has low yield when compared to Upland cotton. Cotton fiber development can be divided into four overlapping stages - intination, elongation (primary cell wall synthesis), secondary cell wall synthesis and maturation (Graves and Stewart 1988). Fiber initiation begins on 2 days before anthesis, and less than 30% of epidermal cells of seed coat differntaite into fiber initials (Basra and Malik 1984). Fiber initials, once formed, start to elongate and continue up to 15- 21 DPA (day postanthesis). Secondary cell wall synthesis takes place at 15 DPA and lasts till 25- 30 DPA. The deposition of cellulose reaches maxmium in the secondary cell wall of fiber cells around 24 DPA. Fiber maturation starts at about 40 DPA (Kim and Triplett 2001). The quality of the fiber depends on, most importantly, the second and the third stages of the development i.e. elongation and secondary cell wall synthesis. It was reported that during the onset of secondary cell wall cellulose deposition there was a steep decline in



protein contents which indicates the critical role of the proteasome proteolytic pathway (Haigler et al. 2009; Meinert and Delmer 1977).

Cloning fiber genes along with microarray expression analyses (Al-Ghazi et al. 2009; Hinchliffe et al. 2010; Hovav et al. 2008; Lee et al. 2007; Taliercio and Boykin 2007; Udall et al. 2006; Wu et al. 2006, 2007) have identified many genes that are expressed in initiation and elongation stages of fiber cell development. Our group has cloned and characterized many fiber genes encoding lipid transfer proteins (Hsu et al. 1999; Liu et al. 2000; Ma et al. 1997; Ma et al. 1995), MYB transcription factors (An et al. 2008; Hsu et al. 2005, 2008;), cell wall proline-rich proteins (Tan et al. 2001), cyclin dependent kinase A (CDKA) (Gao 2004; Gao et al. 2012), ubiquitin conjugating enzymes (E2s) (Zhang 2003), and several ubiquitin E3 ligases (Bandi 2011; Ho et al. 2010).

Ubiquitin-dependent proteolysis is a major pathway for the selective degradation of many abnormal and short-lived proteins in eukaryotic cells. The ubiquitin-proteasome pathway affects many processes in plant growth and development (Vierstra 2009), including hormone signaling (Liu and Stone 2011; Lyzenga et al. 2012; Santner and Estelle 2010; Stone and Callis 2007; Tan and Zheng 2009; Tang et al. 2011), light response, floral and seed development (Liu et al. 2008; Seo et al. 2013), self-incompatibility, disease resistance (Moon et al. 2004), stress tolerance (Bae et al. 2011; Cheng et al. 2012; Qin et al. 2007; Tang et al. 2012; Zhang et al. 2007) and cell cycle progression (Liu et al. 2008). The pathway is mediated through the covalent attachment of ubiquitin, a small protein containing 76 amino acids (a.a), to the targeted protein prior to the degradation by the 26S proteasome. Ubiquitin is a highly conserved protein and is expressed in a variety of eukaryotes. The ubiquitination of target proteins requires the

sequential activity of three enzymes: an ubiquitin-activating enzyme (E1), an ubiquitin-conjugating enzyme (E2), and an ubiquitin ligase (E3) (Pickart 2001). Ubiquitin is first activated by E1 in an ATP-dependent reaction to form ubiquitin adenylate, and the C-terminal Gly residue of ubiquitin is linked to the thiol group of a Cys residue of E1 (Zhang 2003). The activated ubiquitin is then transferred to an internal Cys within an E2 enzyme via a thiol-ester linkage. The E3 enzyme subsequently binds E2 and the target protein, and facilitates the transfer of ubiquitin to the target protein. A polyubiquitin chain is usually formed in target proteins for degradation in which the C-terminus of each ubiquitin unit is linked to Lys48 of the previous ubiquitin.

The regulation of cotton fiber growth and development via protein ubiquitination was first suggested by our research work (Zhang et al. 2003). Our lab had isolated and characterized two cotton E2 genes, *GhUBC1* and *GhUBC2*, which are homologs of yeast *ScUBC4/5*. Both *GhUBC1* and *GhUBC2* encode an E2 enzyme containing 148 aa. *GhUBC1* has 98% identity in aa sequence to *GhUBC2* with only three aa differences. These two E2 genes are expressed in fibers in a developmentally regulated manner. The *GhUBC1/2* transcript levels in fibers reach maxima at 10 DPA (days post anthesis). The cotton *GhUBC1* E2 was used as bait to screen a 10 DPA cotton fiber (*G. hirsutum*) cDNA library with a bacterial two-hybrid system (Dove 2003; Joung 2001). By screening the 10 DPA fiber cDNA library (cloned into pTRG) with pBT-*GhUBC1* as the bait, one fiber cDNA, named *GhRING1*, encoding a RING-type E3 ubiquitin ligase has been cloned (Ho et al. 2010). *GhRING1* has a C3H2C3 RING (RING-H2) domain at the C-terminus. Real-time RT-PCR analysis has revealed that *GhRING1* is differentially expressed in fiber cells; with the highest transcript level in the elongation stage at 15

DPA (Ho et al. 2010). The over-expressed GhRING1 was found to have the ubiquitin E3 ligase activity via an in vitro reconstitution assay (Ho et al. 2010). These observations suggest that protein ubiquitination plays a role in fiber differentiation and development. This suggestion is further supported by Hovav et al. (2008) who found that 600 genes encoding components of the ubiquitin/proteasome protein complex were up-regulated at the early fiber elongation step. Another global gene expression study (Al-Ghazi et al. 2009; Hovav et al. 2008) also showed that many genes involved in the ubiquitin-mediated protein degradation pathway, including the subunits of proteasome, E2, SCF F-box proteins, and RING-type ubiquitin E3 ligases, were highly up-regulated in fiber at the elongation and the secondary cell wall synthesis stages. Our lab has recently conducted microarray experiments to compare gene expression in 10-DPA fiber between two cotton lines CS-B25 and TM-1 (Texas Marker-1). CS-B25 is a chromosome substitution line, generated by replacing chromosome pairs 25 of *G. hirsutum* TM-1 with chromosome pairs 25 from *G. barbadense* double haploid 3-79 lines. CS-B25 has improved traits for fiber quality (increased fiber strength and reduced micronaire) relative to TM-1 (Jenkins et al. 2007; Saha et al. 2006). Via Affymetrix microarray analysis we have identified four RING-type ubiquitin ligases (GhRING2-5) that are up-regulated in 10-DPA CSB25 fiber. Our data along with others strongly suggest that the ubiquitin proteasome pathway regulates fiber growth and development. In this research the full length cDNA and genomic sequences of a RING-type ubiquitin E3 ligase gene *GhRING2* were cloned and characterized. The expression of *GhRING2* in fiber cells of TM-1 and CS-B25 at different developmental stages was determined. The ubiquitin E3 ligase activity of GhRING2 was also assayed and its target protein has been identified.

CHAPTER I  
**LITERATURE REVIEW**

**Cotton fiber Development**

Cotton fibers are seed trichomes and differentiated from epidermal cells of seed coat. Cotton fiber development can be characterized by four overlapping stages - initiation, elongation (primary cell wall synthesis), secondary cell wall synthesis, and maturation (Graves and Stewart 1988a, 1988b; Wilkins and Jernstedt 1999).

**Fiber Initiation**

Fiber development begins at -2 to 1 DPA and less than 30% of outer epidermal cells of cotton seed coat differentiate and protrude marginally above other epidermal cells. These cuboidal cells don't divide and are called fiber initials. Fiber initials can be either in the interphase or undergo cell division in vitro to form multicelled fibers (Hof and Saha 1998). Most of the fiber initiation starts before the pollination and fertilization process, suggesting that cotton fiber development begins at the same time with the expansion and elongation of the petals and style and anthers that occur as a part of anthesis mechanism (Lee et al. 2006). On the day of anthesis the majority of the fiber cells are initiated and these fiber initials continue to elongate at a higher pace (Kechagia 1977).

Fiber initials have numerous vacuoles with phenolic substances that influence the fiber cell physiology when released into the cytoplasm (De Langhe et al. 1978; Ramsey and Berlin 1976). Phenolic substances strongly interfere with indol-3-acetic acid (IAA) oxidase (Leopold and Kriedemann 1975) and inhibits its activity, resulting in the accumulation of IAA in the cytoplasm that subsequently stimulates fiber initiation.

To understand the molecular aspects of the fiber cell initiation various studies have been carried out. Comparative gene expression profiling to identify differentially expressed genes between wild type *G. hirsutum* and a naked seed mutant allowed the identification of activators of patterning genes responsible for cotton fiber development (Lee et al. 2006). One of the gene, *GhMYB2*, was found to be a critical patterning gene for normal trichome development (Lee et al. 2006). Deng et al. (2012) showed that a cotton fiber specific PROTODERMAL FACTOR1 gene (*GbPDF1*) is essential for fiber initiation and ethylene and pectin synthesis (Deng et al. 2012). Recently a fasciclin-like arabinogalactan protein gene (*GhFLAI*) was found to be involved in fiber cell initiation. The *GhFLAI* mutant had a decrease in lint fiber and the abundance of cell initiation specific transcripts (Huang et al. 2013).

Cotton ovule has three layers of tissues - outer integument, inner integument and nucleus where outer integument cells differentiate into fiber initials (Wu et al. 2006). A comparison between outer and inner integuments of cotton ovules revealed that 121 genes were differentially expressed. Among them 66 genes were up-regulated and 55 genes down-regulated (Wu et al. 2006). These expressed genes included patterning or regulatory genes encoding GhMyb25, GhMyb25-like, GhHD1 (Homeobox protein), GhLTP, GhRD22, and genes for GhAsp (L-Asparaginase), GhSus (sucrose synthase),

GhEX1 ( $\alpha$ -Expansin), GhFU1 (glycine-rich protein gene), GhFaEl (fatty acid elongase), GhTMTP (ATP-dependent transmembrane transporter), GhFaEl (Transferase) and GhCycD3 (Cyclin D3) (Ji et al. 2003; Wu et al. 2006; Yu et al. 2000) with structural and functional roles.

Cotton fiber formation in many aspects is analogous to the development of plant leaf trichomes. Many genes involved in fiber initiation have been identified by searching homologous genes involved in the trichome development (Humphries et al. 2005; Suo et al. 2003; Wang et al. 2004). A transcription profiling analysis of the development of Arabidopsis leaf trichomes revealed that three genes of GLABROUS1 (GL1), TRANSPARENTTESTA GLABRA1 (TTG1) and GLABRA were involved in trichome initiation (Schellmann et al. 2002). Suo et al. (2003) reported that several Arabidopsis MYB transcription factors such as AtMYBGL1 and AtWER regulate Arabidopsis trichome formation and they are homologs of cotton GhMYB109.

### **Fiber Elongation**

Fiber initials enter the elongation stage immediately after they are formed and continues to elongate till 25 DPA (Basra and Malik 1984; John and Crow 1992). The primary wall attains the thickness of half micron and fiber cells get constant deposits evenly of the entire primary wall during elongation (Mauney and James 1986). During elongation fiber cells expand rapidly by forming a large vacuole in the cytoplasm surrounding by quickly expanding thin primary cell wall (Graves and Stewart 1988). During this process there is a substantial metabolic activity that is evidenced by a rapid uptake of the substances (Mauney and James 1986). Phytohormones play a crucial role in elongation; gibberellic acid (GA) and auxin together are involved in constant cell

elongation and secondary cell wall synthesis, while abscisic acid (ABA) subverts the action by auxin and GA (Mauney and James 1986).

Microfibril is the basic unit of primary cell wall which lies across inside the cell and parallels outside the cell. Microfibril is 10-30 nm wide, 5-10 nm thick and several microns long (Mauney and James 1986). As elongation proceeds, the transversally adjusted microfibrils in the cell protrude and align parallelly outside of the primary wall, and new microfibrils are synthesized simultaneously at primary wall inside the cell (Albersheim et al. 1977; Roelofsen 1959). Microfibrils and related polysaccharides form a mesh like matrix and get incorporated into the elongating primary cell wall. The matrix substances such as cellulose and other polysaccharides are synthesized by some specific enzymes in the endoplasmic reticulum (Albersheim et al. 1977).

Many genes involved in cotton fiber elongation have been identified and characterized. Most of the genes were involved in osmosis regulation and cell wall loosening (Orford and Timmis 1998). Using differential screening methods our lab identified and characterized two lipid transfer protein genes, *GhLtp3* and *GhLtp6*, that are differentially expressed during cotton fiber elongation (Ma et al. 1995, 1997). Thoma et al. (1994) proposed the involvement of LTPs in cutin synthesis via transporting cutin monomers across the cell membrane to the cutin synthesis region (Ma et al. 1995; Thoma et al. 1994). Two cotton genes, *GhMYB7* and *GhMYB9*, encoding transcriptional factors showed higher levels of gene expression in flowers and elongated fibers and were involved in the regulation of the *GhLtp3* gene (An et al. 2008; Gonda 1998; Hsu et al. 2005). Ji et al. (2003) reported that a cotton gene *GhWBC1* encoding an ATP-binding cassette transporter of the WBC (white/brown complex) subfamily was preferentially

expressed during fiber elongation (Ji et al. 2003). Similarly the cotton genes encoding the MADS-box protein (*GhMADS11*), actin (*GhACT1*) and  $\alpha$ -tubulin (*GhTUA9*) were also differentially expressed during cotton fiber elongation (Li et al. 2005, 2007, 2011). Using the suppression subtractive hybridization (SSH) method, Feng et al. (2004) reported that the genes encoding cell wall structural proteins, PRP2 and PRP4 (proline-rich proteins) and AGP2 and AGP4 (arabinogalactan proteins) reached maximal expression at 10 DPA and played a vital role in restructuring the fiber cell wall (Feng et al. 2004). Wang et al. (2011) found that the transcript level of the gene *ACS2* encoding 1-aminocyclopropane-1-carboxylic acid synthase reached a peak in 10 DPA fiber. They found that *ACS2* was involved in ethylene biosynthesis and regulated by  $\text{Ca}^{2+}$  dependent protein kinase 1 (CPK1) (Wang et al. 2011).

### **Secondary Cell Wall Synthesis**

Secondary cell wall synthesis begins around 15-20 DPA which overlaps with cell elongation and continues till 40 DPA. The maximum cellulose deposition occurs at 25 DPA (Basra and Malik 1984; Meinert and Delmer 1977). The overlapping of elongation and secondary cell wall synthesis represents a smooth transition of corresponding metabolism and gradual increase in cellulose synthesis. During this transition there is a sharp rise in the activity of glucan synthetase which synthesizes beta-1-3-glucan, a possible intermediate for cellulose (Delmer et al. 1977). Since beta-1-3-glucans (such as callose) usually do not have structural role and it was proposed that beta-1-3-glucans are actively converted to cellulose during secondary cell wall synthesis (Delmer et al. 1977). Callose can also act as a lubricating agent when a stress situation is raised during the



overlapping stages of elongation and secondary cell wall synthesis (Mauney and James 1986).

Building of microfibril bundles has a very important role in the synthesis of secondary cell wall than primary cell wall (Willison and Brown 1978). During secondary cell wall synthesis microfibril bundles form transversally in the cell and then protrude out and align alternatively in right-hand and left-hand helices parallelly. Groups of microfibrils are synthesized close together by adding new glucose molecules to the cellulose chains concurrently by tip growth. At the end of microfibril bundles there is a granule attached to them that represents the enzyme complex involved in cellulose deposition (Willison and Brown 1978).

To understand the molecular aspects of the secondary cell wall synthesis various studies have been carried out. In an investigation via microarray analysis of 12,000 ESTs, it was revealed that 2,553 genes were down regulated and 81 genes up regulated during the secondary cell wall synthesis (Arpat et al. 2004). In another investigation the expression of more than 5,000 genes was studied by cDNA arrays and gas chromatography/mass spectrum (GC/MS) analysis (Gou et al. 2007). The expression analysis revealed that most of the genes highly expressed during the secondary cell wall synthesis are involved in signaling and metabolic pathways, which included genes encoding cellulose synthases, pectin esterases, beta-galactosidase, beta-arabinosidase, sucrose synthase, xyloglucan endotransglycosylase and beta-glucosidase (Al-Ghazi et al. 2009; Gou et al. 2007).

## **Maturation**

The last stage of cotton fiber development is dehydration and maturation which occurs at 40-50 DPA (Basra and Malik 1984). As maturation begins the fiber cells start losing the water and become dehydrated. The mature cotton fibers constitute pure cellulose that accounts for 96% in weight (Mauney and James 1986). Maturation also plays a critical role in determining the quality of the fiber. During dehydration microfibrils are bound together by irreversible hydrogen bonds, and improper dehydration can result in the loss of extensibility due to the loss of movement between the fibrils (Ingram et al. 1974).

## **Ubiquitin Proteasome Proteolytic Pathway**

The ubiquitin proteasome proteolytic pathway plays a critical role in the turnover of intracellular proteins, especially regulatory proteins, to indirectly regulate the cell physiology. Plants and animals use this system in all aspects of their growth and development. This pathway works in an ATP dependent manner and mainly constitutes three enzymes; ubiquitin activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin ligase E3 (Hershko and Ciechanover 1998).

The three enzymes E1, E2 and E3 work in a cascade to add a small protein of 76 amino acids called ubiquitin to a substrate. Ubiquitin protein is highly conserved among all eukaryotes. The cascading mechanism of this pathway is initiated through the activation of ubiquitin by E1 in an ATP-dependent manner. In the first step ubiquitin is added to a Cys residue of E1 via a high energy thioester bond to the Gly residue at the carboxyl terminus of ubiquitin. This ubiquitin moiety is then transferred to a Cys residue of ubiquitin conjugating enzyme E2 again through trans-esterification. The third enzyme

ubiquitin ligase determines the specificity by binding it to a particular substrate along with bound E2. The ubiquitin ligase mediates the transfer of ubiquitin from the conjugating enzyme to the substrate.

This pathway can modify proteins by covalent attachment of either one ubiquitin or chain of ubiquitins via monoubiquitination and polyubiquitination, respectively (Ikeda and Dikic 2008). Monoubiquitination often alters the function or the localization of the protein. In polyubiquitination, more ubiquitins are added via one of seven lysine residues to the C-terminal ubiquitin of monoubiquitinated protein. The fate of ubiquitinated proteins is determined by the position of ubiquitin chain linkage. For instance ubiquitin chain through Lysine-48 linkage marks the protein for proteolytic degradation by 26S proteasome while Lysine-63 chain linkage alters biological function of the protein (Hochstrasser 1996).

The E3 ligase acts as a platform to bring the E2 and substrate in close proximity that helps the transfer of activated ubiquitin from E2 to the substrate. In some cases, the activated ubiquitin is transferred from E2 to E3 which is then transferred to the specific substrate (Deshaies and Joazeiro 2009). The domain type of the ubiquitin ligase determines the mode of action by that ligase. Once the substrate is tagged with ubiquitin by the pathway, the fate of the substrate is dependent on the type of ubiquitination (mono- or poly-ubiquitination). Polyubiquitinated substrates are targeted by 26S proteasome and degraded to release the free ubiquitin which then enters the pathway again (Hershko and Ciechanover 1998).

Ubiquitin E3 ligase enzymes can be classified into three groups based on their action mechanism and subunit structure. In plants there are many ubiquitin ligases that

cause diversity of the UPP pathway. Arabidopsis genome has more than 1,300 genes that encode ubiquitin E3 ligases and their subunits (Glickman and Ciechanover 2002; Vierstra 2009). The three groups of ubiquitin E3 ligase are HECT (Homologous to the E6-AP Carboxyl Terminus), U-box, and RING (Really Interesting New Gene) ubiquitin ligases.

### **HECT Protein Family**

HECT (Homologous to the E6-AP Carboxyl Terminus) E3 ligases are usually large proteins having molecular mass more than 110 kDa. They have two units, a regulatory unit that helps in target recognition and a catalytic unit with the HECT domain, that has the ubiquitin ligase activity (Bates and Vierstra 1999; Ditzel et al. 2003). The HECT domain has a conserved C-terminus constituting 350 amino acids. The regulatory unit mediates substrate targeting through domains like C-type lectin-binding, UB-interacting motif, UB associated, and Ub-like protein domains (Hofmann and Falquet 2001). Although the numbers of HECT E3 ligases are low in plants, the knowledge about their function is also particularly limited when compared to animals. *Arabidopsis thaliana* has only seven HECT ubiquitin E3 ligase genes. In one of the studies, the Arabidopsis HECT UPL3 was reported to play a significant role in trichome development (Downes et al. 2003). Miao and Zentgraf (2010) reported that a HECT E3 ligase, UPL5, has a vital function in regulating leaf senescence and trichome development in Arabidopsis by targeting and down-regulating a transcription factor WRKY53.

### **U-box Protein Family**

The U-box protein family is considered as a novel variant of RING-finger domain proteins. They do not have zinc chelating sites but have a structure similar to the RING

domain. The proteins have ubiquitin ligase activity, independent of RING E3 ubiquitin ligase, to elongate ubiquitin chain in the presence of E1 and E2 (Aravind and Koonin 2000). These RING type proteins are named as E4 ubiquitin chain assembly factor as they cannot target a substrate directly but can add ubiquitin to an existing ubiquitinated substrate (Azevedo et al. 2001; Hatakeyama et al. 2001). About 37 genes encoding U box proteins have been identified in the Arabidopsis genome (Azevedo et al. 2001). Recently, one of the U-box ubiquitin ligases was reported to have the ability to directly target a ubiquitinated substrate.

### **RING Protein Family**

Ubiquitin RING (Really Interesting New Gene) E3 ligases containing a RING-finger domain with conserved cysteine-rich sequences were first reported by Freemont et al. (1991). In plants the RING E3 ligase families have numerous variants. Analyses of Arabidopsis and rice genomes revealed that they have 480 (Kosarev et al. 2002) and 425 (Lim et al. 2010) genes encoding the RING-finger domain proteins, respectively. RING-finger domains have a conserved octet with cysteine (Cys) and histidine (His) residues in either of two configurations  $C_3H_2C_3$  (also called RING-H2) or  $C_3H_1C_4$  (RING-HC). These conserved Cys and His residues are buried in the core of the RING-finger domain and they serve to maintain the structure by binding to two zinc atoms and set a globular platform for protein-protein interaction (Borden and Freemont 1996).

Various types of RING ubiquitin E3 ligase play a diverse role in plant physiology during the growth and development. RING family proteins can be classified into two types; single-subunit RING proteins and multi-subunit RING complexes based on their dwelling subunits (Moon et al. 2004).

### **Single-Subunit RING E3**

The RING motif of single subunit RING E3 proteins is directly involved in interaction with the E2 enzyme. The other parts of the protein are involved in binding to the target protein and ancillary factors and accomplish target ubiquitination. Single subunit RING E3 proteins including COP1 (Constitutive Photomorphogenic 1), SINAT5 (Seven in Arabidopsis thaliana 5) and ARC1 (Arm Repeat-Containing 1) have been well studied.

#### *Constitutive Photomorphogenic 1*

Torii et al. (1999) reported that the Arabidopsis *COP1* gene played a critical part in photomorphogenesis. The COP1 influences the photomorphogenic and seedling development by acting as a repressor to down regulate the activator of light response, a basic leucine zipper (bZIP) transcriptional factor HY5 (Torii et al. 1999). In a microarray analysis it was reported that COP1 in dark regulated more than 20 % of the Arabidopsis genome accounting for 28 pathways (Ma et al. 2002). As COP1 is the master regulator of multiple transcriptional factors, COP1 mutant show a pleiotropic phenotype. Even though COP1 is a single subunit RING protein, it however can form protein complexes to attain a broader range of specificity by interacting, via its coiled coil domain, with several other proteins such as RING-finger protein COP1- interacting protein 8 (CIP8), Phytochrome A (phyA) (Moon et al. 2004), LAF1 (Long After Far Red Light1), and SPA (Suppressor of phyA) (Seo et al. 2003).

### *Seven in Arabidopsis Thaliana 5*

SINAT5 is a single subunit RING finger protein that plays a critical role in regulating lateral root formation via down regulation of an auxin response transcription activator NAC1 (NAM/CUC-like protein1) (Xie et al. 2002). It was reported that the enhanced expression of SINAT5 reduced the lateral root formation whereas over expression of SINAT5 with mutations in the RING finger motif resulted in the development of more lateral roots. Moreover, the physiological data affirm protein-protein interaction between SINAT5 and NAC1 and in vitro ubiquitination of NAC1 by SINAT5 (Xie et al. 2002). Lately, Park et al. (2010) reported that SINAT5 was involved in controlling flowering time by ubiquitination of circadian oscillator late elongated hypocotyls (LHY) and the floral repressor FLC. A nuclear protein DET1 stabilizes the LHY protein by inhibiting LHY ubiquitination by SINAT5 (Park et al. 2010).

### *Arm Repeat-Containing 1 (ARC1)*

ARC1 is a U-box protein which has a similar structure as RING finger proteins; it however does not chelate zinc ions. Thirty-seven ARC proteins are encoded by the Arabidopsis genome, yet ARC1 is the only protein that is well characterized (Azevedo et al. 2001). ARC1 is involved in the regulation of a self incompatibility determinant S-receptor kinase (SRK) (Stone et al. 2003). Inhibition of ARC1 reduced self incompatibility, which means the pollination can take place with self pollens. It was reported that ARC1 interacted with phosphorylated SRK which subsequently phosphorylated ARC1 (Stone et al. 2003). Stone et al. (2003) also showed that the activated SRK helps the movement of ARC1 from the cytosol to nucleus, thereby co-localizing with the 26S proteasome subunits.

## **Multiple-Subunit RING E3 Complex**

The ubiquitin RING ligase can also present as a complex that contain more than one subunit. The various subunits of the complex have critical roles from substrate specificity to regulation of the ubiquitin E3 ligase activity. Anaphase Promoting Complex (APC) and Skp-Cullin-F-box (SCF) complex are well characterized multiple-subunit RING E3 complexes that have RING domain subunits.

### *Anaphase Promoting Complex*

Anaphase Promoting Complex is one of the large ubiquitin ligase E3 complex containing eleven highly conserved components. Two of the components, APC2 and APC11, with homology to SCF subunits contain cullin and RING domains, respectively (Tang et al. 2001). This complex is also called cyclosome and abbreviated as APC/C. It plays a crucial role in controlling cell cycle progression. Morgan et al. (1999) reported two WD40 repeat-containing proteins Cdc20/FZ and Cdh1/FZR as the essential factors for the active APC/C complex (Morgan 1999).

Substrates of APC/C have conserved motifs, such as Destruction and KEN boxes, which are recognized through Cdc20/FZ and Cdh1/FZR factors of the active APC/C complex (Capron et al. 2003). The most important function of APC/C is ushering a cell from metaphase to anaphase by targeting A and B-type cyclins and other anaphase inhibitors via ubiquitin-mediated proteolysis. Plant A-type cyclins have three groups A1, A2 and A3. It was showed that mutations in the subunits of APC/C cause plant abnormalities at different stages of development. Reduction of the levels of APC6 or APC10 resulted in defects in vascular development. Defects in embryogenesis were observed in the plant with mutations in the *APC4* subunit. *APC3* and *APC10* were proved



to have an essential role in maintaining cell division, and *APC10* over-expression resulted in increased leaf size due to accelerated degradation of B type cyclins that inhibit mitosis.

Eloya et al. (2012) showed a novel protein SAMBA as a regulator of APC/C that influenced plant early development. By employing a yeast two-hybrid assay and tandem affinity purification (TAP) it was demonstrated that SAMBA interacted directly with APC3b subunit and indirectly with all the subunits of APC/C except for a RING finger-containing APC11 (Eloy et al. 2012). Phenotypical analysis of SAMBA mutant plants revealed the functional significance of SAMBA in plant development. Mutations in the *SAMBA* gene showed the phenotypes with increased size of seeds, roots and leaves. It was also observed that *SAMBA* mutants had reduced fertility. The mutants also caused differential expression of cell cycle regulatory genes, and it was observed that the mutants significantly down regulated B type cyclins and cyclin-dependent kinases (CDKs) and up-regulated cell cycle inhibitors like SMR1, SMR2 and SMR3. With the help of the yeast two hybrid assays and a GUS-GFP reporter system, Eloy et al. (2012) showed that SAMBA interacted with *CYCA2* and *CYCA3* and stabilized the two proteins.

#### *Skp-Cullin-F-box (SCF) Complex*

SCF ubiquitin ligase complex in plants have been well studied. Unlike other eukaryotes, plants can form a large number of SCF complexes with different combination of subunits, thereby expanding their role in plant development. SCF complex has at least four subunits that include SKP1 (S-phase kinase-associated protein 1), Cullin 1, F-box protein, and RING-box 1 (RBX1). Both *Arabidopsis* SKP1, ASK, (a linker protein) and RBX1 (a RING finger protein) bind to the Cullin subunit which acts as a scaffold for complex formation (Zheng et al. 2002). *CUL1* and *CUL2* are two of five basic cullin

proteins found in Arabidopsis that are involved in the SCF complex formation (Cheng et al. 2004; Marrocco et al. 2003). In the Arabidopsis genome, there are more than 700 *F-box* genes (Andrade et al. 2001), two *RING-box* genes (Risseuw et al. 2003), and about 21 *ASK* genes (Liu et al. 2004). The high number of subunits helps in forming numerous SCF complexes that broadens the range of substrate targeting.

In Arabidopsis, only a very few of the 21 *ASK* genes have been characterized. The *ASK* proteins involved in SCF complex formation include *ASK 1, 2, 11* and *19* that interact with *F-box* proteins. From promoter analysis and transcription profiling, it was depicted that the *ASK1* gene is highly expressed among other *ASK* genes (Marrocco et al. 2003). *ASK1* has a crucial role in various biochemical activities during Arabidopsis development, which is consistent with the pleiotropic phenotype associated with the *ask1* mutant with defects in vegetative and floral development. Two SCF complexes, *SCF<sup>COL1</sup>* and *SCF<sup>TIR1</sup>*, were found to be involved in jasmonic acid and auxin responses, respectively, and both have *ASK1* as one of the core subunit and mutation in the *ASK1* gene caused the defects in JA and auxin responses (Gray et al. 1999; Xu et al. 2002).

More than 700 genes in Arabidopsis, accounting for 2.7 % of the genome (Gagne et al. 2002), encode *F-box* proteins that are involved in binding *SKP* subunits to form SCF complex. *F-box* proteins contain *F-box* motifs in the N-terminus of proteins and more than 13 motifs like Leu-rich repeat (LLRs) and WD40 in the C-terminus of the protein are involved in protein–protein interaction. Leu-rich repeats are the most often reported motifs in *F-box* proteins, constituting of about 30% of *F-box* genes in Arabidopsis (Gagne et al. 2002).

## **26S Proteasome**

The 26S Proteasome is a large multi-subunit complex with a size of 2-MDa, mainly containing a cylindrical 20S core protease and 19S regulatory subunits at both ends of the core like lid and cap. The 19S lid contains nine subunits, essentially involved in recognizing the ubiquitinated target and deubiquitinate the target before its complete degradation to recycle the ubiquitin. The 19S base part has several subunits that unfold the ubiquitinated protein before it enter into the 20S core protease (Groll and Huber 2003; Voges et al. 1999). The 20S protease consists of four stacked heptameric ring structures with two peripheral and two central rings. Peripheral rings contain seven alpha subunits and central rings have seven beta subunits, and together they form a cylindrical like structure (Hartmann-Petersen et al. 2003; Xie and Varshavsky 2000).

### **Role of Ubiquitin Proteasome Proteolytic Pathway (UPP) in Plants**

#### **UPP in Hormone Signaling**

In plants, the ubiquitin proteasome proteolytic pathway has a profound role in timely degradation of repressors or activators of transcriptional factors in response to a variety of hormones. Many plant hormones and their response pathways have been found to be associated with the ubiquitin proteasome system.

#### *Abscisic Acid*

Abscisic acid (ABA) is a plant hormone that participates in several processes during plant development. A variety of proteins like kinases, transcriptional factors and phosphatases are involved in sensing ABA signal for plant growth and development. A group of RING-type E3 ligases, ABI3-Interacting Protein (AIP2) and Keep on Going

(KEG), were found to be directly involved in ABA signaling (Santner and Estelle 2010; Tan and Zheng 2009). They regulate the abundance of transcriptional factors ABA-Insensitive3 (ABI3) and ABA-Insensitive5 (ABI5) involved in ABA response. RHA2a is another RING E3 ubiquitin ligase that responds to ABA signaling during seed maturation (Santner and Estelle 2010).

### *Auxins*

Auxins play a significant role in plant growth and development. In the auxin response pathway, the hormone signal is controlled by the short lived repressor proteins AUX/IAA (auxin/indole acetic acid). Most of the AUX/IAA repressor proteins are localized to nucleus and bind to the Auxin response transcriptional factors (ARFs). In the presence of auxin, SCF E3 complex targets AUX/IAA repressor proteins using the TIR1 F-box subunit and down regulates the AUX/IAA repressors to release free ARFs (Dharmasiri et al. 2005). AUX/IAA proteins have a proline-rich Domain II which is responsible for their recognition by LLRs of the SCF<sup>TIR1</sup> complex. In Arabidopsis, the auxin resistance gene 1 (*axr1*) mutant showed a varied auxin response, indicating its importance in auxin signaling. Further studies revealed that AXR1 is vital for auxin response via activation of an ubiquitin-related protein RUB1 (Del Pozo et al. 1998). A RING finger protein SINAT5 plays a crucial part in regulating lateral root formation in coordination with auxin concentration via down regulation of an auxin response transcription activator NAC1 (Xie et al. 2002).

### *Gibberellic Acid*

The gibberellic acid hormone signaling involves temporal degradation of gibberellic acid response (GAR) repressor proteins via the ubiquitin proteasome proteolytic pathway. The abundance of specific transcriptional factors was found to reduce with an increase in the concentration of gibberellic acid in the plants (Fu et al. 2002). These transcriptional factors act as repressor proteins and belong to DELLA protein family that include GA INSENSITIVE, SLR-1, REPRESSOR OF GA1-3 (RGA-3), RGA-LIKE1 (RGL1), RGL2, and RGL3 (Park et al. 2013; Sasaki et al. 2003). In Arabidopsis, gibberellic acid hormone signaling employs the SCF complex to down regulate a DELLA protein RGA-3 with the help of a F-box protein SLY-1 (Sasaki et al. 2003). Park et al. (2013) demonstrated that DELLA proteins interact with RING finger domain proteins Botrytis Susceptible1 Interactor (BOI), BOI-Related Gene 1 (BRG1), BRG2 and BRG3 to repress gibberellic acid response by binding to the promoter of the *GAR* genes (Park et al. 2013).

### *Ethylene*

Ethylene is a gaseous plant hormone and plays a vital role in plant development and stress responses (Johnson and Ecker 1998). It was proposed that ethylene synthesis is regulated by modulating the levels of the enzyme ACC synthase (1-aminocyclopropane-1-carboxylate synthase) during ethylene synthesis by the UPP pathway (Chae et al. 2003). In Arabidopsis, ethylene signaling is regulated by a transcriptional factor EIN3 (ethylene-insensitive3) and its UPP-based degradation is attenuated through ethylene and heightened by sucrose (Gagne et al. 2004). The LRR F-box proteins EBF1 (EIN3-BINDING F-BOX1) and EBF2 in the SCF complex interact with EIN3 and mediates the

ubiquitination (Gagne et al. 2004; Guo and Ecker 2003). EBF1 and EBF2 affect the stability of EIN3 by tightly regulating the ethylene concentration. In the presence of low ethylene concentration EBF1 enhances EIN3 degradation, and at a saturating ethylene concentration EBF2 reduces the stability of EIN3 by optimally maintaining the ethylene levels (Gagne et al. 2004).

### *Jasmonic Acid*

The Arabidopsis COI1 (Coronatine-Insensitive 1) protein containing an F-box motif was found to be required for the response to jasmonic acid (JA)-regulated plant development and defense processes. COI1 associates with Skp1-like proteins ASK1 (or ASK2), CUL1 and Rbx1 to form ubiquitin–ligase complexes SCF<sup>COI1</sup>. JA signaling involves temporal degradation of repressors of JA response elements by the SCF<sup>COI1</sup> complex (Xu et al. 2002). The COI1 mutant is insensitive to JA (Devoto et al. 2002). The target protein RPD3b for SCF<sup>COI1</sup> is a histone deacetylase which acts as repressors of JA response via interaction with LRR of COI1 (Feng et al. 2003). Two RING-type ubiquitin ligases, RGLG3 and RGLG4, have been recently identified to be essential for JA-mediated responses in Arabidopsis (Zhang et al. 2012).

### **Chromosome substitution line**

Cotton lines with high fiber quality and yield could be generated by increasing the frequency of beneficial alleles at several loci via breeding programs. Interspecific introgression to exploit the beneficial exotic traits by conventional breeding often resulted in abnormalities such as distorted segregation (Endrizzi et al. 1985), linkage drag (Brinkman and Frey 1977) and hybrid sterility, etc. To obviate these difficulties and fix

specific desirable traits during interspecific introgression, special breeding strategies are required.

The exotic chromosome substitution by backcross breeding approach (Stelly et al. 2005) is one of the alternative method. Stelly et al. (2005) released 17 Upland cotton germplasm lines disomic for different *G. barbadense* chromosome and chromosome segment substitutions (CS). Each chromosome substitution line of *G. hirsutum* was developed by hypoaneuploid-based backcross substitution of *G. barbadense* chromosome or chromosome segment into the *G. hirsutum* genetic background (Stelly et al. 2005). The high fiber quality and genetic uniformity of *G. barbadense* and readily available hypoaneuploid stocks of *G. hirsutum* TM-1 allowed them to be chosen as parent lines to prepare these chromosome substitution lines.

The chromosome substitution lines with uniform genetic background would provide an opportunity to study genetic effects of the genes present on a particular chromosome or chromosome arm that has been substituted in an uniform *G. hirsutum* background (Shah et al. 1999; Zhen 2001). These substitution lines were designated as CS-B (number) where 'B' and '(number)' refer to the origin of substituted chromosome of *G. barbadense* and its chromosome number, respectively. Saha et al. (2004) compared the agronomic and fiber properties of 13 chromosome substitution lines along with the parent lines TM-1 and Pima (3-79) and found that all the CS-B lines had intermediate boll size less than the two parent lines TM1 and 3-79 except for CS-B06 and CS- B12sh lines. In comparison to TM-1, the CS-B25 line, which had chromosome 25 from *G. barbadense* substituted into TM-1, exhibited improved fiber traits with higher strength, increased length, and lower micronaire compared to TM-1, the parental *G. hirsutum* line

(Saha et al. 2004). The chromosome 25 in CS-B25 has been identified to associate with fiber strength quantitative trait locus (QTL) (Kohel et al. 2001).



## CHAPTER II

### MATERIALS AND METHODS

#### Plant Materials

Two cotton (*Gossypium hirsutum*) lines, Texas Marker-1 (TM-1) and Chromosome Substitution Line CS-B25, were used in this research and planted in the field at Agricultural Research Service of USDA (United States Department of Agriculture) at North Farm of Mississippi State University. Cotton flowers were tagged one day before anthesis (-1 DPA) to avoid cross pollination. Cotton bolls were collected at different developmental stages (5, 10, 15 and 20 DPA), and leaf and flower were also collected.

#### Cloning and characterization of *GhRING2* gene

##### Cloning Full-length *GhRING2* Gene

A partial *GhRING2* cDNA sequence (360 bp) and the entire *GhRING2* gene along with its 5'- and 3'-flanking regions were previously isolated in our laboratory (Bandi 2011; Ma unpublished data). The *GhRING2* gene and its 5'- and 3'-flanking regions (3,040 bp) were cloned by using an inverse PCR genomic walking method (Triglia et al. 1988). The gene was re-amplified by PCR using high fidelity Pusion DNA polymerase, cloned into pGEM-T Easy vector and sequenced using a fluorescent dye-based cycle sequencing method.

### *Cloning GhRING2 into pGEM-T Easy Vector*

PCR amplification of the 3,040 bp *GhRING2* fragment using Phusion DNA polymerase produced a blunt ended PCR product. The PCR product was first purified with a QIAquick PCR purification kit and Taq DNA polymerase containing terminal deoxynucleotidyl transferase (TdT) activity was used to add 3' A overhangs to the PCR product by mixing 44 µl of *GhRING2* fragment with 5 µl of 10x Taq PCR buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM MgCl<sub>2</sub>, pH 8.3) and 1 µl of 10 mM dATP and then incubating at 72°C for 20 min. The *GhRING2* amplicon with 3' A overhangs was then ligated with pGEM-T Easy vector by incubating a ligation mixture containing 1 µl (5 units) of T4 DNA ligase, 5 µl of 2X Rapid ligation buffer, 3.5 µl (1µg) of *GhRING2* fragment with 3' A overhangs and 0.5 µl (400 ng) of pGEM-T Easy vector at 4°C overnight. *E. coli* XL-1 blue cells were transformed with the ligated *GhRING2*/pGEM-T sample and recombinant pGEM-T Easy/*GhRING2* plasmid was isolated from *E. coli* colonies grown on LB/ampicillin plates using a Quick mini preparation kit (Qiagen).

### *Preparation of E. coli XL1-Blue Competent Cells*

An *E. coli* XL-1 Blue cell stock from Stratagene was streaked on a fresh LB agar plate and incubated overnight (around 16 hr) at 37°C. A single colony was picked and inoculated into 2 ml of LB media and incubated overnight at 37°C in a shaking incubator (225 rpm). About 200 µl of overnight culture were inoculated into 20 ml of fresh LB media and incubated at 37°C in a shaking incubator until optical density at a wavelength of 600 nm (OD<sub>600</sub>) reached 0.4- 0.6 (about 3 hr to enter log phase). Cells in the log phase were centrifuged at 8,000x g for 10 min with a Sorvall RC-5B Refrigerated Superspeed Centrifuge. The supernatant was removed, and the pellet was re-suspended in

10 ml of ice cold 0.1 M CaCl<sub>2</sub> and incubated on ice for 15 min. Cells were centrifuged at 8,000x g for 10 min, and the pellet was re-suspended in 2 ml of ice cold 0.1 M CaCl<sub>2</sub> and used as competent cells for transformation.

#### *Transformation of E. coli*

Transformation of competent *E. coli* XL1-blue cells with recombinant plasmid was performed using the CaCl<sub>2</sub> method. The plasmid DNA was added to 100 µl of CaCl<sub>2</sub> treated competent cells in a micro-centrifuge tube, gently mixed, and incubated in the ice for 30 min. The cells were then subjected to heat shock at 42°C for 2 min and immediately chilled on ice for 2 min. To the mixture, 500 µl of fresh LB media were added and cells were then incubated at 37°C in a shaking incubator (225 rpm) for 1hr. Cells in 25, 50, and 100 µl aliquots were spread on LB/amp plates and incubated overnight at 37°C. Transformed cells formed colonies on the selective LB agar plates with corresponding antibiotic.

#### *DNA Sequencing*

The recombinant pGEM-T Easy/*GhRING2* plasmids were sequenced by the dideoxynucleotide chain termination method using a Big Dye terminator V1.1 cycle sequencing kit (Applied Biosystems). The reaction mixture for Cycle DNA sequencing was prepared by mixing 4 µl of TRR (Terminator Ready Reaction) mix with 2 µl of 5X BigDye Sequencing Buffer, 1 µl of 10 µM *GhRING2* specific primer, 2 µl (800 ng) of recombinant plasmid, and 11 µl of double distilled water in a 0.2 ml thin wall tube. The mixture was subjected to PCR amplification by using the following conditions: pre-denaturation at 96°C for 2 min and following by 25 cycles at 96°C for 10 sec, 50°C for 5

sec and 60°C for 4 min. The PCR product (total volume 20 µl) was precipitated by adding 50 µl of 96% ethanol and 2 µl of 4 M sodium acetate and incubating at room temperature for 15 min. The sample was centrifuged at 13,000 rpm using Eppendorf Centrifuge 5415 D for 10 min and the supernatant was carefully removed by pipetting. The DNA pellet was washed with 70 % ethanol, centrifuged at 13,000 rpm for 5 min, and vacuum freeze dried for 15 min by using a Savant Speed Vac Concentrator. The pellet was re-suspended in 20 µl of Hi-Di Formamide, subjected to heat denaturation at 98°C for 2 min, and chilled on ice. The sample was then loaded onto an ABI Prism 310 Genetic Analyzer for sequence determination.

### **Homology Modeling of GhRING2 protein**

The structure of the GhRING2 protein was modeled based on homology modeling using Modeller 9.12 (Eswar et al. 2006). The Basic Modeling protocol was used to create four python scripts with Python programming language. All the scripts were executed using the Python GUI program (Sanner 1999). Each script has its role in building GhRING2 protein structure. The first script was used to search for the structures related to GhRING2 and the second script selected the best template for GhRING2 from the structures retrieved from Protein Data Bank (PDB) having high percentage of identity. The third script aligned the amino acid sequence of GhRING2 with the template, and the fourth script was the core script that generated five GhRING2 protein models and calculated the Discete Optimized Protein Energy (DOPE) and GA341 scores for each model. DOPE score represented the statistical possibility of atoms in the protein depart from the native structure. A protein model with minimum DOPE score will have the structure that is more comparable to the native structure. GA341 corresponded to the

degree of coverage of the model structure to the template protein, EL5, an ubiquitin ligase of rice (*Oryza sativa*) (Kato et al. 2003) which has the highest amino acid sequence identity with the GhRING2 protein.

### **Expression Analysis of *GhRING2* Gene**

#### **Isolation of Total RNAs from Cotton Tissues**

Total RNA was isolated from cotton flower, leaf and fiber at different developmental stages (5, 10, 15, and 20 DPA) from Texas Marker-1 and Chromosome Substitution Line CS-B25 using a hot borate extraction method (Wan and Wilkins 1994). One gram of cotton tissue (fiber, leaf and flower) was ground into fine powder in liquid nitrogen with a pre-chilled mortar and pestle. The powder was then transferred into a 50 ml high-speed centrifuge tube containing 5 ml of hot (80°C) borate extraction buffer containing 200 mM sodium borate decahydrate, pH 9.0, 1% (w/v) SDS, 10 mM DTT, 30 mM EDTA, 2% (v/v) PVP40, 1% (w/v) sodium deoxycholate, and 0.5% (v/v) NP-40. To the tissue-buffer mixture 100 µl of proteinase K (25 mg/ml) were added, mixed and then incubated at 42 °C in a shaking incubator (with 100-120 rpm) for 1.5 hr. To the homogenate 952 µl of 1 M KCl were added, chilled on ice for 1 hr, and centrifuged with the Sorvall RC-5B centrifuge at 12,000x g for 20 min. The supernatant was transferred into a fresh 50 ml high-speed centrifuge tube, and RNA in the supernatant was precipitated by adding 1.5 ml of 8 M LiCl and incubating the sample overnight (16 hr) on ice. The supernatant was then centrifuged at 12,000x g for 20 min to collect the RNA precipitate. RNA pellet was washed 3 times using 5 ml of 2 M ice-cold LiCl, re-suspended in 2 ml of 10 mM Tris-HCl (pH 7.5), and followed by centrifugation at 12,000x g for 10 min to remove insoluble materials. Supernatant was transferred into a

fresh 50 ml centrifuge tube and 83  $\mu$ l of 5 M KAc (pH 5.5) were added and incubated on ice for 15 min. The sample was centrifuged at 12,000x g for 10 min, and the supernatant was then transferred into a new 50 ml centrifuge tube. The RNA was precipitated by adding 2.5 volumes of ice cold 96% ethanol and incubating at -20°C for several hr. RNA pellet was obtained by centrifugation at 12,000x g for 20 min and then rinsed with 5 ml of 70% ethanol. The RNA pellet was finally freeze dried under vacuum for 10 min and re-suspended in 200  $\mu$ l of DEPC-treated double distilled water.

### **Synthesis of First-stand cDNA from RNA Samples**

Two  $\mu$ g of cotton total RNA were mixed with 1  $\mu$ l of oligo (dT) primer (5'-CCCCAAAGCTTTTTTTTTTTTTTTT-3', 10  $\mu$ M), 1  $\mu$ l of 10 mM dNTP mix and appropriate volume of distilled water to make a final volume of 13  $\mu$ l. Initially this mixture was heated to 65°C for 5 min, quick chilled on ice, and followed by addition of 4  $\mu$ l of 5x first stand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl<sub>2</sub>) and 2  $\mu$ l of 0.1 M DTT. The sample was then incubated to 37 °C for 2 min and 1  $\mu$ l (200 units) of M-MLV RT was added and followed by incubation at 25°C for 10 min. The mixture was incubated at 37°C for 1 and ½ hr and heat inactivated at 70°C for 15 min (Jonsdottir et al., 2004). The final product of the first stand cDNA was diluted by three fold by adding 40  $\mu$ l of double distilled water.

### **Real Time RT-PCR Analysis of *GhRING2* in Different Cotton Tissues**

The transcript level of *GhRING2* in TM-1 and CS-B25 tissues (leaf, flower, and fiber at different developmental stages 5, 10, 15, and 20 DPA) was determined by Real-Time RT-PCR. The first strand cDNA samples were utilized as templates to amplify a

178 bp cDNA fragment of the *GhRING2* gene by using two primers RT-R1 (5'-TTATGGTACGCCACGAATCA-3') and RT-R2 (5'-CCGATTGCTCTGCCTCTATC-3') while performing Real-Time RT-PCR. As a reference to analyze *GhRING2* expression, the cotton polyubiquitin cDNA, was amplified using two universal ubiquitin primers UBQ F (5' - GAGTCCTCAGACACCATTGACAAC - 3'), UBQ R (5' - GTGCTCTCCTTCTGGATGTTGTAG - 3'). The reaction mixture was prepared by mixing 2 µl of diluted first stand cDNA sample with 5 µl of real-time RT-PCR master mix (SsoFast™ EvaGreen® Supermix bought from Bio-Rad), 0.2 µl (10 µM) each of RT-R1 and RT-R2 and double distilled water to a final volume of 10 µl. These reaction mixtures were subjected to PCR amplification with initial denaturation at 95°C for 3 min and followed by 45 cycles at 95°C for 15 sec, 55°C for 10 sec and 72 °C for 15 sec using the BIO-RAD CFX96/C1000 Real Time PCR system. The abundance of the *GhRING2* transcript was calculated using the formula  $2^{-\Delta\Delta CT}$ , where  $\Delta\Delta CT$  (Cycle Threshold) =  $(CT_{GhRING2} - CT_{Ubiquitin}) - (CT_{Calibrator} - CT_{Ubiquitin})$ .

### **In-vitro Auto ubiquitination Assay of GhRING2 E3 Ligase Activity**

#### **Construction of Recombinant pGEX-4T-1 Plasmid**

The open reading frame (ORF) of *GhRING2* was cloned into the pGEX-4t-1 vector to express the GhRING2 protein in fuse with the tag protein glutathione S-transferase (GST). Two primers Ring2-P2 (5'- CGCGGATCCATG CGCCATGGCGCGCACTATAC-3') and RING2-P3 (5'- CGCGAATTCTT AGCAAGGGTTATCATCACC-3') were used to amplify the ORF of *GhRING2* by PCR using Phusion DNA polymerase. The *Bam*H 1 and *Eco*R1 restriction sites were incorporated into the 5' end of RING2-P2 and RING2-P3, respectively. The *GhRING2*

PCR product (1.5 µg) and the plasmid pGEX-4t-1 (400 ng) were digested with *Bam*H1 and *Eco*RI (20 units each) at 37°C for one hr in the presence of 1x NEB buffer 2 (10 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 50 mM NaCl, 1 mM DTT, pH 7.9). Digested *GhRING2* PCR product and pGEX-4t-1 vector were then subjected to electrophoresis on a 1% agarose gel. After electrophoresis, the gel was illuminated with long wave UV light and DNA bands corresponding to the digested *GhRING2* and pGEX-4t-1 vector were cut and purified using a QIAquick gel Extraction kit from QIAGEN. The *GhRING2* was then cloned into pGEX-4t-1 vector by a ligation reaction conducted at 4°C overnight in a mixture containing 1 µl (400 units) of T4 DNA ligase, 2 µl of 10x T4 DNA ligase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 1 mM ATP, 10 mM DTT), 2 µl (1 µg) of *GhRING2*, 1 µl (600 ng) of pGEX-4t-1 and 14 µl of ddH<sub>2</sub>O. *E. coli* XL1 blue cells were then transformed with the ligation mixture. A transformed colony containing the recombinant plasmid pGEX-4t-1/*GhRING2* was cultured in 2 ml of LB/ampicillin and the recombinant plasmid pGEX-4t-1/*GhRING2* was then isolated from the cell culture using a QIAprep Spin Miniprep Kit.

### **Expression and Purification of GST-GhRING2 Fusion Protein**

The recombinant plasmid pGEX-4t-1/*GhRING2* was first transformed into *E. coli* BL21 (DE3) cells. A BL21 (DE3) colony containing the recombinant plasmid was selected, inoculated into 10 ml of LB-ampicillin media (100 µg/ml of ampicillin), and incubated overnight at 37°C in a shaking incubator (225 rpm) to prepare a primary culture. Ten ml of the primary culture were then inoculated into 1 L of fresh LB-ampicillin medium (100 µg/ml of ampicillin) and incubated at 37°C in a shaking incubator until optical density at 600 nm (OD<sub>600</sub>) reached 0.6. The expression of GST-



GhRING2 fusion protein was then induced by 1mM IPTG and the culture was incubated for additional 4 hr. Cells were harvested by centrifuging the culture (using the Sorvall RC-5B Refrigerated Superspeed Centrifuge) at 3,000x g for 10 min. The cell pellet was resuspended in 40 ml of ice cold PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.3) containing 1% of Triton-x 100 and sonicated with a 550 Sonic Dismembrator (Fisher Scientific) by employing 23 units of PULSAR 15 sec each with 30 sec interval for a total of 5 min. Cell lysate was then centrifuged at 12,000x g for 10 min and the supernatant was transferred into a new 50 ml tube and 1 ml of pre-equilibrated Glutathione Sepharose 4 Fast Flow resin (Amersham Biosciences) was added and incubated for 1 hr at 4°C with rotation. The mixture was then slowly transferred into a 15 ml column by allowing the GST resin to settle down and flow through to pass the column. Resin was washed using 5 column volumes of PBS buffer (75 ml) and the GST-RING2 fusion protein was subsequently eluted from the GST resin with 2 ml of glutathione elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM reduced L-glutathione, 1mM DTT, 1mM PMSF).

### **Expression and Purification of Histidine-Tagged GhUBC Proteins**

*E. coli* BL21 (DE3) cells were individually transformed with recombinant plasmids pET28a (+)/GhUBC1 and pET28a (+)/GhUBC2 using the standard CaCl<sub>2</sub> method and plated on LB agar medium containing 100 µg/ml of kanamycin. A transformed colony of *E. coli* BL21 (DE3) cells with pET28a (+)/GhUBC1 and pET28a (+)/GhUBC2 vectors was selected and each was inoculated into 10 ml of fresh LB-kanamycin media (100 µg/ml of kanamycin) and incubated overnight at 37°C in a shaking incubator (225 rpm) to prepare the primary culture. Five ml of the primary

culture were then inoculated into 500 ml of fresh LB-kanamycin medium (100 µg/ml of kanamycin) and incubated at 37°C in a shaking incubator until optical density at 600 nm (OD<sub>600</sub>) reached 0.6. IPTG was added to the culture to a final concentration of 1 mM and the culture was continuously incubated at 37°C for 4 hr. Cells were harvested by centrifuging the culture (Sorvall RC-5B Refrigerated Superspeed Centrifuge) at 3,000x g for 10 min. Cell pellet was then resuspended in 20 ml of ice cold Ni-NTA affinity column binding buffer (20 mM Tris-HCl, pH 7.9, 5 mM imidazole, 0.5 M NaCl) and sonicated on ice by employing the Fisher Scientific 550 Sonic Dismembrator using 23 units of PULSAR 15 sec each with 30 sec interval for a total of 5 min. Cell lysate was then centrifuged at 12,000x g for 10 min and the supernatant was transferred into a new 50 ml tube. One ml of pre-equilibrated Ni-NTA Agarose (Qiagen) was added to the tube and incubated for 1 hr at 4°C with rotation. The sample mixture was transferred into a 15 ml column slowly by allowing the Ni-NTA Agarose resin to settle and flow through to pass the column. Resin was then washed with 5 column volumes (75 ml) of washing buffer (20 mM Tris-HCl, pH 7.9, 20 mM imidazole, 0.5 M NaCl) and subsequently the His-GhUBC1 (or His-GhUBC2) fusion protein was eluted from the Ni-NTA Agarose resin using 2 ml of elution buffer containing 20 mM Tris-HCl, pH 7.9, 300 mM imidazole, 0.5 M NaCl, 1mM DTT, 1mM PMSF).

### **In-vitro Ubiquitination Assay of GhRING2 Using Wheat Germ Extract**

The ubiquitin E3 ligase activity of GhRING2 was determined by employing an in vitro auto ubiquitination assay using wheat germ extract. Wheat germ extract provides two enzymes, ubiquitin activating E1 and conjugating enzyme E2, for auto-ubiquitination of GhRING2. One µl of wheat germ extract (Promega) was individually added into six of

25  $\mu$ l aliquots containing 500 mM Tris-HCl pH 7.5, 25 mM MgCl<sub>2</sub>, 1 M DTT, 100 mM ATP, 68 ng GST-GhRING2, and 7  $\mu$ g ubiquitin (Sigma). The aliquots without GST-GhRING2, wheat germ extract or ATP were used as negative controls for the assay. Each reaction mixture was incubated for a different amount of time 0, 5, 15, 30, 60 and 120 min, and the reaction was then terminated by adding 6  $\mu$ l of 6x SDS loading dye (12% SDS, 300 mM Tris-HCl pH 6.8, 60% (w/v) glycerol, 0.6% (w/v) bromphenol blue, 600 mM DTT). The samples were boiled for 5 min and centrifuged for 5 min at 13,000 rpm. The samples were then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using an 8 % (w/v) resolving polyacrylamide gel covered with a 4% (w/v) stacking polyacrylamide gel.

#### **SDS Polyacrylamide Gel Electrophoresis and Eletroblotting**

The resolving gel solution was prepared by mixing 2.33 ml of double distilled water, 1.25 ml of 1.5 M Tris-HCl, pH 8.8, 50  $\mu$ l of 10% (w/v) SDS, and 1.34 ml of 30% (w/v) polyacrylamide (acrylamide: N', N'-bis-methyleneacrylamide = 29: 1). After adding 25  $\mu$ l of APS (10%) and 5  $\mu$ l of TEMED, the gel solution was mixed and immediately poured into the space between two pre-assembled glass plates to the level one inch below the top of the glass plates. The gel solution was then covered with 200  $\mu$ l of butanol to prevent the oxidation of free radicals and allowed to polymerize at room temperature for 30 min. After polymerization the resolving gel was rinsed with distilled water to remove butanol. A stacking gel (4 %) solution comprising 3.05 ml of double distilled water, 1.25 ml of 0.5 M Tris-HCl, pH 6.8, 50  $\mu$ l of 10% (w/v) SDS, 0.65 ml of 30% (w/v) polyacrylamide, 25  $\mu$ l of APS and 5  $\mu$ l of TEMED was poured on top of the resolving gel and a 1 mm thick 10-well comb was inserted immediately into the stacking

gel solution. The comb was slowly removed after the stacking gel was polymerized and the wells were washed with SDS-PAGE 1X TGS running buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine, 0.1% (w/v) SDS). Protein samples were loaded onto the wells and subjected to electrophoresis at 100 V for 90 min using the Bio-Rad Mini- PROTEAN Tetra cell system.

After electrophoresis, the gel was rinsed and soaked in a transfer buffer containing 25 mM Tris-HCl, pH 8.3, 192 mM glycine, 20% (v/v) methanol for 15 min. A polyvinylidene difluoride (PVDF) membrane (Amersham Hybond-P, GE Healthcare Lifesciences), about the same size of the SDS PAGE gel, was soaked in 100 % methanol for one minute and then in the transfer buffer for 15 min. A fiber pad soaked in the transfer buffer was placed on the black panel of gel holder cassette and covered with three pieces of transfer buffer-saturated Whatman 3MM papers. The gel was placed on top of the 3MM papers and PVDF membrane was then laid on it. The air bubbles trapped between gel and membrane were removed gently and covered with three more pieces of Whatman 3MM papers saturated with the transfer buffer. A second fiber pad soaked in the transfer buffer was placed on the red panel of gel holder cassette. The assembled cassette was then placed in the tank for electroblotting with the gel side (black panel of gel holder cassette) facing the cathode. Ice-cold transfer buffer was poured into the tank to immerse the cassette, and electroblotting was carried out overnight at 30 V at 4°C.

### **Immunodetection of Polyubiquitinated GST-GhRING2 Proteins with Anti-GST**

After electroblotting, the membrane was carefully removed from the cassette, rinsed with 1X TBS buffer (50 mM Tris-HCl, pH 7.5. 150 mM NaCl, 0.1% (v/v) Tween-20), and incubated in 20 ml of blocking solution (5% (w/v) non-fat milk, 1X TBS buffer,

0.1% (v/v) Tween-20) at room temperature for 1 hr to block non-specific binding sites. The membrane was washed three times each with 20 ml of TBS-T buffer for 10 min. Primary antibody solution (1:2000) was prepared by adding 5  $\mu$ l of Anti-GST tag/mouse monoclonal IgG antibody (1 mg/ml) (Millipore) into 10 ml of blocking solution. Membrane was incubated with the primary antibody solution for 1 hr at room temperature and then rinsed three times each with 20 ml of TBS-T buffer for 10 min. Secondary antibody solution (1:5000) was prepared by adding 2  $\mu$ l of Anti-IgG mouse horseradish peroxidase conjugated antibody (10  $\mu$ g/mL) (Thermo Scientific Pierce) into 10 ml of blocking solution. Membrane was incubated with the secondary antibody solution for 1 hr at room temperature and rinsed three times each with 20 ml of TBS-T buffer for 10 min. Immunodetection was done using the substrate LumiSensor Chemiluminescent HRP (GenScript). Solutions A and B of the substrate were mixed, poured onto the membrane in a box and incubated for 3 min at room temperature. Membrane is then drained, covered with Saran wrap and exposed to three sheets of Amersham Hyperfilm ECL films in a cassette for 30 sec, 1 min and 2 min at room temperature. The films were developed with an automated Kodak M35A X-Omat processor.

## **Identification of Target Protein of GhRING2**

### **Preparation of Yeast Two-hybrid cDNA Library**

One  $\mu$ l of CDS III Primer (10  $\mu$ M) (Oligo-dT Primer) and 1.3  $\mu$ g of 10 DPA fiber RNA from TM-1 were mixed in a 0.6 ml centrifuge tube to a final volume of 4  $\mu$ l with sterile distilled water. The tube was incubated at 72°C for 2 min, chilled on ice for 2 min, and then spun down briefly at 14,000x g for 30 sec. Two  $\mu$ l of 5X First-Strand Buffer

(250 mM Tris-HCl, pH 8.3; 375 mM KCl; 15 mM MgCl<sub>2</sub>), 1.0 µl of DTT (100 mM), 1.0 µl of dNTP Mix (10 µM) and 1.0 µl (200 units) of SMART MMLV reverse transcriptase were added to the tube and mixed. The sample was then incubated at 42°C for 10 minutes, centrifuged for 30 sec after 30 min, and followed by adding 1 µl of 10 µM SMART III-modified oligo (5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGG-3'; Clontech). The first-strand cDNA was synthesized by incubating the mixture at 42°C for 1 hr and the reaction was terminated by heating at 75°C for 10 min (Jonsdottir et al. 2004). The final product was diluted two-fold with 10 µl of double distilled water and then used as template for PCR amplification to generate double-strand cDNAs. The PCR reaction mixture was prepared by mixing 2 µl of 5' PCR Primer (5'-TTCCACCCAAGCAGTGGTATCAACGCAGAGTGG-3', 10 µM ), 2 µl of 3' PCR Primer (5'-GTATCGATGCCACCCTCTAGAGGCCGAGGCGGCCGACA-3', 10 µM ), 2 µl of First-Strand cDNA, 70 µl of distilled water, 10 µl of 10X Advantage® 2 PCR buffer, 2 µl of 50X dNTP (10 mM), 10 µl of 10X Melting Solution, and 2 µl of 50X Advantage 2 Polymerase Mix (Clontech). The PCR was conducted via pre-denaturation at 95°C for 30 sec, following by 18 cycles at 95°C for 10 sec (denaturation) and 68 °C for 6 min (annealing and elongation), and a final extension at 68°C for 5 min.

The double-stand cDNA PCR product was purified using the CHROMA SPIN TE-400 Column (Clontech) to remove contaminants including small DNA molecules that are less than 400 bp. The quality of the ds cDNA PCR product was analyzed by electrophoresis on a 1% agarose gel containing 0.5 µg/ml ethidium bromide. The high quality cDNAs with sizes larger than 400 bp were then used for large scale transformation of yeast Y187 cells to make the yeast two-hybrid library.

### **Preparation of Yeast Competent Cells and Transformation**

A yeast cell (Y187) stock from Clontech was used to streak on a fresh YPDA (yeast peptone dextrose adenine) agar plate (10 g/liter bacto yeast extract, 20 g/liter bacto peptone, 20 g/liter glucose monohydrate, 40 mg/liter adenine hemisulfate, 20 g/liter bacto agar, pH 6.5) and incubated overnight at 30°C until colonies appeared (around 3 days). A single colony was picked, inoculated into 3 ml of YPDA medium, and incubated at 37°C in a shaking incubator (225 rpm) for 8–12 hr. About 5 µl of the culture were inoculated into 50 ml of fresh YPDA in a 250 ml flask and incubated at 30 °C in a shaking incubator until optical density at 600 nm (OD600) reached 0.15–0.3 (about 16–20 hr). The cells were then centrifuged at 700x g with SS-34 rotor in a Sorvall RC-5B Refrigerated Superspeed Centrifuge for 5 min. The supernatant was removed, and the pellet was re-suspended in 100 ml of YPDA and incubated at 30°C in a shaking incubator until the OD600 reached 0.4–0.5 (about 3–5 hr). The cells were centrifuged at 700x g for 5 min, and the cell pellet was washed twice with 30 ml of double distilled water by resuspension and centrifugation. The cells were further washed in 1.5 ml of 1xTE/LiAc (1x TE and 0.1 M LiAc), centrifuged, and finally resuspended in 1.2 ml of 1xTE/LiAc.

Large scale transformation of yeast (Y187) competent cells with linearized pGADT7-Rec plasmid and fiber ds cDNA was performed to construct the yeast two-hybrid library. In a 15 ml tube 3 µg of pGADT7-Rec plasmid DNA (Clontech), 5 µg of ds cDNA and 0.2 mg Yeastmaker Carrier DNA were mixed with 600 µl of yeast (Y187) competent cells. To the mixture 2.5 ml of PEG/LiAc (40% PEG and 0.1 M LiAc) were added, mixed and incubated for one hr at 30 °C in a shaking incubator. After adding 20 µl of dimethyl sulfoxide (DMSO), the sample was heated in a water bath at 42°C for 20

min. The cells were mixed every 5-10 min by gently vortexing during the heating step. The cells were then harvested by centrifugation at 700x g for 5 min, and the cell pellet was re-suspended in 3 ml of YPD Plus Medium (Clontech), centrifuged, and finally suspended in 15 ml of 0.9% (w/v) NaCl solution. Cells were then spread onto 50 of 150 mm plates containing Minimal Synthetic Defined Double Dropouts medium (SD-Trp/-Leu) and incubated at 30°C until colonies appeared (about 3–5 days).

Plates were chilled at 4°C for about 3–4 hr and cells were harvested using 400 ml of Freezing Medium (8 ml for each plate). The cell density of the yeast two-hybrid library was adjusted with the medium to exceed  $2 \times 10^7$  per ml. The library was then aliquoted into 1.5 ml centrifuge tubes and stored at –80°C.

### **Screening Yeast Two-hybrid Library to Identify GhRING2 Interacting Proteins**

A yeast mating method was used to identify GhRING2 interacting proteins by screening the yeast two-hybrid library constructed in the Y187 strain. Mating of two haploid yeast cells from opposite mating strains, Y2HGold and Y187, which harbor pGBKT7 or pGADT7 plasmid, resulted in the formation of doubly transformed diploid zygotes. The diploid zygotes can proliferate on double (-Trp and – Leu,) drop out SD medium and they can also grow on quadruple drop out (-Trp, – Leu, -His and –Ade) SD medium if the bait and prey proteins interact.

Yeast Y2H gold cells were transformed with the bait plasmid pGBKT7/*GhRING2* using the yeast transformation protocol as described previously. Transformed cells were plated on –Trp single drop out SD media and incubated at 30°C until colonies appeared (about 3–5 days). A single colony was then picked, inoculated into fresh 50 ml of –Trp SD medium and incubated at 30°C until the OD at 600 reached 0.8 (about 16–20 hr).



Cells were centrifuged at 1,000 g for 5 min and pellet was re-suspended in 4–5 ml of – Trp SD medium. One ml of yeast two hybrid library cells (Y187 strain cells) and 4-5 ml of bait cells (Y2H gold strain) were inoculated into 45 ml of 2x YPDA in 1 L conical flask. The cells were mixed and incubated at 30°C in a slowly shaking incubator (50-60 rpm) for about 24 hr. Mated yeast cells were collected by centrifugation at 1,000 xg for 5 min, washed with 50 ml of 0.5x YPDA, and re-suspended in 10 ml of 0.5xYPDA containing 50 µg/ml kanamycin. Cells were then plated on single (-Trp or – Leu), double (-Trp and – Leu) and quadruple drop out (-Trp, – Leu, -His and –Ade) SD agar plates and incubated at 30°C until colonies appeared (about 3–5 days). Putative positive colonies that appeared on quadruple drop out SD agar plates were analyzed by yeast colony PCR using a Matchmaker Insert Check PCR Mix 2 kit (Clontech Laboratories, Inc.). The PCR product was purified using a QIAquick PCR purification kit (QIAGEN) and then sequenced using either the 5' PCR Primer (10 µM; 5'-TTCCACC CAAGCAGTGGTATCAACGCAGAGTGG-3') or 3' PCR Primer (10 µM; 5'-GTATCGATGCCCACCCTCTAGAGGCCGAGGCGGCCGACA-3') from Clontech.

### **Plasmid Swap Assay**

A plasmid swap assay with pGBKT7/*GhRING2* and pGADT7/*GhPDF1* was employed to further test the interaction between GHRING2 and GhPDF1 that was previously identified during the screening of the yeast two-hybrid library using pGADT7/*GhRING2* as bait. The restriction sites of *EcoRI* (GAATTC) and *BamHI* (GGATCC) were incorporated into the 5'-end of primers Ring2-T2 (5'-CGCGAATTCATGCGCCATGGCGCGCACTATAC -3'), GFP-1 (5'- CGCGAATTCATGGTAAATATCCGATGCTCT-3'), Ring2-T3 (5'- CGCGGATCCTTAGCAAGGGTT

ATCATCACC-3'), and GDFP-2 (5'- CGCGGAT CCTTACTGCCCAAGAATTTCCCAG-3'). The ORF of *GhPDF1* and *GhRING2* was amplified with the primer pairs GDFP-1/GDFP-2 and Ring2-T2/Ring2-T3, respectively using fiber cDNA as template by Phusion DNA polymerase. The *GhPDF1* and *GhRING2* PCR products were cloned into the *EcoRI* and *BamHI* restriction sites of pGBKT7 and pGADT7 vectors, respectively. The yeast two-hybrid system was then used to confirm the interaction between GhPDF1 and GhRING2 by co-transformation of the two recombinant plasmids into yeast Y2H gold cells using the LiAc/SS carrier DNA/PEG method (Gietz et al. 1995).

### **Pull Down Assay**

Ten µg of GST-tag and GST-GhRING2 fusion proteins were bound to 200 µl of Glutathione Sepharose 4 Fast Flow resin (Amersham Biosciences), respectively, and packed in a 10 ml Poly-Prep Chromatography Column (BIO-RAD). Ten µg of GhPDF1-MBP fusion protein were loaded onto the columns and the samples were incubated at 4 °C for two hr. The resin was then washed 5 times at 4°C each with 20 ml of wash buffer (50 mM Tris-HCl pH, 8.0, 300 mM NaCl, 1% Triton X-100) (Kraft 2007). The GST- tag and GST-RING2 fusion proteins were eluted from the GST resin using multiple 200 µl of glutathione elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM reduced L-Glutathione, 1mM DTT, 1mM PMSF). Eluted protein samples were quantified using nano spectrophotometer), subjected to SDS PAGE, and analyzed by Western blotting using mouse anti-GST tag monoclonal IgG antibody (1 mg/ml) from Millipore.

## Promoter analysis of *GhRING2*

### PCR Amplification of *GhRING2* Promoter

The full length and 5' truncated promoter fragments of *GhRING2*, named *R2GUS1* (1410 bp), *R2GUS2* (910 bp) and *R2GUS3* (410 bp), were amplified by PCR using three primer pairs GUS-F1/GUS-R2, GUS-F2/GUS-R2 and GUS-F3/GUS-R2, respectively. The nucleotide sequences of GUS-F1, GUS-F2, GUS-F3 and GUS-R2 primers are listed below, and restriction sites *HindIII* (AAGCTT) and *XbaI* (TCTAGA) designed at the 5'-end of the four primers are underlined:

GUS-F1 5'-CCCAAAGCTTAAAGCATGCTTTATTGGTTC-3'

GUS-F2 5'-CCCAAAGCTTGCCTTTCTCTCATCTCAATC -3'

GUS-F3 5'-CCCAAAGCTTCTCCCACTCGACAAATCT -3'

GUS-R2 5'- GCTTAGAAATAGTCAGCTGGTTTTGTG-3'

The PCR amplification was conducted by using Phusion DNA polymerase under the following conditions: pre-denaturation at 94°C for 3 min and following by 30 cycles at 94°C for 30 sec, 55°C for 15 sec, and 72°C for 90 sec.

### Construction of Recombinant pBI121 Plasmids

The PCR amplified promoter fragments of *GhRING2* were cloned into the binary vector pBI121 to replace cauliflower mosaic virus (CaMV) 35S promoter (835 bp) using the restriction enzymes *HindIII* and *XbaI*. One µg each of pBI121, *R2GUS1*, *R2GUS2* and *R2GUS3* was digested with 20 units of *HindIII* and *XbaI* (New England BioLabs) in 1x NEB buffer 2 (10 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 50 mM NaCl, 1 mM DTT, pH 7.9) at 37°C for one hr. The digested promoter fragments and pBI121 were separated by electrophoresis on a 1% (w/v) agarose gel, excised and purified using a QIAquick gel

extraction kit (QIAGEN). The three promoter fragments *R2GUS1*, *R2GUS2* and *R2GUS3* were individually ligated to pBI121 and transformed into *E. coli* XL1-Blue competent cells. The transformed colonies containing desired promoter constructs were then confirmed by colony PCR using corresponding primers. The colonies were cultured in 2 ml LB/kanamycin medium and recombinant plasmids pBI121-*R2GUS1* (*R2GUS2* and *R2GUS3*) were isolated by using a Plasmid Miniprep Kit (from QIAGEN).

### **Transformation of Recombinant pBI121 into *Agrobacterium tumefaciens***

The recombinant pBI121 plasmids containing *GhRING2* promoter fragments upstream of the GUS reporter gene were transformed into *Agrobacterium tumefaciens* C58 cells by using a freeze-thaw method (Walkerpeach and Velten 1994). A single colony of C58 cells was picked and inoculated into 2 ml of LB medium with streptomycin (200 µg/ml) and incubated overnight at 28°C in a shaking incubator (225 rpm). The overnight-grown culture was then inoculated into 50 ml of fresh LB medium with streptomycin (200 µg/ml) and incubated at 28°C in a shaking incubator until OD at 600 nm reached 0.5- 1.0. The cells were then centrifuged at 3,000x g (using ss-34 rotor and Sorvall RC-5B Refrigerated Superspeed Centrifuge) for 10 min at 4°C. The supernatant was removed, and the pellet was re-suspended in 1 ml of ice-cold 20 mM CaCl<sub>2</sub> and 230 µl of 80% (v/v) glycerol, incubated on ice for 15 min, and used as competent cells for transformation. For transformation, 100 µl of *Agrobacterium* competent cells were mixed with 1 µg of recombinant pBI121 plasmids and frozen in liquid nitrogen. The transformation mixture was then immediately transferred into a 37 °C water bath and incubated for 5 min. To the cell mixture 1 ml of LB medium was added and incubated at 28°C for 3 hr in a shaking incubator (225 rpm). The cells were

harvested by centrifugation at 13,000 x g for 30 seconds and the pellet was re-suspended with 100 µl of LB medium. Cells in 25 and 50 µl aliquots were spread on LB/streptomycin plates and incubated for 2-3 days at 28°C until transformed colonies appeared.

### ***Agrobacterium*-Mediated Floral Dip Transformation of *Arabidopsis thaliana***

*Arabidopsis thaliana* was transformed with recombinant pBI121 plasmids via an *Agrobacterium*-mediated floral dip method (Clough and Bent 1998). Wild type *A. thaliana* (ecotype Columbia) seeds were placed in a microfuge tube, rinsed with 200 µl of 70 % ethanol, and then washed three times with 1 ml of sterile distilled water. After adding 200 µl of Clorox bleach the seeds were incubated at room temperature for 5 min and then washed three times each with 1 ml of sterile distilled water. Surface sterilized seeds were re-suspended in 1 ml of 0.08% agarose and gently spread on Gamborg B5 agar medium plates containing 3.2 g/liter Gamborg B5 basal medium (Phytotechnology Laboratories TM), 0.5 g/liter MES, 20 g/liter sucrose, 9 g/liter phyto agar, pH 5.7 (adjusted with 1M KOH). Plates were placed in a cold room at 4°C for two days to synchronize the germination of seeds. *Arabidopsis* seedlings were grown in a 22°C plant growth chamber (Percival, Model 1-35LLVL) with 16 hr photoperiod for 7 to 10 days. The seedlings were then transplanted onto the soil and grown in a plant growth chamber (Percival - Intelius Environmental Controller) with 16 hr photoperiod for several weeks till the plants start to flower.

*Agrobacterium* C58 cells transformed with recombinant pBI121 vectors were cultured separately in 500 ml LB media containing 50 µg/ml kanamycin and 200 µg/ml streptomycin at 28°C in a shaking incubator (225 rpm) until they reached the stationary

growth phase. Cells were harvested by centrifugation at 3,000x g with GSA rotor in a Sorvall RC-5B Refrigerated Superspeed Centrifuge for 10 min, and cell pellet was re-suspended in 5% (w/v) sucrose to OD 0.8 at 600 nm. To this cell suspension the surfactant Silwet L-77 (Osi Specialties) was added to a final concentration of 0.05% (v/v). The inflorescence bolts of *Arabidopsis* plants were gently dipped into the *Agrobacterium* solution by holding the pot for 10-15 sec. *Arabidopsis* plants were then placed in a closed tray, kept in the darkness for 24 hr, and transferred to a plant growth chamber (Percival, Model 1-35LLVL) with 16 hr photoperiod.

### **Seed Collection and Screening of Transgenic *A. thaliana* Plants**

Matured seeds (T1) were collected from transformed *Arabidopsis* plants (T0) with each promoter construct and stored in 1.5 ml centrifuge tubes at 4°C. About 250-500 seeds were placed in a 1.5 ml centrifuge tube and surface sterilized as previously described. Sterilized seeds were re-suspended in 1 ml of 0.08 % agarose and gently spread on B5 medium plates containing 50 µg/ml kanamycin. Plates were then placed in a cold room at 4°C for two days and transferred to a plant growth chamber (Percival, Model 1-35LLVL) with 16 hr photoperiod for 7 to 10 days.

Transgenic *A. thaliana* seedlings (T1) grown in the B5 medium plates were identified by their appearance of green cotyledon and long roots when compared with non-transgenic plants that turned into pale yellow on the plates with kanamycin (50 µg/ml). These transgenic *A. thaliana* seedlings were then transplanted onto the soil and transferred to a plant growth chamber (Percival - Intelius Environmental Controller) with 16 hr photoperiod for several weeks. The transgenic *A. thaliana* plants (T1) were further confirmed by PCR using respective primers for different promoter constructs to eliminate

false positive plants. The T2 seeds were collected from transgenic T1 *A. thaliana* plants and stored in 1.5 ml centrifuge tubes at 4°C.

### **Histochemical GUS assay**

The promoter activity of *GhRING2* was monitored in transgenic *A. thaliana* plants using a histochemical GUS assay (Sessions et al. 1999). The seedling, leaf, flower, stem and root of transgenic *A. thaliana* plants (T2) were placed in 1.5 ml centrifuge tubes and 1 ml of GUS staining solution containing 100 mM sodium phosphate pH 7.0, 1 mg/ml of X-Gluc (Gold BioTechnology), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA and 0.1% Triton X-100 was then added. The tubes were gently inverted for 5-6 times and incubated at 37°C overnight. The chlorophyll pigment was removed by extraction of the plant tissues once with 50 % ethanol for 5 min, 70 % ethanol for 5 min and three times each with 96 % ethanol for 1 hr. The plant tissues were then maintained in 100 mM sodium phosphate buffer (100 mM K<sub>2</sub>HPO<sub>4</sub> and 100 mM KH<sub>2</sub>PO<sub>4</sub>; pH 7.4) and observed using a Nikon SMZ1000 stereomicroscope.

## CHAPTER III

### RESULTS

#### **Cloning and characterization of *GhRING2* gene**

An inverse PCR genomic walking method was used to clone the *GhRING2* gene along with its 5' and 3'-flanking regions (3,040 bp). The nucleotide and derived amino acid sequences of *GhRING2* are shown in Fig. 1. The gene has no introns and encodes a protein of 132 amino acids with a predicted molecular mass of 15 kDa. The GhRING2 protein contains a RING H2 domain with conserved cysteine and histidine residues and is classified as a C<sub>3</sub>H<sub>2</sub>C<sub>3</sub> (RING-H2) type protein. The six cysteine and two histidine residues in the H2 domain are involved in Zn binding.

A protein Blast (blastp) search was performed to find RING proteins homologous to GHRING2 from plants, and eleven closely related ubiquitin E3 ligases with their amino acid sequences, including *Theobroma cacao* (TcRING), *Fragaria vesca* (FvRING), *Ricinus communis* (RcRING), *Populus trichocarpa* (PtRING), *Solanum lycopersicum* (LeRING), *Vitis vinifera* (VvRING), *Prunus persica* (PpRING), *Cicer arietinum* (CaRING), *Medicago truncatula* (MtRING), *Arabidopsis lyrata* (AIRING) and *Arabidopsis thaliana* (AtRING), were retrieved. A multiple sequence alignment was performed for these eleven sequences along with GhRING2 using the ClustalW2 program from EMBL-EBI. Sequence alignment results indicated that 6 cysteine and 2 histidine residues of the C<sub>3</sub>H<sub>2</sub>C<sub>3</sub> (RING-H2) motif were highly conserved among all the



12 plant RING finger proteins (Fig. 2), suggesting that the RING finger domain is evolutionarily conserved in plant ubiquitin ligases. A phylogenetic analysis was conducted using the amino acid sequences of the RING proteins from the twelve plants, and a phylogenetic tree was constructed and shown in Fig. 3. The RING proteins TcRING from *Theobroma cacao* and PtRING from *Populus trichocarpa* are the two most closely related E3 ubiquitin ligases to the GhRING2 protein.

Structural analysis of the GhRING2 protein was performed by finding the closest protein homologue with a solved structure. A BLAST search of Protein Data Bank (PDB) indicates that amino acid sequence of the GhRING2 protein has the highest identity to EL5, an ubiquitin ligase of rice (*Oryza sativa*) (Kato et al. 2003). The RING H2 domain of GhRING2 has 57% amino acid sequence identity with EL5 and the structure of GhRING2 modeled using Modeller 9.12 is shown in Fig. 4.



Ring2-P1 →

ATGCTGGTTTCTGTTTATTAGCCCTTTTCTCCCATGCGCTGGCATGAGTGCTGTTTTTC 60  
M L V S V L L A L F L P C A G M S A V F 20

ATTGTTTATATCTGTCTTTTATGGTACGCCACGAATCACCGGGCTGATAATTCAGGGTCG 120  
I V Y I C L L W Y A T N H R A D N S G S 40

TTATCGGTTAAGCAGGCTGGTGAGAAAGGGTTGTCCGTTTCGGAGCTTGAGAAGCTGCCC 180  
L S V K Q A G E K G L S V S E L E K L P 60

AAAGTTACCGGAAGGATCTTGTGTTGGGGACTGAGTGTGCGGTTTGTCTCGATGAGATA 240  
K V T G K D L V L G T E C\* A V C\* L D E I 80

GAGGCAGAGCAATCGGCTAGAATGGTTCCCGGTTGTAATCACGGCTTTCATTTACAATGT 300  
E A E Q S A R M V P G C\* N H\* G F H\* L Q C\* 100

GCTGATACTTGGCTTTCAAAGAACTCGGTCTGTCCTGTTTGTAGGGCCAAGCTTGAGCCT 360  
A D T W L S K N S V C\* P V C\* 120

AAGTTGTTTCGATCCACCTGGTGATGATAACCCCTGCTAA ← Ring2-P3 399  
K L F D P P G D D N P C - 132

Figure 1 (Continued)

The promoter region and ORF were designated from -1 to -1506 nt and 1 to 399 nt. The RING domain of GhRING2 is underlined and the eight conserved Cys and His residues in the domain are marked with stars. The primer pairs GUS F1/GUS R2, GUS F2/GUS R2 and GUS F3/GUS R2 were used to amplify the promoter fragments R2GUS1, R2GUS2 and R2GUS3, respectively, for GUS assays. The primers Ring2-P1 and Ring2-P3 were used to amplify the *GhRING2* ORF for GhRING2 GST fusion protein expression.



```

TcRING      -----MRHGGHYTPPLLSPSPSPALNSTTTAGSHHPMLVSVFLALFLPCAGM 48
FvRING      -----MLFSI FLALFLPCVGM 16
RcRING      MHNGVQYAPLTPHPPLLISPSPPASLSIPPPPITAAAAANQNMLLSVFLALFLPCVGM 60
PtRING      -----MLVSVFLALFLPCAGM 16
LeRING      -----MLVSVFLALFLPCVGM 16
VvRING      -----MLLSI FLALFLPCAGM 16
PpRING      -----MLVSVFMAILLPSVAI 16
CaRING      -----MLDAVLLALFLPCLGM 16
MtRING      -----MLDTVLLALFLPCLGM 16
AlRING      -----MHYTRI SPVLVSPPTTAVESGGGPMIATVFMALLPCVGM 43
AtRING      -----MHYTRI SPALVPSLPTAAAESGGGTMIATVFMALLPCVGM 43
GhRING2     -----MLVSVLLALFLPCAGM 16
                                         *: :::::****. .:
TcRING      SAVFIVYICLLWYATNYRTG---NSGSLAIKQVAEKGLSVSELEKLPKVTGKELVLG--- 102
FvRING      SAVFVVYICLLWFASNSRSA---VAPQLPVKHVSEKGLSASDLEKLPKTSKGELIRG--- 70
RcRING      SAI FLIYICLLWYAANHHHH---SEIVTPVKPSAEKGLSASDLEKLPKMTGKELILG--- 114
PtRING      SAVFIVYICLLWYTANNQPE---IR--LPVKIVTEKGLSSSELEKLPKVTGKELVLG--- 68
LeRING      SAVFLVYICLLWYAAAYQSAGPGAENYHSQKTNQELGLSAAQLDKLPKITGNELLMG--- 73
VvRING      SVV FVVYICLLWYALGQ-Q---PELPAPVKPESEKGLSASDLKLPKLAGKDLVVG--- 69
PpRING      SAVFVVYICLLWYATNNQE-Q---PEVRLPVKQVSQKGLSASELDKLPKITGKELTTER--- 71
CaRING      SAI FIIYMCLLWYATTHRPR--NNINAIQPVKPVTDNGLSPSDLQKLPITITGKDLLAA--- 72
MtRING      SAI FVVYMCLLYATTHRSD--SNP---PLKPVTEKGLSPLDLEKLPKITGKELLAP--- 68
AlRING      CIVFLIYLFLLWCSTRRRRIER--LRFAEPVKPVTGKGLSVLELEKIPKLTGKELAI IARS 101
AtRING      CIVFLIYLFLLWCSTRRRRIER--LRFAEPVKPVTGKGLSVLELEKIPKLTGRELAVIARS 101
GhRING2     SAVFIVYICLLWYATNHRAD---NSGSLSVKQAGEKGLSVSELEKLPKVTGKDLVLG--- 70
. :****: **: : : : * **** :*:*:*. :*:**
TcRING      TECAVCLDEIEAEQPARVVPGCNHGFHLCADTWLSKHSVCPVCRAKLEPD-----QLFD 157
FvRING      TECAVCLDEIEPEQPARVVPGCNHGFHLCADTWLSKNSCCPVCRAKLQPPQ-----QIYA 125
RcRING      TECAVCLDEIESEQPARLVPGCNHGFHLECADTWLSNHSVCPVCRAKLDS-----QFFN 168
PtRING      TECAVCLDDIESEQVARMVPGCNHGFHLECADTWLTKRPVCPVCRAKLDA-----QLFS 122
LeRING      NDAVCLDEIENEQIARVVPGCNHGFHLECADTWLSKNPICPVCRTKLEP-----EFFN 127
VvRING      AECAVCLDEIESDAPARLI PGCNHGFHLCADTWLSKHSVCPVCRAILAP-----EFFN 123
PpRING      TECAVCLDEIEGEQVARLVPGCNHAFHLLCADTWLSKHSFCPVCRAKLQLHQDLPOSSVT 131
CaRING      PECAVCLDEIGLDQSVRVI PGCNHSFHLECADTWLSRNP LCPVCRAKLDA-----NFFI 126
MtRING      TECAVCLDDIVDEQPARLLPGCNHAFHLCADTWLSKHPMCPLCRAKLDP-----QSLN 122
AlRING      TECAVCLDEIESGQSRLLVPGCNHGFHRLCADTWLSNHTVCPVCRAELAP-----NIPQ 155
AtRING      TECAVCLDEIESGQSTRLVPGCNHGFHLCADTWLSNHTVCPVCRAELAP-----NLPQ 155
GhRING2     TECAVCLDEIEAEOSARMVPGCNHGFHLCADTWLSKNSVCPVCRAKLEP-----KLFD 124
:*****:.* :*:*****.* *****:..* :*:*:*. *
TcRING      AS-----DENPC 164
FvRING      SS-----DDNPC 132
RcRING      AS-----TDNPC 175
PtRING      TPPPPAQENNTC 134
LeRING      PP-----ESNPC 134
VvRING      TS-----ENPC 129
PpRING      CS-----ENPC 137
CaRING      ASSS----LNSC 134
MtRING      SES-----PC 127
AlRING      CNE-----NQSPC 163
AtRING      CNE-----NQSPC 163
GhRING2     PPG-----DDNPC 132
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Figure 2 Comparison of amino acid sequences of twelve plant RING proteins

Multiple sequence alignment was performed using Clustal method. The RING proteins are from: *Gossypium hirsutum* (GhRING2), *Theobroma cacao* (TcRING) with GI: 508723217, *Fragaria vesca* (FvRING) with GI: 470110114, *Ricinus communis* (RcRING) with GI: 255539054, *Populus trichocarpa* (PtRING) with GI: 224083141, *Solanum lycopersicum* (LeRING) with GI: 460385383, *Vitis vinifera* (VvRING) with GI: 225459738, *Prunus persica* (PpRING) with GI: 462422985, *Cicer arietinum* (CaRING) with GI: 502126436, *Medicago truncatula* (MtRING) with GI: 357467061, *Arabidopsis lyrata* (AlRING) with GI: 297790413, *Arabidopsis thaliana* (AtRING) with GI: 15238323. Dashed line represents the gaps introduced to increase the identities. A colon (: ) and a period ( . ) corresponds to homologous and non-homologous substitutions of amino acids respectively. Asterisks (\*) represents the identical amino acids conserved in all sequences. RING domain is underlined and the eight conserved Cys and His residues in C3H2C3 (RING-H2) motif are highlighted using grey color.

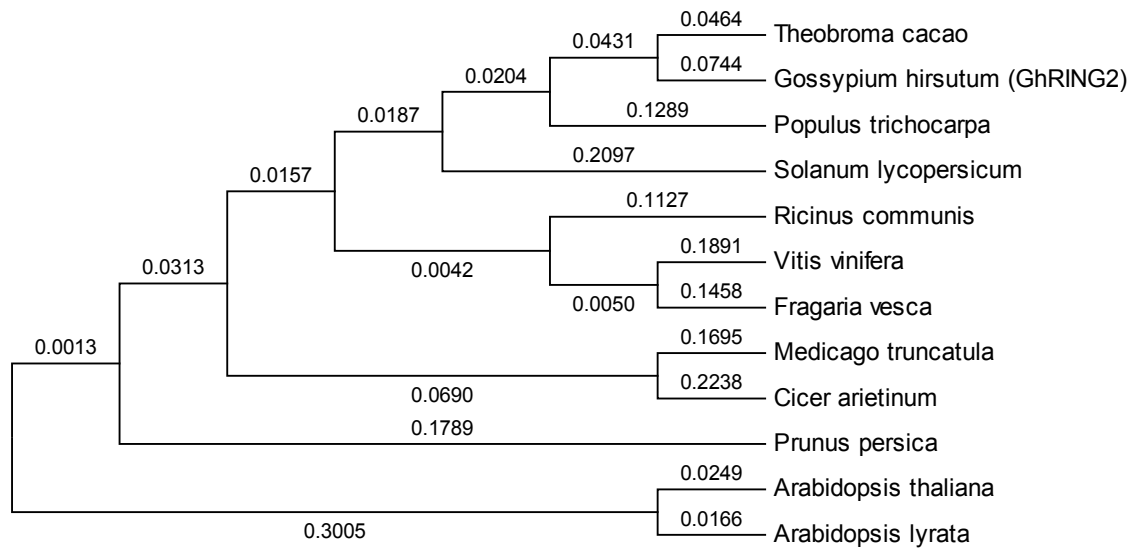


Figure 3 Phylogenetic analysis of E3 ubiquitin ligases from *Gossypium hirsutum* and 11 other plants.

The evolutionary history of *GhRING2* was inferred using the Neighbor-Joining method (Saitou and Nei 1987). Using the Clustal method (Thompson et al. 1997) the phylogenetic tree was constructed based on amino acid sequences of E3 ubiquitin ligase gene from *Gossypium hirsutum* (*GhRING2*) and 11 other plants (*Theobroma cacao* with GI: 508723217, *Fragaria vesca* with GI: 470110114, *Ricinus communis* with GI: 255539054, *Populus trichocarpa* with GI: 224083141, *Solanum lycopersicum* with GI: 460385383, *Vitis vinifera* with GI: 225459738, *Prunus persica* with GI: 462422985, *Cicer arietinum* with GI: 502126436, *Medicago truncatula* with GI: 357467061, *Arabidopsis lyrata* with GI: 297790413, *Arabidopsis thaliana* with GI: 15238323). The evolutionary distances were calculated by MEGA5 using the Poisson correction method (Tamura K. et al. 2011). The branch length with numbers represents the evolutionary distance between the homologous genes.

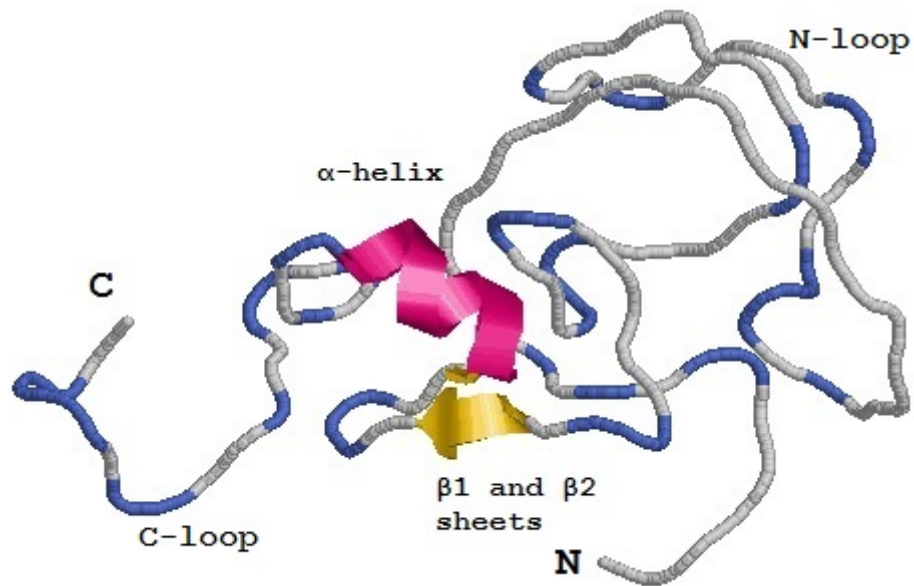


Figure 4 The GhRING2 structure

The RING-H2 domain has two beta sheets ( $\beta 1$  and  $\beta 2$ ) and one alpha helix ( $\alpha 1$ ) connected by N and C terminal loops.

The hydrophobicity of GhRING2 was analyzed using the tool ProtScale (Gasteiger et al. 2005) from the ExPASy Server. The hydrophobicity plot (Fig. 5) indicated that GhRING2 has both hydrophilic and hydrophobic amino acids and the N-terminal residues have higher hydrophobicity than C-terminal residues. The ProtScale analysis suggests that GhRING2 is a trans-membrane protein with hydrophobic N-terminal (Breton et al. 2003).

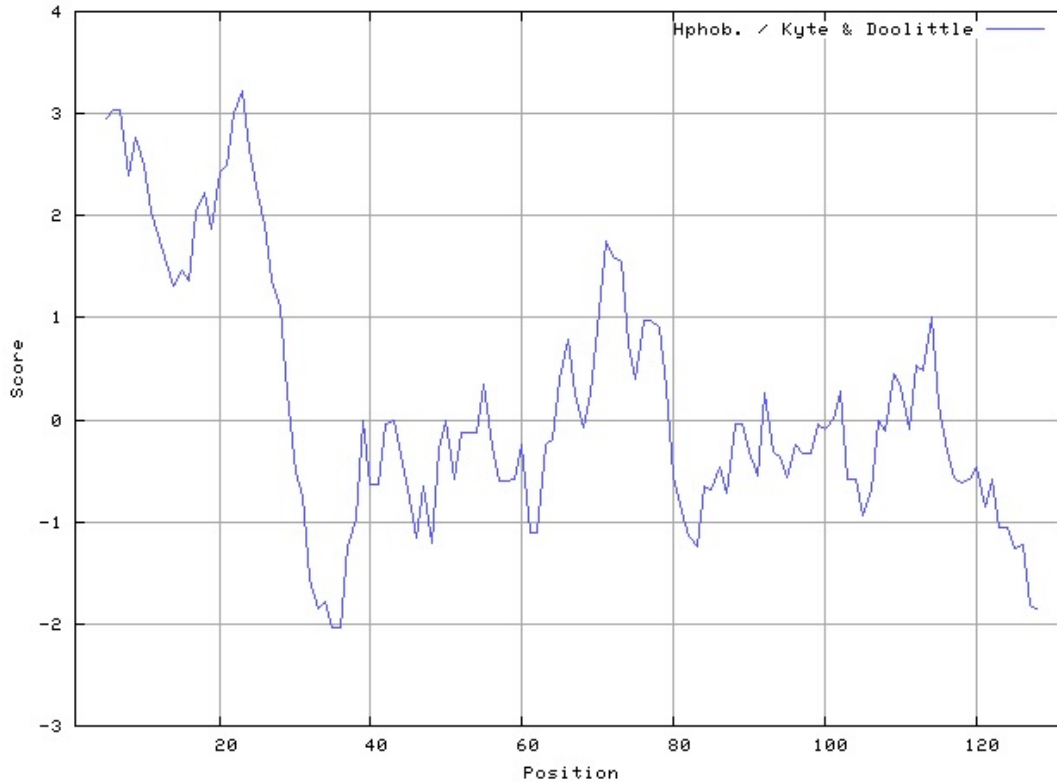


Figure 5 Hydrophobicity analysis of GhRING2 protein

The hydrophobicity plot is represented with the Kyte-Doolittle scale. The horizontal axis (x-axis) represents the amino acid residues and the vertical axis (y-axis) indicates the degree of hydrophobicity of each respective residue.

Cotton (*Gossypium hirsutum*) is a disomic tetraploid with A- and D- genomes obtained from diploid ancestors. The *GhRING2* gene was amplified by PCR from both A2 (*G. arboreum*) and D5 (*G. raimondii*) genomes using the forward primer Ring1-P2 (5'- CGCGGA TCCATGCTGGTTTCTGTTTTAT-3') and reverse primer RING2-P3 (5'- CGCGAATT CTTAGCAAGGGTTATCATCACC-3'). Sequence analysis of the PCR products revealed that the *GhRING2* gene from A2- and D5- genomes has only two base pair difference in the nucleotide sequence, which changes amino acid residues of GhRING2 at two positions (Fig. 6). The aa residues at positions 41 and 110 in the A2-

genome encoded GhRING2 are Leucine and Valine, whereas in the D5-genome they are Serine and Isoleucine.

```

A-Genome,      1 MLVSVLLALFLPCAGMSAVFIVYICLLWYATNHRADNSGSLSVKQAGEKGLSVSELEKLP
D-Genome,      1 MLVSVLLALFLPCAGMSAVFIVYICLLWYATNHRADNSGSSSVKQAGEKGLSVSELEKLP
                *****
A-Genome,      61 KVTGKDLVLGTECAVCLDEIEAEQSARMVPGCNHGFHLQCADTWLSKNSVCPVCRAKLEP
D-Genome,      61 KVTGKDLVLGTECAVCLDEIEAEQSARMVPGCNHGFHLQCADTWLSKNSICPVCRAKLEP
                *****
A-Genome,      121 KLFDPGDDNPC
D-Genome,      121 KLFDPGDDNPC
                *****

```

Figure 6 Amino acid sequence alignment of GhRING2 proteins encoded by A- and D- genomes.

Amino acid residues differing between A- and D-genome encoded GhRING2 proteins are marked in red color. The RING- H2 motifs are underlined.

### Expression analysis of *GhRING2* gene

The transcript level of *GhRING2* in TM-1 and CS-B25 tissues was determined by Real-Time RT-PCR. The TM-1 and CS-B25 RNA samples isolated from leaf, flower, and fiber at different developmental stages (5, 10, 15, and 20 DPA) were used for synthesis of first strand cDNA using oligo(dT) primer and M-MLV reverse transcriptase (Heid et al. 1996; Livak and Schmittgen 2001). The first strand cDNA samples were then utilized as templates for Real-Time RT-PCR using two primers RT-R1 (5'-TTATGGTACGCCACGAATCA-3') and RT-R2 (5'-CCGATTGCTCTGCCTCTATC-3') to amplify a 178 bp cDNA fragment of the *GhRING2* gene. As a reference to analyze *GhRING2* expression, the cotton *polyubiquitin* cDNA, was amplified using two universal ubiquitin primers UBQ F (5' – GAGTCCTCAGACACCATTGACAAC - 3'), UBQ R (5'



– GTGCTCTCCTTCTGGATGTTGTAG - 3'). The Real Time RT-PCR results showed that the transcript levels of *GhRING2* increased at the late elongation and secondary wall synthesis stages (Fig. 7). The *GhRING2* mRNA reached a maximal level in 20-DPA fiber, and the transcript levels in TM1 were higher than those in CS-B25.

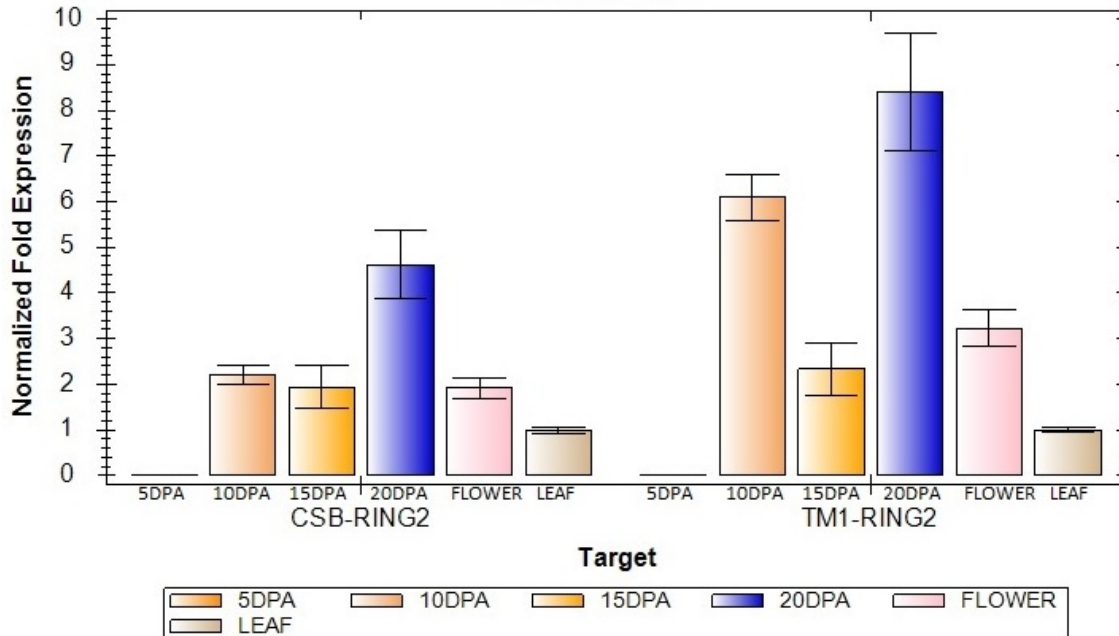


Figure 7 Comparison of the relative expression of *GhRING2* between CS-B25 and TM1 in different tissues.

The different tissues were leaf, flower and fiber at different developmental stages (5, 10, 15 and 20-DPA).

### In vitro auto-ubiquitination assay of GhRING2 E3 ligase activity

#### Purification of GST-GhRING2 fusion protein

The open reading frame (ORF) of *GhRING2* was cloned into the pGEX-4t-1 vector to express the GhRING2 protein in fuse with the glutathione S-transferase (GST) protein. The recombinant plasmid pGEX-4t-1 containing the *GhRING2* insert was transformed into *E. coli* BL21 (DE3) cells, and the expression of the GST-GhRING2

fusion protein in transformed BL21 (DE3) cells was induced by 1 mM IPTG. *E. coli* cells were harvested and lysed, and a 58 kDa GST-GhRING2 fusion protein was purified using GST-resin affinity chromatography (Fig. 8).

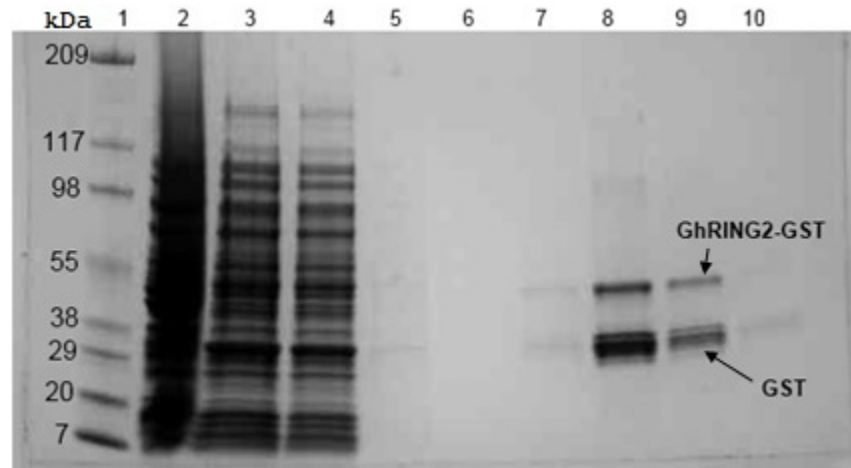


Figure 8 Purification of GhRING2-GST fusion protein using GST•Bind Resin from Novagen.

Lanes 1, 2, 3, 4, 5 and 6 are protein marker (band size represented in kDa), pellet, supernatant, flow through, wash 1 and wash 2, respectively; lanes 7, 8, 9 and 10 are elutions in order in four tubes.

### ***In vitro* auto-ubiquitination of GST-GhRING2 using wheat germ extract**

The ubiquitin E3 ligase activity of GhRING2 was determined by employing an *in vitro* auto-ubiquitination assay as described by Zhao et al. (2012). The GST-GhRING2 protein was mixed with wheat germ lysate, ATP, and ubiquitin and incubated at 37°C for 0-120 min. The reaction mixtures were then fractionated on a SDS-PAGE gel and probed using anti-GST antibody. The results of Western analysis showed that high molecule weight polyubiquitinated GhRING2 proteins were formed (Fig. 9), indicating that GhRING2 had ubiquitin E3 ligase activity (Zhao et al. 2012). The higher molecular

weight polyubiquitinated GhRING2 proteins were progressively accumulated with the increase in incubation time from 0-120 min (Fig. 9).

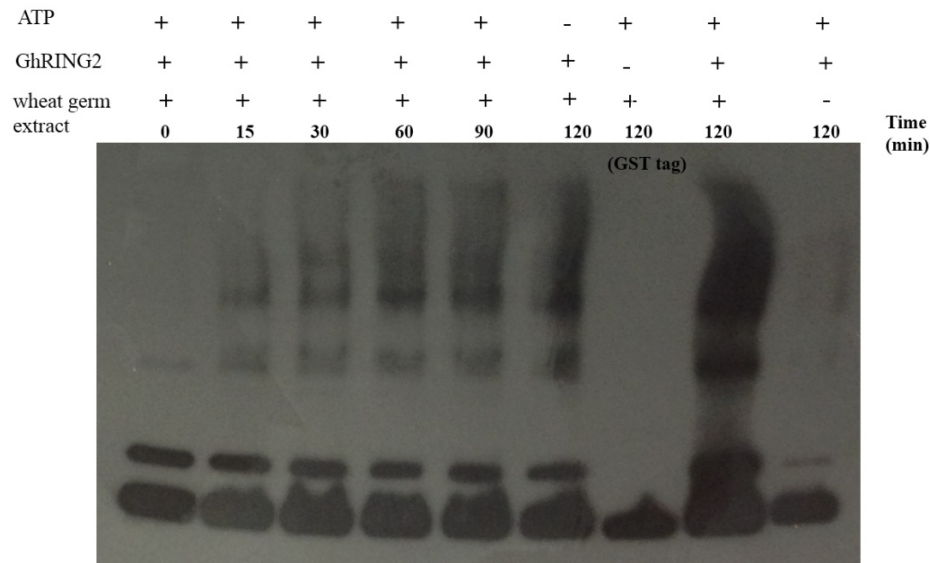


Figure 9 Western analysis of in vitro auto-ubiquitination of GST-GhRING2 using wheat germ extract

Anti-GST antibody (1 to 5000 dilutions) was used as the probe

### Identification of target protein of GhRING2

Ubiquitin E3 ligases have a critical role in ubiquitin-mediated proteolytic pathway by targeting specific proteins via protein-protein interaction for polyubiquitination (Jackson et al. 2000). A Yeast Two-hybrid assay (Legrain and Selig 2000) was used to identify the interacting target protein of the RING-type ligase GhRING2 as most of protein-protein interactions published to date were based on this assay (Zhuang et al. 2012). Identification of the target protein not only helps to gain more insight into GhRING2 function but also reveals the direction of the ubiquitin proteolytic pathway in cotton fiber cells.

In the Yeast Two-hybrid assay, target proteins were fused with either one of two domains of yeast transcriptional factor GAL4, activation domain (AD) and DNA binding domain (BD). These two domains were brought to close proximity when two fused target proteins interact and make GAL4 active (Fields and Song 1989). The interaction becomes evident as activated Gal4 turns on the transcription of four reporter genes, *AURI-C* (confers resistance to Aureobasidin A), *HIS3* (permits growth on –His minimal medium), *ADE2* (permits growth on -Ade minimal medium) and *MEL1* (encodes alpha-galactosidase) (Ito et al. 2001).

Y2H gold yeast cells containing pGBKT7/GhRING2 were used as bait to identify the target proteins of GhRING2 with a yeast mating method. The yeast two-hybrid library cells (haploid Y187 strain) were mated with bait cells (Y2H gold strains with pGBKT7/GhRING2). Before the mating haploid Y187 and Y2H Gold strains appear as single cells (Fig. 10A) and in budding form (Fig. 10B) under the microscope. After mating for 24 hr diploid zygotes with a 3-lobed structure were observed (Fig. 10C and 10D), indicating the occurrence of mating efficiency of the two haploid cells.

## Mating of yeast haploid Y187 and Y2H Gold cells

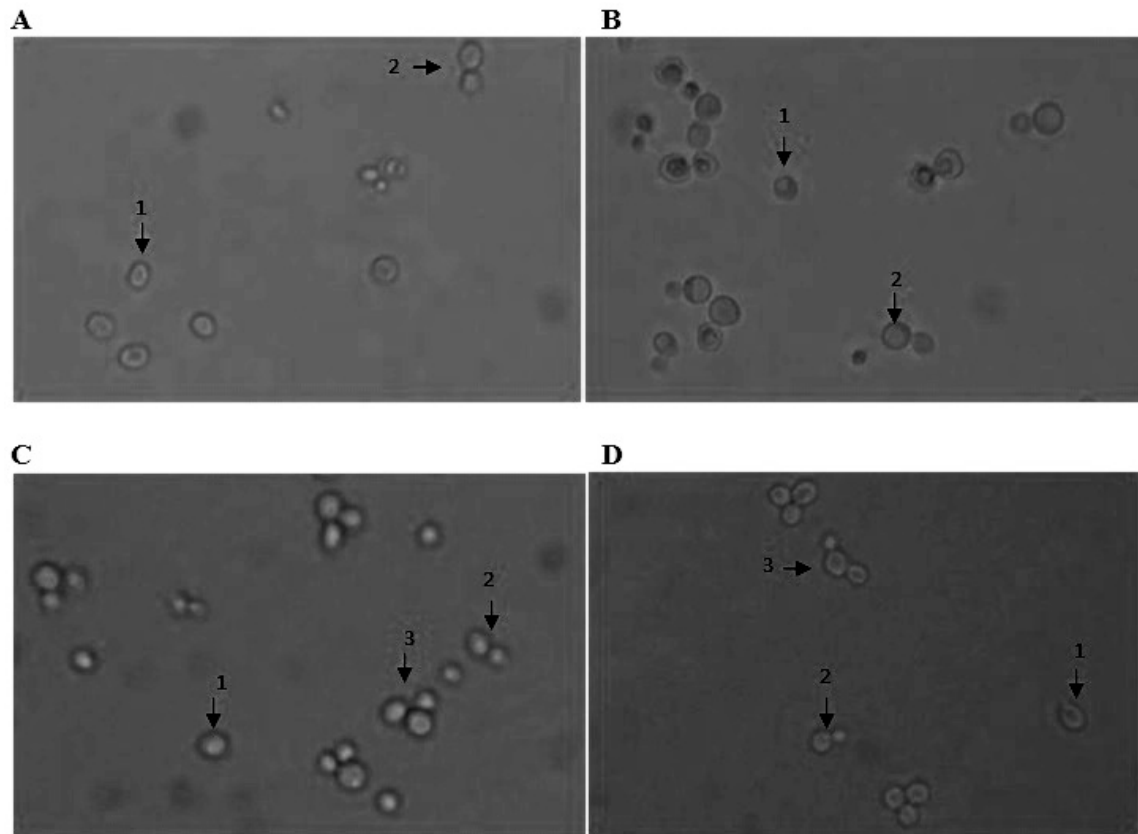


Figure 10 The formation of zygotes via mating of two opposite mating types of yeast haploid Y187 and Y2H Gold cells.

The images were captured using a Motic BA410 phase contrast microscope (40X). A and B represent the cells of Y187 and Y2H Gold strains, respectively and the numbers 1 and 2 in the images indicating the single and budding yeast cells. C and D represents the diploid zygotes (mating cells after 24 hr) of Y187 and Y2H Gold strains that form a 3-lobed structure indicated by the number 3.

A PROTODERMAL FACTOR1 (GhPDF1) protein and the 7- $\beta$ -like subunit of 20S protease were identified as the target proteins by screening a yeast two-hybrid fiber cDNA library using GhRING2 as bait. The *GhPDF-1* gene was subsequently amplified by PCR with primers GDFP-1 and GD-FP-2 (primer were designed from the GbPDF-1 gene sequence from *G. barbadense*) and sequenced. The nucleotide and derived amino

acid sequences of GhPDF-1 are shown in Fig. 11, and the *GhPDF-1* gene encodes a protein containing 494 amino acids.

→ GFP-1  
 ATGGTTAATATCCGATGCTCTAATGGTTCAAATTTACGGTTAGGACCAAACCTCGAATCA 60  
 M V N I R C S N G S K F T V R T K L E S 20  
 ACCATTGGATCTTTCAAAGTGTFTTAGCTCAGAGTTGCGATATCCCAGCTGATCAGCAA 120  
 T I G S F K V V L A Q S C D I P A D Q Q 40  
 CGATTGATTTATAAAGGCCGAATCTTAAAAGACGACCAAACCCTTCAAAGTTACGGTTTG 180  
 R L I Y K G R I L K D D Q T L Q S Y G L 60  
 CAAGCTGATCATACTGTTTCATATGGTCCGTAGTTTTGCCCATCTTCATCAGCCCTCCT 240  
 Q A D H T V H M V R S F A P S S S A P P 80  
 CCTGTTGCTACTACAAATGTGGAAC TCCTAATACTACACCAGGGTCACTCGTGGTGT 300  
 P V A T T N V G T P N T T P G V T R G V 100  
 GGTCTAATGAGGGTGTGGTCTGGGAGCATCAATTTCCCTGGACTTAATCTGCTTGGT 360  
 G S N E G A G L G A S I F P G L N L L G 120  
 GGTAACGGAGGTAGTTTTGGTTTGTGGATCAGGACTTCCTGAATTTGAACAAGTACAG 420  
 G N G G S F G L F G S G L P E F E Q V Q 140  
 CAACAATACTCAAATCCCAACATGATGAGGGAAATAATGAATACACCTGCTATTCAA 480  
 Q Q L T Q N P N M M R E I M N T P A I Q 160  
 AGTTTGGTGAATAACCCAGAGTTAATGCGCACTTTGATTATGAGCAATCCCCTAATGCGT 540  
 S L V N N P E L M R T L I M S N P Q M R 180  
 GAGATTATGACCGAATCCAGAGCTTGGGCATATACTTAATGATCCTAGCATTCTGCGA 600  
 E I I D R N P E L G H I L N D P S I L R 200  
 CAGACTTTGGAAGCTGCTAGGAATCCGAACTCATGCGTGAAATGATGCGAAACACTGAC 660  
 Q T L E A R N P E L M R E M M R N T D 220  
 AGGGCTATGAGTAAATTGAATCCTC TCCTGAGGGATTTAACA TGCTCAGGCGTATGTAT 720  
 R A M S N I E S S P E G F N M L R R M Y 240  
 GAAAATGTT CAGGAGCCATT TTTGAA TGCTACA ACTATGGCTG GAAATAATG GAAACT 780  
 E N V Q E P F L N A T T M A G N N G N T 260  
 CCAGTTCAAACCCATTGCGAGCTCTTTGGGCAATCAAGGCGTTCTCAAGGTGTAACC 840  
 P G S N P F A A L L G N Q G G S Q G V T 280  
 CAGACCAATACTACTGCGAGGTCAAATCCTGCTGGGGATGCTAGGACACCAGGTATTGGT 900  
 Q T N T T A R S N P A G D A R T P G I G 300  
 GGTCTGGGAGGTCCCGGGCTCCAGACATGCCACCCATGTTGAATGGAATGCCAGATGCT 960  
 G L G G P G L P D M P P M L N G M P D A 320  
 TCTCAGTTGACTCAATTGTTGCAAAA TCCAGCTATATCACAGATGATGCAGAGCATAATG 1020  
 S Q L T Q L L Q N P A I S Q M M Q S I M 340  
 TCTAATCCCAATAATGAATCAGAT TATGAACCTCAACCCCAACTTCGTGAAATGTTT 1080  
 S N P Q Y M N Q I M N L N P Q L R E M F 360  
 GATTTGAATCCTCAATTGAGAGAAATGATGCAAAACCAGAAGTACTTCGTGCTGTTGTTT 1140  
 D L N P Q L R E M M Q N P E V L R Q L F 380  
 TCCCCTGATACAATGCAGCAAATGCTATCTTTACAGCAGTCTCTTATGTCTCAGCTCAAT 1200  
 S P D T M Q Q M L S L Q Q S L M S Q L N 400  
 CGACAGCAAACATCCAAAGATTCAACACAGACAAGTGGTACTACAGGAGCACCCCTACT 1260  
 R Q Q T S Q D S T Q T S G T T G A P P T 420  
 GCTAGTCTGGATTTGTTGATGAATATGTTTGGTGGTCTAGGTGCTGGCGCCTCAGTGT 1320  
 A S L D L L M N M F G G L G A G G L S V 440  
 CCCAACCAACCTGATGTTCCCCAGAGAAGTGTATGCCACGCAATTATCACAACTCCAA 1380  
 P N Q P D V P P E E L Y A T Q L S Q L Q 460  
 GAAATGGGCTTCTATGATACACAAGAGAATA TCAGAGCACTTCGTGCTACTGCGGGAAAC 1440  
 E M G F Y D T Q E N I R A L R A T A G N 480  
 GTCCATGCTGCAGTTGAGAGACTCCTGGGAAATTCTGGGCAGTAA ← GFP-2 1485  
 V H A A V E R L L G N S G Q - 494

Figure 11 Nucleotide and derived amino acid sequences of cotton *GhPDF1* Gene.

The primers GFP-1 and GFP-2 were used to amplify the *GhPDF1* ORF for GhPDF1 MBP fusion protein expression



## Testing interaction of GhRING2 and GhPDF1

The interaction between GhRING2 and GhPDF1 was further confirmed with the yeast two-hybrid system by the swap of the two plasmids pGADT7 and pGBKT7 to clone *GhRING2* and *GhPDF1*. Transformed Yeast Y2H Gold cells containing pGBKT7/*GhPDF1* and pGADT7/*GhRING2* plasmids (Fig. 12 section 2) were able to grow on the quadruple dropout synthetic medium plates (Fig. 12B and C) and express the alpha galactosidase activity (Fig. 12C).

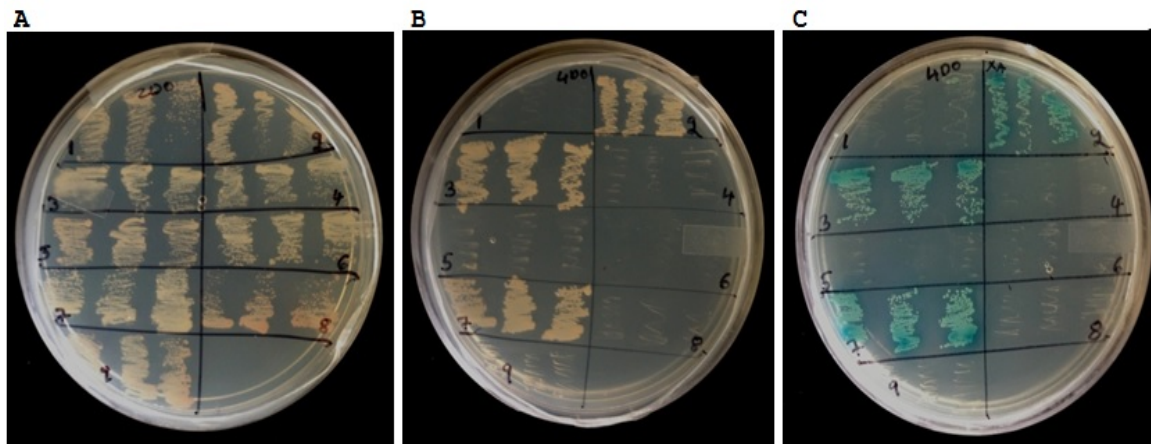


Figure 12 Testing interaction of GhRING2 and GhPDF1.

Yeast Y2H Gold cells were co-transformed with (1) pGADT7-GhRING2/pGBKT7, (2) pGADT7-GhRING2/ pGBKT7-GhPDF1, (3) pGADT7-GhPDF1/pGBKT7-GhRING2, (4) pGADT7-GhPDF1/pGBKT7, (5) pGADT7/ pGBKT7-GhRING2, (6) pGADT7/ pGBKT7-GhPDF1, (7) pGADT7-T / pGBKT7-53, (8) pGADT7-GhRING2 / pGBKT7-53 and (9) pGADT7 / pGBKT7. A, B, and C represent plates containing double drop out (-Trp and - Leu), quadruple drop out (-Trp, - Leu, -His and -Ade), and quadruple drop out with alpha-gal minimal synthetic defined media, respectively. The blue color in C represents alpha-galactosidase activity which degrades the substrate alpha-gal into blue product.



### **Purification of MBP and MBP-GhPDF1 fusion protein**

The open reading frame (ORF) of *GhPDF1* was cloned into the pMAL-c5X vector to express the GhPDF1 protein in fuse with maltose-binding protein (MBP). The recombinant plasmid pMAL-c5X containing the *GhPDF1* insert was transformed into *E. coli* BL21 (DE3) cells. The transformed BL21 (DE) cells were induced for the expression of MBP-GhPDF1 fusion protein by 0.3 mM IPTG. The MBP-GhPDF1 fusion protein was purified from *E. coli* lysates using amylose affinity chromatography (amylose resin from New England BioLabs). Similarly, the pMAL-c5X vector without the insert was also used for expression and purification of the MBP protein (Cao et al. 2003). The purified MBP and MBP-GhPDF1 proteins were analyzed by SDS-PAGE and are shown in Fig. 13A and 13B, respectively. Four distinct protein bands were observed in the purified MBP-GhPDF1 fusion protein (Fig. 13B), and the highest molecular weight band (top band) represents the intact MBP-GhPDF1 fusion protein (107 kDa) and the lowest molecular weight band is MBP (52 kDa). The two middle bands between MBP and MBP-GhPDF1 (Fig. 13B) may represent partially degraded MBP-GhPDF1 fusion proteins. The presence of MBP and MBP-GhPDF1 proteins were further confirmed by Western analysis using anti-MBP rabbit antiserum (New England BioLabs) antibody as the probe (Fig. 14).

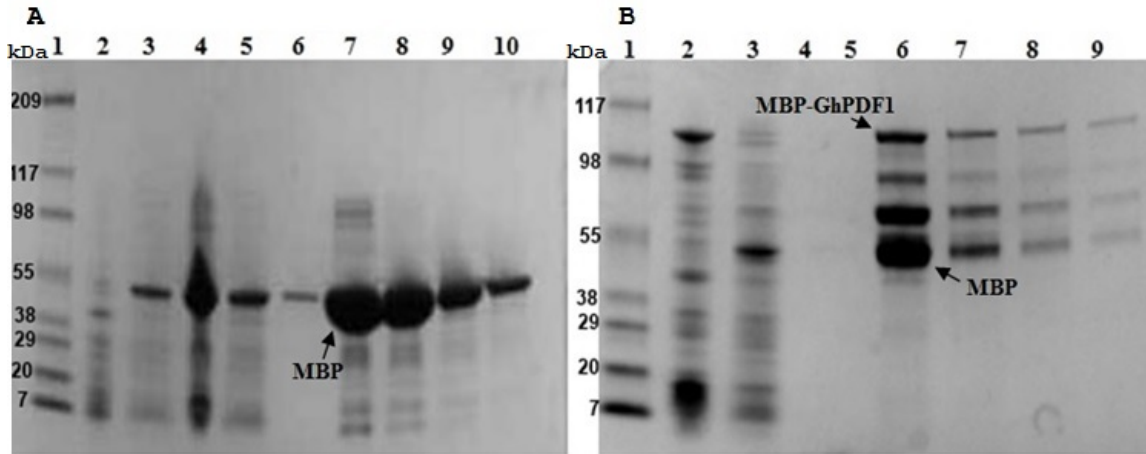


Figure 13 Analysis of purified MBP (A) and MBP-GhPDF1 (B) fusion proteins by SDS-PAGE.

Lanes 1, 2, 3, 4, 5 and 6 in A are protein marker (individual band represented in kDa), pellet, supernatant, flow through, wash 1 and wash 2, respectively; lanes 7, 8, 9 and 10 are MBP elutions in four tubes. Lanes 1, 2, 3, 4 and 5 in B represent protein marker (individual band represented in kDa), pellet, supernatant, wash 1 and wash 2, respectively; lanes 7, 8, 9 and 10 are MBP-GhPDF1 elutions in four tubes.

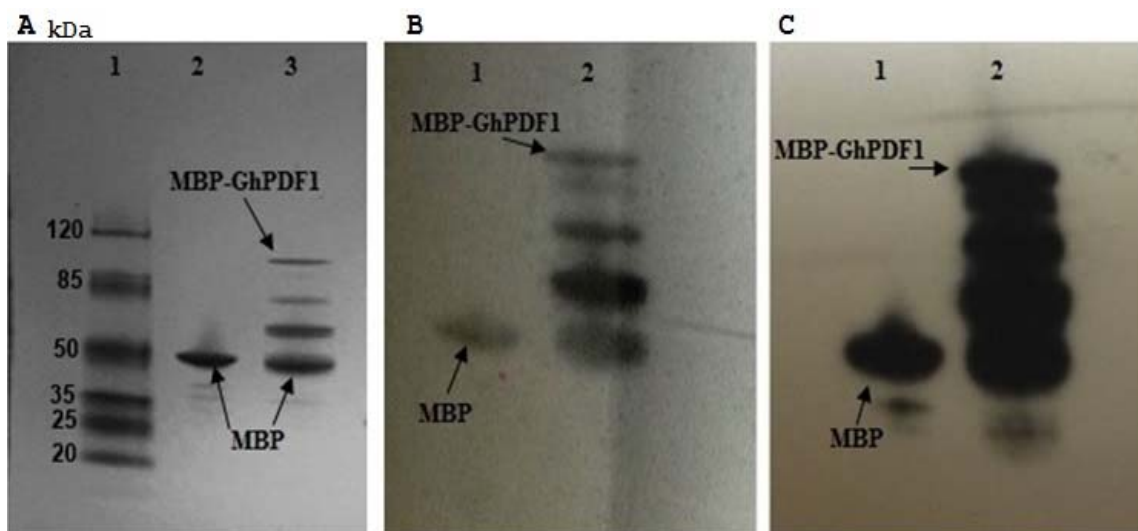


Figure 14 Western analysis of MBP and GhPDF1-MBP fusion proteins.

The MBP and MBP-GhPDF1 proteins were separated by SDS-PAGE and subjected to Western analysis using anti-MBP rabbit antiserum antibody (1:1000 dilutions, New England Biolabs) as the probe. Image A shows results of SDS-PAGE analysis, and lanes 1, 2 and 3 are protein marker (band size represented in kDa), MBP (52 kDa) and GhPDF1-MBP fusion protein (107 kDa), respectively. Images B and C represent Western blotting results with 10 sec and 1 min exposure time, respectively. Lanes 1 and 2 in images B and C are MBP and GhPDF1-MBP fusion proteins.

### Pull Down Assays

The *in vitro* pull down assays were performed to confirm the protein-protein interaction between GhRING2 and GhPDF1. GST-tag and GST-GhRING2 fusion proteins pre-bound to Glutathione Sepharose 4 Fast Flow resin (Amersham Biosciences) was individually incubated with MBP and GhPDF1-MBP fusion protein at 4°C for two hr. The resin-bound GST-tag incubated with GhPDF1-MBP fusion was served as a negative control. The resin bound GST-GhRING2 incubated with the GhPDF1-MBP fusion protein was used to demonstrate protein-protein interaction between GhRING2 and GhPDF1 proteins.

The pull down assays via Western (Fig. 16) and SDS-PAGE (Fig. 15) analyses clearly demonstrated that GhRING2 interacted with GhPDF1. The co-purification of MBP-GhPDF1 fusion protein with GST-GhRING2 fusion protein was observed in the elution from the resin bound GST-GhRING2 incubated with GhPDF1-MBP, but not the resin bound GST-tag incubated with GhPDF1-MBP. The enrichment of the intact MBP-GhPDF1 fusion protein (highest molecular weight band) in the second elution (Fig. 15, lane 9) indicated that the intact GhPDF1 fusion protein was more efficient in binding to GhRING2 than partially degraded GhPDF1-MBP proteins (two smaller protein bands).

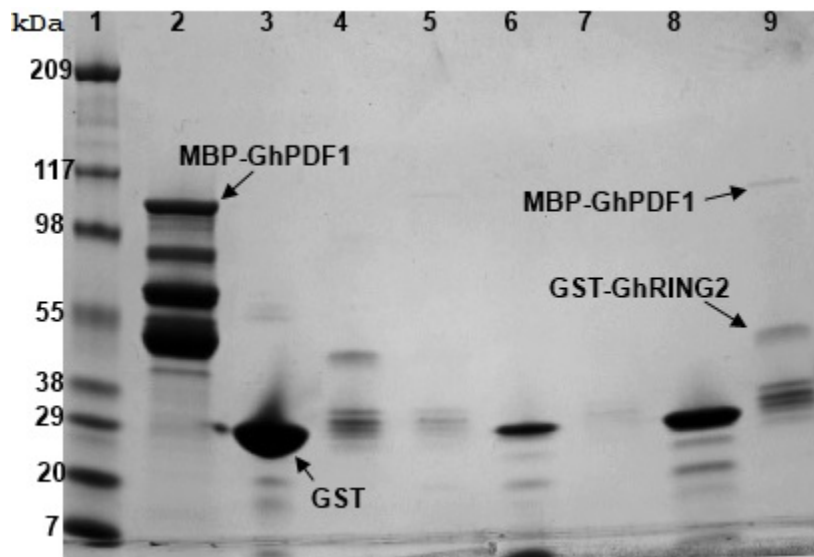


Figure 15 Testing of interaction between GhRING2 and GhPDF1 by pull down assays.

Lanes 1, 2, 3 and 4 contain protein marker (band size represented in kDa), purified MBP-GhPDF1 fusion protein, GST-tag and GST-GhRING2 fusion protein, respectively; lanes 5, 9 and 7 are three elutions in order from the resin bound GST-GhRING2 incubated with GhPDF1-MBP; and lanes 6 and 8 are two elutions from the resin bound GST-tag incubated with GhPDF1-MBP.

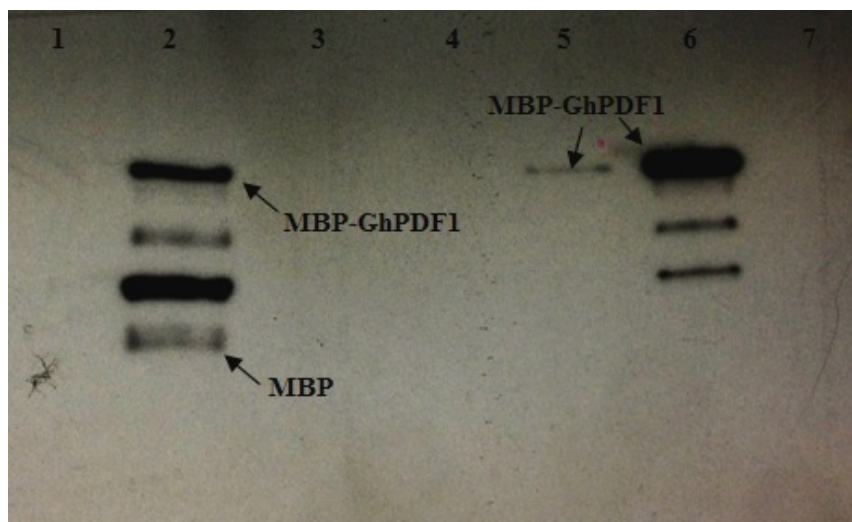


Figure 16 Western analysis of the MBP-GhPDF1 elution protein.

Anti-MBP rabbit antiserum (New England Biolabs) antibody (1:1000 dilution) was used as the probe. Lanes 1 and 2 are purified GST-GhRING2 and MBP-GhPDF1 fusion proteins, respectively; lanes 3 and 4 are two elutions from the resin bound GST-tag incubated with GhPDF1-MBP; lanes 5, 6 and 7 are three elutions in order from the resin bound GST-GhRING2 incubated with GhPDF1-MBP.

### Promoter analysis of *GhRING2*

The promoter of a gene has a critical role in regulating the expression of the gene. A GUS reporter system was used for analysis of the promoter of the *GhRING2* gene via constructing transgenic *Arabidopsis* plants. The GUS system allows detection of the tissue specific localized expression of the reporter beta-glucuronidase under the control of the *GhRING2* promoter. The full length and two 5' truncated promoter fragments R2GUS1, R2GUS2 and R2GUS3 were amplified using GUS F1/GUS R2, GUS F2/GUS R2 and GUS F3/GUS R2 primers, respectively, and cloned into pBI121 at the upstream region of the GUS reporter gene (Fig. 17). Transgenic *Arabidopsis thaliana* containing full length and 5' truncated *GhRING2* promoter fragments (Fig. 18) were generated and used for GUS assays by following the protocol of (Jefferson 1987).

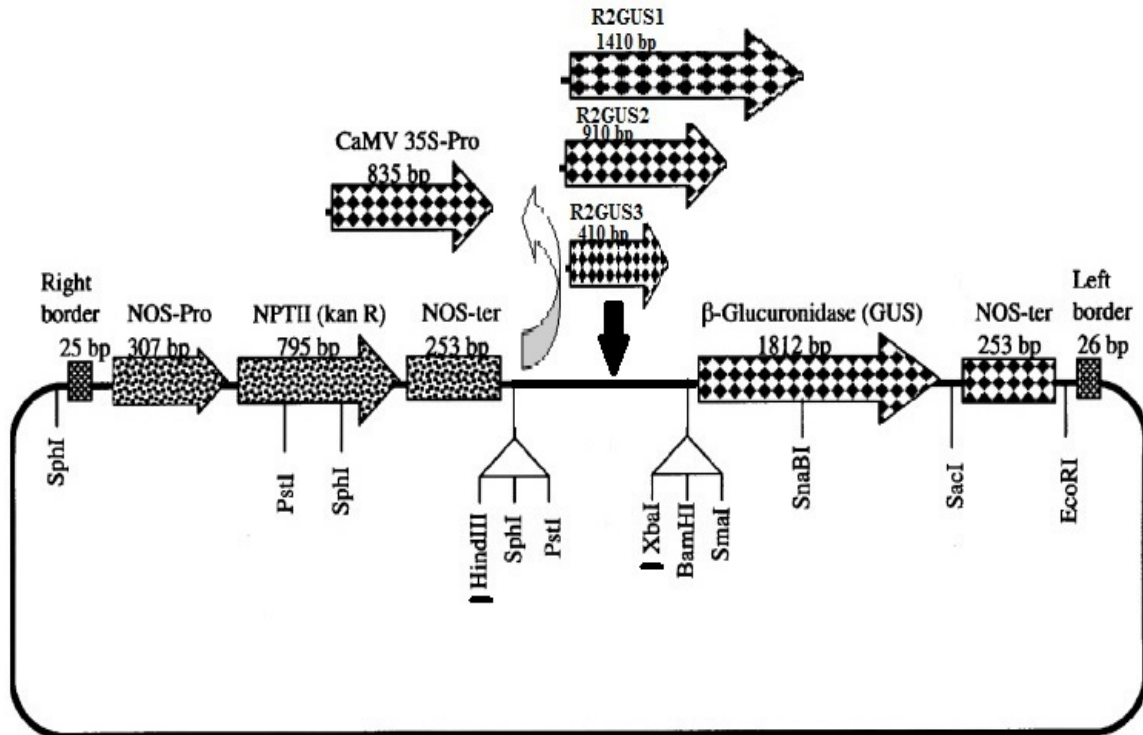


Figure 17 Map of the binary vector pBI121.

The recombinant pBI121-R2GUS1, pBI121-R2GUS2 and pBI121-R2GUS3 vectors were constructed by replacing the cauliflower mosaic virus (CaMV) 35S promoter (835 bp) from pBI121 with restriction enzymes *HindIII* and *XbaI*. NOS-pro and NOS-ter are the promoter and terminator regions of nopaline synthase gene of T-DNA. Neomycin phosphotransferase II (NPT II) gene confers the kanamycin resistance. Right (25 bp) and left (26 bp) borders of the Ti plasmid are indicated.



Figure 18 Generation of transgenic *Arabidopsis thaliana* plants containing the *GhRING2* promoter.

*A. thaliana* seedlings having green leaves and long roots (represented by arrows) represent transformed plants that are resistance to kanamycin (Harrison et al. 2006).

Gus expression was not detected in the leaf tissue of transgenic *Arabidopsis thaliana* containing the full length promoter R2GUS1 (Fig. 19A) and 5' truncated promoter fragments R2GUS2 and R2GUS3 (Fig 19B and 19D).



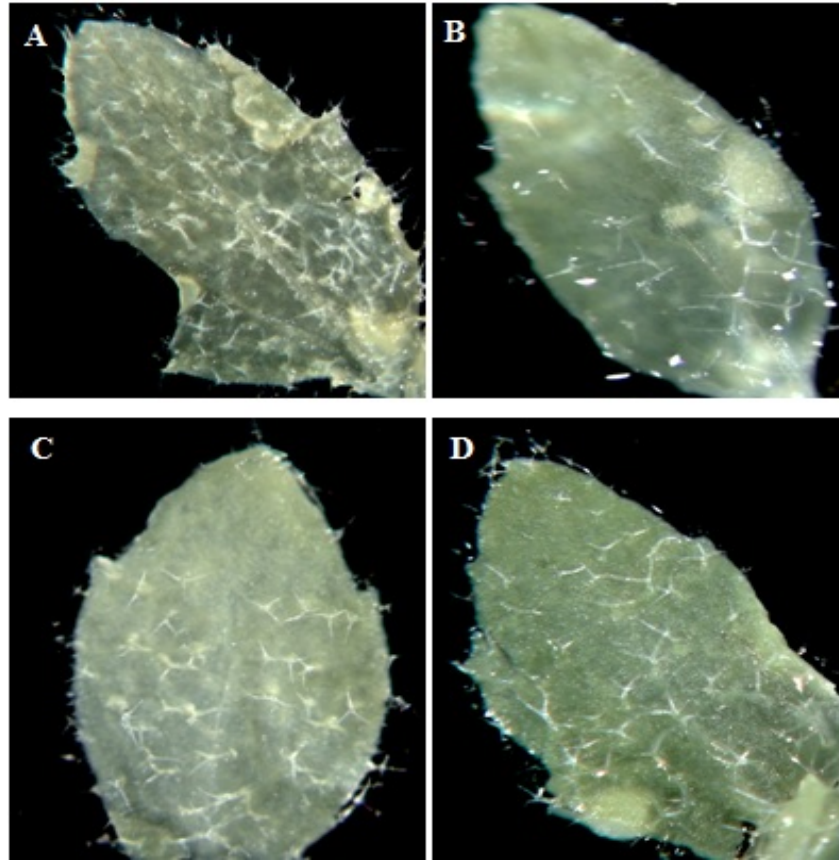


Figure 19 Histochemical analysis of *GhRING2* promoter driven GUS expression in leaves of transgenic and wild type *Arabidopsis thaliana*.

Images A, B and C represent a leaf from transgenic *Arabidopsis thaliana* plants containing the full length and 5' truncated promoter fragments R2GUS1, R2GUS2 and R2GUS3, respectively. Image D represents the leaf of wild type *Arabidopsis thaliana* (Columbia (Col-0)).

The GUS expression, in contrast, was detected in the hypocotyls of transgenic *Arabidopsis thaliana* seedlings containing the promoter fragments R2GUS1 (Fig. 20A and 20B) and R2GUS2 (Fig. 20C and 20D). No GUS activity, however, was detected in transgenic seedlings containing the short promoter fragment R2GUS3 (Fig. 20G and 20H). GUS expression was also not detected in the roots of transgenic seedlings containing the full length and 5' truncated promoter fragments (Fig. 20C, 20E, 20F). These observations suggest that the *GhRING2* promoter regions (R2GUS1 and R2GUS2)



from -607 to -1506 nt are crucial for GUS expression during hypocotyl development. Because of rapid elongation of cells in hypocotyl tissue, the *GhRING2* gene may play a functional role in cotton fiber cell elongation.

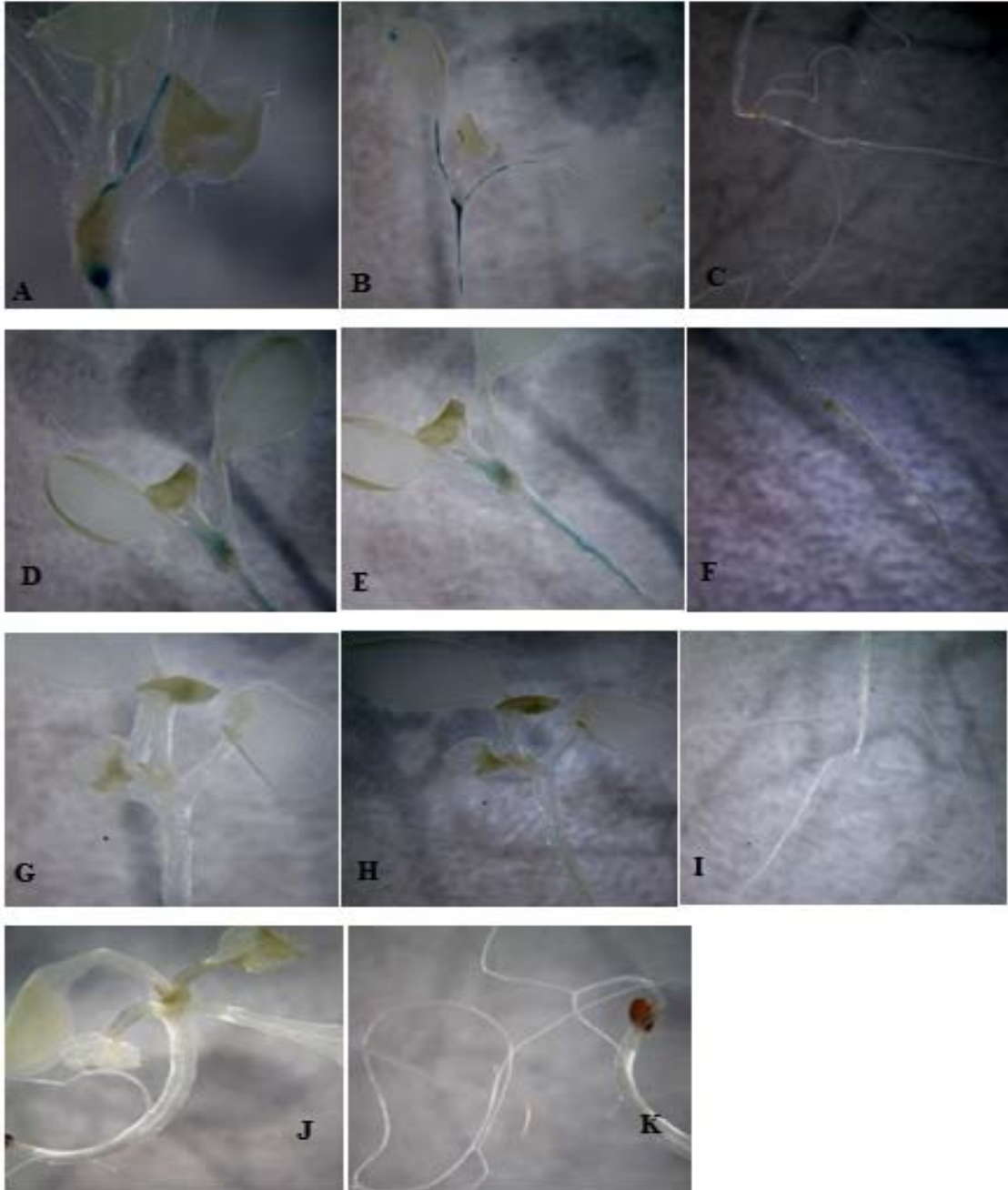


Figure 20 Histochemical Analysis of *GhRING2* promoter driven GUS expression in 12 day old seedlings of transgenic and wild type *Arabidopsis thaliana*.

The images A, B, C; D, E, F and G, H and I represent transgenic *Arabidopsis* plants containing the promoter fragments R2GUS1, R2GUS2 and R2GUS3, respectively. Images J and K represent wild type *Arabidopsis thaliana* seedlings. GUS activity was not detected in transgenic *Arabidopsis* seedlings containing the R2GUS3 promoter fragment (G, H, and J). No GUS expression was observed in the root tissue of seedlings with all three promoter fragments (C, F, and I).

The GUS activity was also detected in the young stem of five week old transgenic *Arabidopsis* plants containing the full length and 5' truncated promoter fragments R2GUS1 (Fig. 21A), R2GUS2 (Fig. 21B) and R2GUS3 (Fig. 21C), respectively. As the negative control no GUS activity was detected in the stem of the wild type *Arabidopsis thaliana* plant (Fig. 21D).

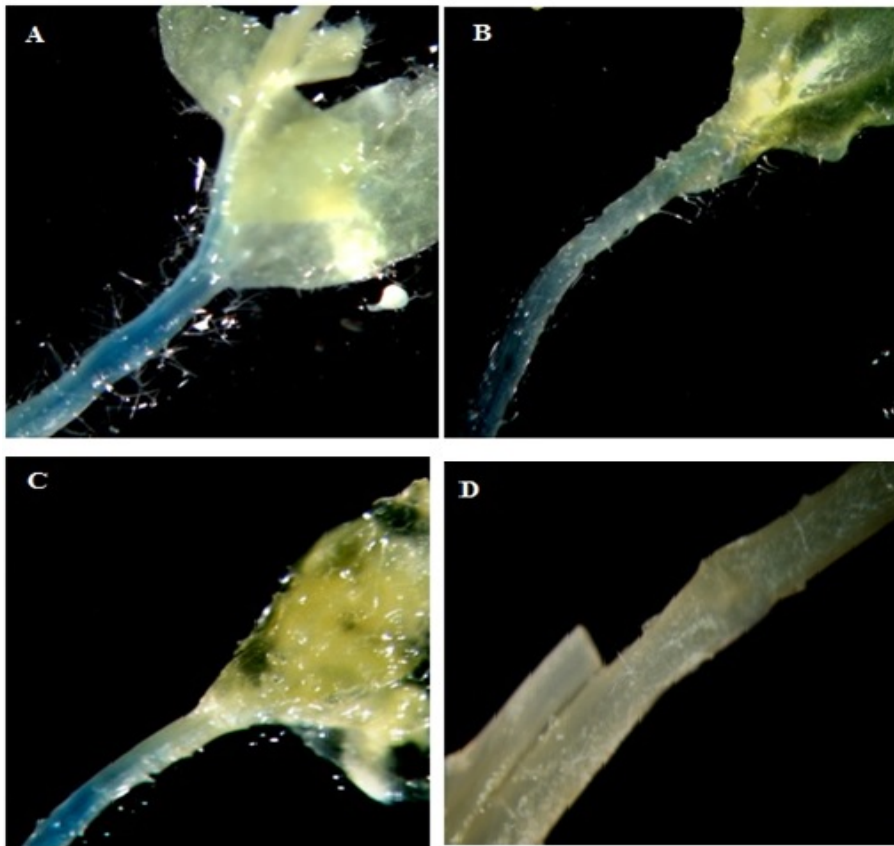


Figure 21 Histochemical analysis of *GhRING2* promoter driven GUS expression in the young stem of transgenic and wild type *Arabidopsis thaliana* plants.

Images A, B and C represents young stems of transgenic *Arabidopsis thaliana* plants containing the promoter fragments R2GUS1, R2GUS2 and R2GUS3, respectively. Images D represent the young stem of the wild type *Arabidopsis thaliana*.

The cross section of young and mature stems of five week old transgenic *Arabidopsis thaliana* plants containing the full length and 5' truncated promoter fragments R2GUS1, R2GUS2 and R2GUS3 were then analyzed to determine the localization of GUS activity. It was observed that GUS activity was localized to xylem and cambium cells of young stems (Fig. 22A-C) but not in mature stems (Fig. 22D and 22E) of transgenic *Arabidopsis thaliana* plants.

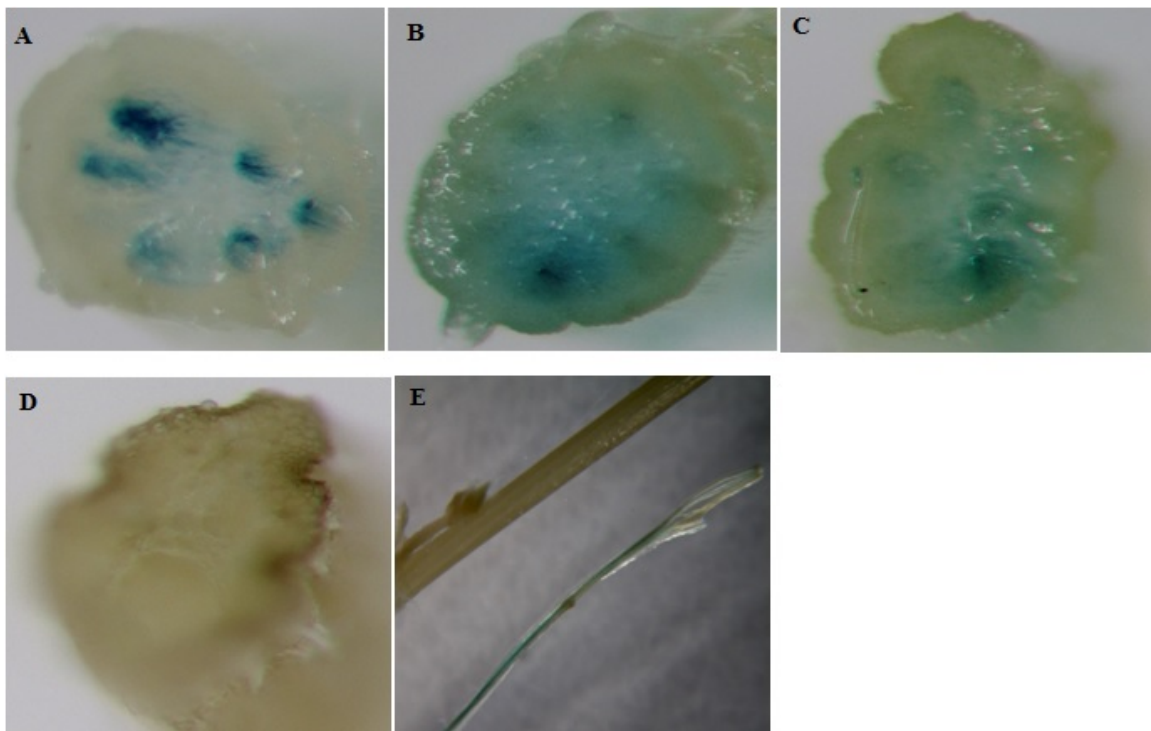


Figure 22 Cross section of young and mature stems of transgenic *Arabidopsis thaliana* showing GUS activity driven by *GhRING2* promoter fragments.

Images A, B and C represent the cross-section of young stems of transgenic *Arabidopsis thaliana* plants containing the promoter fragments R2GUS1, R2GUS2 and R2GUS3, respectively. Image D represents the cross-section of the mature stem with the plants containing the R2GUS1 promoter fragment. GUS expression was only detected in the young stem (image E at bottom) but not in mature stems (image E on top) of transgenic *Arabidopsis* plants containing the RGUS1 promoter fragment.

## CHAPTER IV

### DISCUSSION

Cotton fiber development has four overlapping stages and the development is governed by a large number of genes. The timing and duration of each stage influence the fiber characteristics, like length and strength of the fiber. Expressed sequence tag (EST) and microarray analyses have been used to identify the genes that are preferentially expressed in a stage specific manner using wild type and lintless or reduced fiber lines (Al-Ghazi et al. 2009; Albornos et al. 2012; Haigler et al. 2009; Hinchliffe et al. 2010; Hovav et al. 2008; Jackson et al. 2000; Ji et al. 2003; Lee et al. 2007; Li et al. 2002; Li et al. 2013; Taliercio and Boykin 2007; Udall et al. 2006; Wu et al. 2007; Wu et al. 2006). Cotton fiber is single cell and is a good model to study cell elongation, cell wall formation and cellulose synthesis (Basra and Malik 1984).

The ubiquitin proteasome proteolytic (UPP) pathway plays an important role in all aspects of plant growth and development. The components of this pathway represent more than 5% of the total proteome in plants, which is more than double in yeast, *Drosophila*, mice, and human. Arabidopsis genome analysis revealed that there are more than 1300 genes encoding putative E3 subunits (Smalle and Vierstra 2004). In the recent years, considerable amount of research had been done to study the UPP pathway in plant growth and development. Understanding this pathway in economically important crops like cotton is crucial.

The regulation of cotton fiber growth and development via protein ubiquitination was first suggested by Zhang et al. (2003). They isolated and characterized two cotton E2 genes, *GhUBC1* and *GhUBC2*, which were expressed in fibers in a developmentally regulated manner. The *GhUBC1/2* transcript levels in fibers reach the peak at 10 DPA. Ho et al. (2010) cloned one fiber cDNA, named *GhRING1*, encoding a RING-type E3 ubiquitin ligase. Real-time RT-PCR analysis revealed that *GhRING1* is differentially expressed in fiber cells; with the highest transcript level in the elongation stage at 15 DPA (Ho et al. 2010). The expressed GhRING1 was found to have the ubiquitin E3 ligase activity (Ho et al. 2010) via an in vitro reconstitution assay. These observations suggest that protein ubiquitination plays a role in fiber differentiation and development. This suggestion is further supported by a report (Hovav et al. 2008) that 600 genes encoding components of the ubiquitin/proteasome protein complex are up-regulated at the early fiber elongation step. Another global gene expression study (Al-Ghazi et al. 2009) has also shown that many genes (encode the subunits of proteasome, E2, SCF F-box proteins, and RING-type ubiquitin E3 ligases) involved in the ubiquitin-mediated protein degradation pathway are highly up-regulated in fiber at the elongation and the secondary cell wall synthesis stages. Han et al. (2012) reported the importance of the ubiquitin mediated protein degradation pathway via hormone (auxin) signaling in cotton fiber development. They proposed that Auxin could modulate the interaction of SCF<sup>TIR1</sup> (an ubiquitin E3 ligase) complex with Aux/IAA proteins, and resulting in down regulation of Aux/IAA proteins via ubiquitin mediated protein degradation and release of auxin response factors (ARFs). The activated ARFs subsequently bind to Auxin-responsive cis elements (AuxREs) (Han et al. 2012).

Identification and characterization of cotton ubiquitin E3 ligases will provide an insight into the ubiquitin proteasome proteolytic pathway and its functional role in cotton fiber development. Cotton (*G. hirsutum*) is a disomic tetraploid with A- and D- genomes derived from diploid ancestors. The initially cloned *GhRING2* gene was from the A-2 genome. The amino acid sequence alignment of GhRING2 from A-2 and D-5 genomes revealed that there are only two amino acid changes at aa positions 41 and 110. The amino acid at position 110 changed from valine in A-2 to isoleucine in D-5. Since both valine and isoleucine are hydrophobic aa they probably wouldn't affect the E3 ligase activity even they are located in the RING-H2 domain. The amino acid at position 41, however, changed from a hydrophobic leucine into a polar serine. This change also might not have a significant effect on the E3 activity as it is not located in the RING-H2 motif region.

The expression level of *GhRING2* in the leaf tissue of TM1 and CS-B25 was almost identical. In contrast, *GhRING2* was expressed in higher levels in the flower of TM-1 than CS-B25. Based on the *GhRING2* expression pattern in TM-1, it is proposed that *GhRING2* expression in cotton fiber had two phases, with maximal expression at 10 and 20 DPA. It suggests that *GhRING2* expression was possibly regulated by at least two transcriptional factors. These observations clearly demonstrate that the *GhRING2* is involved in late elongation and secondary wall synthesis stages.

The length of cotton fiber depends on the rate and duration of elongation stage which in turn depends on the number of days before elongation ceases (Applequist et al. 2001). Lower expression of *GhRING2* in CS-B25 than TM-1 at 10 and 20 DPA might reduce the protein that positively regulates the elongation and secondary wall synthesis

stages. Therefore, the CS-B25 line might have longer duration in these stages and subsequently resulted in high fiber length and strength. This hypothesis based on the expression data strongly agrees with the empirical data reported by Saha et al. (2004). Similar results were reported by Li et al. (2013) who performed RT-PCR analysis of the QTL primers that are preferentially expressed between *G. hirsutum* Emian22 and *G. barbadense* 3-79 during fiber development. Expression of *FPG012* primer, tightly linked with a QTL marker, reached a peak at 20 DPA in Emian22, while the expression peak appeared at 25 DPA in 3-79. Based on the expression data between the two lines, Li et al. (2013) suggested that the delay in gene expression may be responsible for the high fiber strength of 3-79 than Emian 22.

The protein-protein interaction between an ubiquitin E3 ligase and its target substrate determines the specificity (Jackson et al. 2000). Identification of the substrate therefore is crucial and helps to gain more insight into the function of the ubiquitin E3 ligase (Zhuang et al. 2012). The identification of the GhRING2 target, the PROTODERMAL FACTOR1 protein and 7- $\beta$ -like subunit of 20S protease, will help us understand the function of GhRING2 in the ubiquitin proteasome proteolytic pathway and its role in cotton fiber development.

Lee et al. (2006) carried out expression profiling studies on TM-1 and naked seed mutant (N1N1) cotton lines. They reported that 117 and 30 genes were expressed differentially in ovules at 0 DPA and 3 DPA in TM1 and N1N1. *GhPDF1* was reported to be one of the patterning genes and its expression reached the peak at 5 dpa (Lee et al. 2006). Tu et al. (2007) also showed that *GbPDF1*, a GhPDF1 homologue from *G. barbadense*, was involved in cell fate determination. Recently, the *GbPDF1* gene has



been cloned and characterized by Deng et al. (2012) who revealed the functional role of *GbPDF1* in fiber cell initiation process. The identification of the interaction between *GhRING2* and *GhPDF1* via the yeast two-hybrid and pull down assays in this study further support that GhRING2 regulates fiber cell initiation and fiber length by targeting GhPDF1 (Deng et al. 2012).

The GhRING2 protein interacts directly with 20S protease via the 7-beta like subunit. This interaction allows the GhRING2 ligase to direct its substrates that are ubiquitinated to the 20S protease. The same mechanism was previously reported by Löscher et al. (2005) who studied the mechanism underlying the transportation of ubiquitinated targets to the proteasome. They proposed that the ubiquitin E3 ligase SNEV (senescence evasion factor) in mice was involved in escorting the ubiquitinated proteins to the 20S protease via binding to the beta 7 subunit (Loscher et al. 2005). This mechanism was also used in yeast where the ubiquitin E3 ligase Prp19 interacted directly with 20S protease via a 7-beta like subunit. These observations indicate that this mechanism is evolutionarily conserved. In contrast, ubiquitinated targets are also recognized by the proteasome through the polyubiquitin moiety (Hochstrasser 1996).

The promoter region of the gene governs its expression temporally in specific tissues. The *E. coli* GUS ( $\beta$ -glucuronidase) reporter system has been one of the best methods to determine the promoter activity of the gene. The development of transgenic cotton plants for analysis of the *GhRING2* promoter is difficult because cotton shows very low efficiency of transformation. In addition, relatively long time is required for regeneration and growth of cotton plants. Transgenic *A. thaliana* plants harboring recombinant PBI121 were therefore generated via *Agrobacterium tumefaciens* mediated

transformation and the promoter activity of *GhRING2* was subsequently monitored using the histochemical GUS assay (Sessions et al. 1999).

Promoter analyses indicated that GUS was expressed in the hypocotyl and apical ends of the cotyledons in transgenic *Arabidopsis* seedlings, suggesting that the *GhRING2* promoter region (R2GUS1 and R2GUS2) located from -607 to -1506 nt is crucial for GUS expression in the seedling. Albornos et al. (2012) reported that *Arabidopsis AtBGAL12* ( $\beta$ -galactosidase gene) promoter directed GUS expression in the root, hypocotyl and apical end of the cotyledons of transgenic *Arabidopsis* plants. They proposed that *AtBGAL12* participated together with expansins in cell wall extension or remodelling (Albornos et al. 2012). Unlike the *AtBGAL12* promoter, the GUS expression was not detected in the roots of transgenic seedlings containing the full length and 5' truncated promoter fragments of *GhRING2*. Based on these observations, it is proposed that at least one transcriptional factor binding site is present between -607 to -1506 nt of the *GhRING2* promoter region. The hypocotyl cells with GUS activity in transgenic *Arabidopsis* seedlings may correspond to rapidly elongating cells, and the apical end cells of the cotyledons may represent cotyledons forming cells (Albornos et al. 2012). *GhRING2* may be involved in cell wall remodeling and coordination with expansins, and may play a functional role in cotton fiber cell elongation.

The GUS activity was observed in young stems of transgenic *Arabidopsis thaliana* plants for all three *GhRING2* promoter fragments which conflicts with the previous observation that the GUS activity was detected in transgenic *Arabidopsis* seedlings only containing R2GUS1 and R2GUS2 promoter fragments. This may be due to the fact that the *GhRING2* promoter region has more than one transcription factor

binding site; one being present in the R2GUS3 promoter fragment and the other located between -607 to -1506 nt. The *GhRING2* gene may be regulated by more than one transcriptional factor (TF) that is expressed temporally at different stages of plant development. For instance, one TF may be expressed in the seedling stage and the other during the development of young stems in transgenic *Arabidopsis* plants.

The GUS activity of the *GhRING2* promoter was localized to xylem and cambium cells of young stems but not in mature stems of transgenic *Arabidopsis thaliana* plants. Persson et al. (2007) studied the expression of an *Arabidopsis IRX8* gene encoding glycosyltransferase and found that the gene was essential for secondary wall formation. The *IRX8* gene promoter directed GUS expression in developing fiber and young xylem cells where secondary cell walls are deposited. The *irx8* mutant plants had reduced levels of glycan and homogalacturonan, collapsed xylem vessels and displayed a dwarfed phenotype (Persson et al. 2007). The observation of GUS expression in xylem cells of transgenic *Arabidopsis* plants containing the *GhRING2* promoter suggests that the *GhRING2* gene may be involved in cotton fiber secondary cell wall thickening.

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