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Genome editing in kenaf: Initial studies and target gene characterization

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Genome editing in kenaf: Initial studies and target gene characterization

By

Kangqi Li

A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Biological Sciences
in the Department of Biological Sciences

Mississippi State, Mississippi

May 2020

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2020

Genome editing in kenaf: Initial studies and target gene characterization

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The potential for *Hibiscus cannabinus* L. (kenaf) improvement via genome editing using the CRISPR/Cas9 system to generate gene knock-outs was explored. Studies included target gene identification, target guide RNA (gRNA) selection, plant tissue (explant) choice and media composition for plant regeneration. A putative kenaf phytoene desaturase gene (*pds*, GEED01047592.1) was identified in the kenaf transcriptome, and molecularly confirmed. Kenaf seedling tissues were transformed via *Agrobacterium tumefaciens* containing the *cas9* gene (endonuclease required for gene knock-out) and each gRNA separately; putative transgenic calli and adventitious shoots arose on a medium containing 1-naphthaleneacetic acid, thidiazuron and silver nitrate. Tissues appeared chlorotic/albino and shoots remained diminutive/dwarf-like. These unique morphologies had also been noted by researchers who successfully knocked out the *pds* gene in other plant species. *Cas9* DNA was detected in these putative transgenic kenaf tissues, but initial DNA sequencing analysis did not confirm knock-out/mutations in targeted areas of the *pds* gene.

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CHAPTER I

LITERATURE REVIEW

1.1 *Hibiscus cannabinus* (Kenaf)

Kenaf (*Hibiscus cannabinus* L.) is an important fiber crop. It is a diploid ($2N=2X=36$) herbaceous annual plant in the Malvaceae family. Believed to have originated in northern Africa, domesticated kenaf was introduced into India, Russia and China as recently as 200 years ago. Interest in growing kenaf in the United States (US) began when the US Department of Agriculture (USDA) determined that it could be an important cellulose fiber source (Dempsey, 1975; Webber and Bledsoe, 2002).

There are two main fiber sources in kenaf stems (stalks), the inner core and outer bast fibers. The bast fibers are primarily composed of phloem fibers rich in cellulose and lignin, and have been successfully incorporated into production of paper, textiles, mats, etc. (Goforth and Fuller, 1994). The core fibers are also a source of paper pulp, but were also used to develop commercial absorbent products because of their high absorptive capacity (Webber and Bledsoe, 2002).

Kenaf cultivars that have been grown in Mississippi include Everglades 41 (E41) and E71, Guatemala 4 (G4), G45 and G48, Cubano and Cuba 108 (C108), Tainung 1 (Tai1), and Tai2 (Lim, 1997). Whitten is a new variety developed by Baldwin et al. (2006) displaying enhanced fiber yield; it was selected from progeny arising from a cross of E41 \times G45. There is continued interest in improving fiber (cellulose) yield and more energy-efficient extraction of it

in kenaf and other fiber crops used to generate lignocellulosic biomass (Jung et al., 2012; Sticklen, 2006). An overall goal in these crops, including kenaf, would be to reduce the amount of lignin in bast fibers. A genetic engineering approach would entail the use of plant tissue culture, plant transformation and genome editing procedures. In vitro regeneration procedures have been developed for kenaf which will be discussed in a later section, as well as limited plant transformation methodologies. Genome editing has not been demonstrated in kenaf, to date. Prior to attempting to reduce lignin in kenaf bast fibers, a combined protocol for generation of genome-edited kenaf will need to be developed.

1.2 In Vitro Culture – Plant Tissue Culture

1.2.1 History

The field of plant tissue culture has been possible due to the ability of most somatic plant cells to be totipotent – have the ability/capacity to form complete adult plants (Trigiano and Gray, 1999). The overall technology uses cells/tissues/organs to grow, manipulate and maintain cultures under controlled conditions which could be differentiated (actually, redifferentiated) into plants (Dodds and Roberts, 1987). It is a vital experimental system for studying plant development, asexual propagation and genetic improvement.

The origin of plant tissue culture has been attributed to Haberlandt, the first scientist who attempted to culture plant tissues in vitro (Dodds and Roberts, 1987). In the late 19th century, calli, undifferentiated proliferated cell masses, were known to form on plant tissues as a result of wounding. These calli were later found capable of very efficient cell divisions in vitro and had the potential to differentiate (Dodds and Roberts, 1987). This differentiation from calli, or original explants, is highly dependent on providing cells with the correct chemical and

environmental cues. Important chemical cues are plant hormones/plant growth regulators (discussed below) (Sugiyama, 1999).

1.2.2 Exogenous plant growth regulators (PGRs)

Auxins and cytokinins are the most important classes of hormones used in plant tissue culture to generate desired growth (Woodward and Bartel, 2005). They and/or their synthetic counterparts, plant growth regulators (PGRs), are typically added to tissue culture media alone, or in combination. Early experiments by Miller and Skoog in the 1950s demonstrated that different plant organs could be obtained in tissue culture by adjusting the types of auxin and cytokinin used, in addition to their individual concentrations (auxin to cytokinin ratio, a/c) in the media. In tobacco cultures, when a/c was low, shoot organogenesis was predominant. A high a/c yielded root organogenesis, and a/c ~1 yielded undifferentiated (calli) growth (Dodds and Roberts, 1987). Therefore, the type of auxins and cytokinins used as well as their overall concentrations directly influence differentiation in vitro (Gaspar et al., 1996).

Auxin is a family of hormones that are known to be responsible for many aspects of plant growth, such as root growth and development, and enforcing apical dominance. Indole-3-acetic acid (IAA) is a naturally occurring hormone in this family. It regulates plant organ development probably through inducing cell elongation and cell division (Zhao, 2010). A commonly used synthetic PGR is 1-naphthaleneacetic acid (NAA) which is widely used as a regulator of in vitro organogenesis (Woodward and Bartel, 2005).

Cytokinin is a family of hormones that induces cell division and is involved in the regulation of root and shoot growth, as well as helping to overcome apical dominance. Zeatin is a hormone in this family. Commonly used synthetic PGRs include 6-benzylaminopurine (BAP)

which has been demonstrated to induce shoot multiplication (Dixon and Gonzales, 1994) and the newer thidiazuron (TDZ).

TDZ, N-phenyl-N'-1,2,3-thiadiazol-5-ylurea, is a newer synthetic plant growth regulator (Huetteman and Preece, 1993). TDZ has been utilized as a cytokinin for in vitro embryogenesis and organogenesis. Reviewed by Lu (1993), inclusion of TDZ in tissue culture media induced somatic embryogenesis, as well as adventitious and axillary shoot formation from different tissues. They concluded that TDZ showed greater efficiency in inducing adventitious shoot compared to adenine-type cytokinins (like BAP). Guo et al. (2011) noted greater transformation frequencies when using TDZ to enhance the activity of regenerated shoots. A review by Dewir et al. (2018) indicated that using TDZ enhanced flower induction in vitro in various species. They also noted the increased stability of TDZ compared to other types of cytokinins, since it was not inhibited by cytokinin oxidase activity.

1.2.3 Kenaf tissue culture and regeneration

For this section, refer to Table 1.1 Regeneration procedures have been developed for a number of kenaf cultivars (Liu, 1994; Lim, 1997; Young, 2000). Liu (1994) used leaf explants from in vitro-maintained plants of kenaf cultivars E41 and G45; explants were cultured on a Murashige and Skoog (1962; MS) based medium plus PGRs NAA (0.1 mg/l) and TDZ (3.0 or 3.5 mg/l) to initiate shoot organogenesis. All tissues were cultured at room temperature (21-27°C) with a 16:8 photoperiod.

Kenaf leaf explants from cultivars C108, E41, E71, G45, G48 and Tai2 were used for shoot regeneration experiments by Lim (1997). Leaf explants (5 x 5 mm) were harvested from different seed clonal lines (SCLs) of each cultivar maintained in vitro. The regeneration

efficiencies included below represent the greatest percentage of explants yielding shoots per cultivar for any of the SCL tested, since regeneration efficiencies varied among them.

Lim (1997) compared medium TN12 (Liu, 1994; contained 0.1 mg/l NAA and 3.5 mg/l TDZ) to two NH-based (Nitsch and Nitsch, 1969) media, NH10 and NH11, (contained NAA at 0.1 mg/l plus TDZ at 2.2 or 3.0 mg/l, respectively). In E41, TN12 yielded the fastest response (callus), but shoot primordia first appeared on NH10 (60% regeneration efficiency) and NH11. The regeneration efficiencies varied among the other cultivars tested. NH10 yielded the best regeneration efficiency for C108 (20%), G45 (26.7%) and G48 (26.7%); TN12 was ideal for E71 (20%). For all cultivars, most of shoot primordia first appeared after 4 - 6 weeks in culture, and regeneration efficiency results were recorded after 12 weeks on the media. Regenerated shoots were transferred to MSO medium (no PGRs) for root induction.

Six media containing different concentrations of TDZ were tested for the best regeneration efficiency using cultivars Cubano, E41, E71 and G45 (Young, 2000). 5 x 5 mm leaf sections were harvested and used as explants. After 4 months in culture, no shoots were observed on any media for Cubano or G45. E41 explants yielded shoots on NH10 (20%) and 6.7% of E71 explants yielded shoots on NH11.

Silver nitrate (AgNO_3) is known to reduce ethylene activity (Beyer, 1976). Reviewed by Kumar et al. (1998), AgNO_3 is used as an ethylene inhibitor to enhance in vitro organogenesis in many plant species. Lim (1997) and Young (2000) included silver nitrate in their shoot initiation media in attempts to increase the number of responding explants and shoots generated.

Lim (1997) evaluated the effect of silver nitrate (5, 10, 20, 50 mg/l) on six cultivars. Addition of 20 mg/l AgNO_3 led to the best improvement in shoot production in cultivars E41 and G45. E41 displayed a higher regeneration efficiency (80%) and a greater number of shoots

generated per explant on NH10 containing 3% (w/v) glucose instead of sucrose plus 20 or 50 mg/l AgNO₃. NH10 was best for G45 (5 mg/l AgNO₃), as well as C108 and G48 (20 mg/l AgNO₃). TN12 was best for Tai2 (20 mg/l AgNO₃) and E71 (50 mg/l AgNO₃).

Young (2000) also tested the effects of AgNO₃ (5.1, 10.2, 20.4 mg/l) on leaf explants using cultivars Cubano, E41, E71 and G45. Cubano and G45 did not regenerate shoots after 4 months in culture on any media. Shoots of E41 were first observed after 8 weeks in culture and the greatest explant response (80%) was obtained on MS-based media containing 0.1 mg/l NAA and 0.35 mg/l TDZ plus 10.2 mg/l AgNO₃. For E71, the same highest shoot regeneration rates were comparable (7%) on three media containing AgNO₃ (5.1 to 10.2 mg/l).

It can be concluded from research of Liu (1994), Lim (1997) and Young (2000) that optimal TDZ concentration (0.35 to 3.5 mg/l) and AgNO₃ concentration (5 to 50 mg/l) varied and depended on the cultivar used. The new cultivar Whitten has never been studied for in vitro responses.

Khatun et al. (2003) added Pluronic F-68 to their media to enhance regeneration efficiency. Pluronic F-68 works as a non-ionic surfactant in cell culture and has been used to promote jute cotyledon and transformed root growth (Khatun et al., 1993). Cotyledons with attached petioles were harvested from seedlings of cultivar HC-2 when the shoot-tip emerged from the center of cotyledons. Shoot regeneration results were taken 49 days after culture initiation. The MS-based medium supplemented with 0.5 mg/l IAA and 5.0 mg/l BAP yielded the greatest regeneration efficiency; with addition of 0.5% Pluronic F-68, the shoot regeneration rate increased from 59% to 81%.

Samanthi et al. (2013) also use excised seedling cotyledons as the explant for cultivars G4 and V36. The best callus induction medium for both cultivars was MS-based containing 0.05

mg/l indole-3-butyric acid (IBA) and 1.5 mg/l BAP, with incubation under continuous darkness. Calli were harvested and placed on a shoot regeneration medium, that contained the same PGRs with the addition of 0.3 mg/l gibberellic acid (GA₃). On this medium, the best shoot regeneration rate, determined after 9 weeks, was 68.7%.

Shamsunnaher et al. (2013) also used seedling explants for regeneration of kenaf. Cotyledons with attached petioles were harvested from 10 day-old seedlings of cultivars HC-2 and HC-95. There were no significant cultivar differences; the best medium was MS-based containing 0.5 mg/l IAA and 3.0 mg/l BAP which yielded the greatest callus induction rate (92%) and shoot regeneration rate (88%). It took an average of 7.5 days for callus induction and 15.7 days for initial shoot regeneration on this medium.

Table 1.1 Summary of kenaf shoot regeneration procedures

Cultivar	Explant	PGRs in best medium for		Reference
		shoot regeneration (mg/l)	Other additives	
E41 and G45	Leaf section	0.1 NAA and 3.5 TDZ	-	Liu (1994)
C108, E41 and G48	Leaf section	0.1 NAA and 2.2 TDZ	20 mg/l AgNO ₃	Lim (1997)
E71	Leaf section	0.1 NAA and 3.5 TDZ	50 mg/l AgNO ₃	Lim (1997)
G45	Leaf section	0.1 NAA and 2.2 TDZ	5 mg/l AgNO ₃	Lim (1997)
Tai2	Leaf section	0.1 NAA and 3.5 TDZ	20 mg/l AgNO ₃	Lim (1997)
E41 and E71	Leaf section	0.1 NAA and 0.35 TDZ	10.2 mg/l AgNO ₃	Young (2000)
HC-2	Cotyledon with attached petiole	0.5 IAA and 5.0 BAP	0.5% Pluronic F-68	Khatun et al. (2003)
G4 and V36	Cotyledon	0.05 IBA and 1.5 BAP	0.3 mg/l GA ₃	Samanthi et al. (2013)
HC-2 and HC-95	Cotyledon with attached petiole	0.5 IAA and 3.0 BAP	-	Shamsunnaheer et al. (2013)

1.3 Genetic Transformation Using *Agrobacterium tumefaciens*

1.3.1 Background information

Several methods can be used to transform plant protoplasts or intact plant cells using a plasmid carrying foreign genes for introduction, and lead to generation of genetically altered (transformed/transgenic) plants. They include biotic-based (using *Agrobacterium*-mediated DNA delivery) and abiotic (using particle bombardment/biolistics) procedures (Husaini et al., 2010). The focus will be on *Agrobacterium tumefaciens*-mediated transformation.

A. tumefaciens is a gram-negative, rod-shaped parasitic bacterium capable of infecting plant cells. It is composed of genomic DNA and plasmid DNA, called tumor-inducing (Ti) plasmid (Hooykaas et al., 1984). The Ti plasmid is large (~206 kilobase pair, kbp) and belongs to the *repABC* family. It also contains a region (~25 kbp) of DNA that is transferred into plant cells, T-DNA. This T-DNA is bordered by 25 bp direct repeats (LB and RB; Gelvin, 2003).

In nature, the transfection of *A. tumefaciens* into wounded plant tissues happens spontaneously. By transferring and integrating T-DNA into host cells, *A. tumefaciens* induces the formation of a tumor (crown gall); those plant cells produce opines that only *A. tumefaciens* can use as a nitrogen source to support its growth (Tzfira and Citovsky, 2006). *A. tumefaciens*-mediated plant transformation is a complex process, including the recognition and attachment of *A. tumefaciens* to plant cells, secretion and induction of plant signaling molecules, activation and expression of genes in the virulence region (*vir* genes), replication of T-DNA and transfer of a single-stranded copy (T-strand), and integration and expression in plant cells (Gelvin, 2003; Lorence and Verpoorte, 2004).

The molecular process that facilitates this DNA transfer was described by Christie and Gordon (2014). VirB/VirD4 conjugation systems in the Ti plasmid are responsible for

transporting/secreting T-DNA into the host cells. The T-strand is processed by DNA transfer and replication proteins (Dtr) and forms a relaxosome, then binds to a type IV coupling protein (T4CP) to form a transfer intermediate which is delivered through a secretion channel composed of VirD4 T4CP and VirB. After delivery into the plant cell, a T-complex is formed by the T-strand, VirD2 and VirE2 (Gelvin, 2012). VirD2 and VirE2 contain nuclear localization sequences (NLS) to facilitate integration of the DNA into the plant nuclear genome (Christie and Gordon, 2014).

Since *A. tumefaciens* naturally mediates plant cell transformation it is frequently used due to its simple operation, high transformation efficiency, stability of inserts and less transgene copy numbers incorporated into the host plant's genome (Husaini et al., 2010). Plant tissue wounding initiates the process. The T-DNA can be engineered to deliver foreign genes of interest into plant cells to create transgenic plants. This procedure is quite efficient in eudicots (Nester and Kosuge, 1981).

Most *A. tumefaciens*-mediated genetic transformation methods are based on the fact that removal of the original DNA situated between T-DNA borders (making it “disarmed”) does not influence the efficiency of transformation (Gelvin, 2003). So, insertion of genes of interest between the T-DNA borders enable *A. tumefaciens* to efficiently transfer genes of interest into plant cells. Two types of disarmed Ti vectors are used in plant transformations, cointegrate and binary. Cointegrate vectors contain a T-DNA region where genes of interest can be inserted into, and Vir region/genes. The binary vector system contains two plasmids, Ti to provide Vir, but lacks a T-DNA region. A second smaller plasmid contains the T-DNA borders where DNA of interest is inserted between them (Lorence and Verpoorte, 2004).

1.3.2 Selectable marker and reporter genes

Marker genes play an important role in plant genetic engineering. Their role in genetic transformation is to distinguish between transformed and non-transformed cells to help screen and identify transformed cells, tissues and transgenic plants.

Selectable marker genes provide cells that receive this type of gene (generally impart specific chemical resistance) the selective advantage to grow on media containing that chemical. Key genes include those that impart resistance to antibiotics and herbicides mostly through inactivation, thereby enabling transformed cells to continue to grow (Rosellini, 2011).

The most widely used selectable marker gene is neomycin phosphotransferase II (*nptII*), derived from the *aphA2* gene of *E. coli*. It imparts resistance to the antibiotics kanamycin, neomycin, and geneticin/G418. NPTII catalyzes the transfer of a phosphate group from ATP to the antibiotic that prevents target site binding, thereby inactivating the antibiotic (Goldman and Northrop, 1976). Like *nptII*, another selectable marker gene hygromycin phosphotransferase II (*hptII*) was also isolated from *E. coli*. It provides hygromycin resistance by phosphorylating and inactivating hygromycin (Htwe et al., 2014). The phosphinothricin acetyltransferase gene (*pat*) derived from *Streptomyces viridochromogenes* imparts resistance to phosphinothricin (PPT), a herbicide, by acetylating and inactivating PPT (Strauch et al., 1994).

Reporter genes do not impart resistance to a chemical, but rather provide visual markers to identify the cells/tissues that have received the gene, thereby indicating successful delivery of that gene – an indicator of successful transformation.

The β -glucuronidase (*gus*) gene isolated from *E. coli* is capable of hydrolyzing a derivative of optically active β -guanidine-glucuronide (Jefferson et al., 1987). Using X-GLUC as a substrate to perform histochemical assays, a blue stain (indigo) can be visually observed.

Using 4-methylumbelliferone-D-glucuronide (4-MUG) as a substrate to perform a fluorometric assay, the resulting product 4-methylumbelliferone (4-MU) fluoresces (Jefferson et al., 1987). To express the *gus* gene in transformed plants, it is usually driven by a constitutive promoter like 35S from cauliflower mosaic virus (CaMV).

The green fluorescent protein (*gfp*) gene was isolated and purified from *Aequorea victoria* (jellyfish); GFP is a 238 amino acid protein that is capable of excitation by UV (360–400 nm) or blue (440–480 nm) light which induces the emission of green light (Leffel et al., 1997). Compared to GUS, GFP does not require additional substrate or reagent to show visible results nor is it a destructive assay. Its specificity and convenience enabled researcher to develop and utilize GFP as an efficient and reliable reporter tool, and is widely used as a reporter gene in numerous plant species (Leffel et al., 1997).

Modified *gfp* genes have also been used for detection in plant tissues as well as whole plants. For example, Pérez-Clemente et al. (2005) used *sgfp*, a re-engineered *gfp* gene that provided high GFP expression in plants, to screen for transgenic tissues in regenerated peach shoots. Cormack et al. (1996) developed the *egfp* gene that increased the fluorescence intensity 100-fold. Rajasekaran et al. (2005) constructed a vector that contained *egfp* as a marker gene for *in situ* (on-site) inoculation. Similar to *gus*, *gfp* genes are also usually driven by the CaMV 35S promoter.

1.3.3 Kenaf transformation

Banks et al. (1993) first confirmed that kenaf could be successfully transformed with *A. tumefaciens* using the β -glucuronidase (GUS) transformation system from Clontech Laboratories Inc. *A. tumefaciens* strain LBA4404:pBI121 was used. Binary plasmid pBI121 contained a *gus* reporter gene controlled by the CaMV 35S promoter and the *nptII* gene controlled by the *A.*

tumefaciens nopaline synthase (nos) promoter. Hypocotyl explants (5 to 8 mm length) were harvested from seedlings of kenaf cultivar Indian, 3 days after germination. Explants were pre-cultured for 3 days on a MS-based medium containing 0.75 mg/l MgCl₂, 0.1 mg/l 2,4-D and 0.5 mg/l kinetin, inoculated with LBA4404:pBI121 1 min, blotted dry, then placed back onto pre-culture medium for an additional 3 days. MS-based callus initiation media contained 0.75 mg/l MgCl₂, 2 mg/l NAA and 0.1 mg/l kinetin plus 1000 mg/l amoxicillin, 500 mg/l potassium clavulanate and 50 mg/l kanamycin. Amoxicillin and potassium clavulanate were added to inhibit/kill *A. tumefaciens*, and kanamycin was used to select transformed cells. GUS activity was observed in calli arising from transformed hypocotyl explants 30 days post-transformation.

Young (2000) developed procedures that resulted in generation of transgenic plants. Four cointegrate plasmids were tested. Each contained *gus* controlled by the CaMV 35S promoter and *nptII* gene controlled by the nos promoter. Disarmed *A. tumefaciens* strain C58C1, containing non-oncogenic Ti plasmid pGV3850, was used. Leaf explants harvested from E41 and E71 plants maintained in vitro were pre-cultured for 2 days on a MS PGR-free medium, inoculated 10 min with *A. tumefaciens* broth culture (OD_{600nm}=0.8) containing 3.92 mg/l acetosyringone (AS). Explants were blotted dry before co-culturing on a shoot regeneration medium (MS-based containing 0.1 mg/l NAA, 0.35 mg/l TDZ, 10.2 mg/l AgNO₃ plus 19.62 mg/l AS) for 2 days. AS was added to enhance the transformation efficiency. Tissues were rinsed with 500 mg/l cefotaxime, and placed on the shoot regeneration medium described above (but no AS) containing 10-15 mg/l geneticin and 500 mg/l cefotaxime. Cefotaxime was used to inhibit the growth of *A. tumefaciens*. Shoots were transferred to MS PGR-free medium containing 5.0 mg/l geneticin and 500 mg/l cefotaxime for rooting. Tissues were screened via histochemical GUS assays to measure transient GUS expression. Transient transformation frequencies (percentage of

area covered by GUS-positive sectors) ranged from 11% to 46% for E41, and 23% to 61% for E71; expression differences were attributed to the different plasmids used. Polymerase chain reaction (PCR) analyses confirmed generation of transgenic plants.

Herath et al. (2005) used GUS expression to measure factors that might influence *A. tumefaciens* transformation. Shoot tips were harvested from the 7-10 day-old seedlings of kenaf cultivar Tai2. Two binary plasmids, pIG121-Hm and pEC:GUS, were used with *A. tumefaciens* strain EHA105. Plasmid pIG121-Hm contained *gus* driven by the CaMV 35S promoter, and two selectable marker genes, *nptII* and *hptII*. Plasmid pEC:GUS contained an enhanced CaMV 35S promoter controlling *gus* with an untranslated leader sequence from kyuri green mottle mosaic virus, and a *nptII* gene. Explants were wounded twice with a sterile needle before being inoculated with *A. tumefaciens*; 39.24 mg/l acetosyringone was added to *A. tumefaciens* 30 min prior to inoculation. They determined that the best duration for explant pre-culture was 2 days, and the best duration of co-culture was 2 - 3 days; MS-based pre-culture medium contained 1.98 mg/l BAP, and the co-culture medium contained 2.97 mg/l BAP and 39.24 mg/l acetosyringone. Explants were washed with 500 mg/l carbenicillin, blotted dry, and transferred to a medium containing 1.98 mg/l BAP and 500 mg/l carbenicillin for 2 weeks. Explants were then placed on the selective medium (1.98 mg/l BAP, 100 mg/l kanamycin and 250 mg/l carbenicillin). Histochemical GUS assays were used to test transient GUS expression 3 days after co-culture, and regenerated shoots were counted 2 months after explants were placed on selective media. The best combination of pre-culture and co-culture conditions, described above, yielded the highest GUS transient expression rate (92%) and the greatest number of regenerated shoots.

Shoot apices of Tainung 2 were used as explants to compare efficiencies of three *A. tumefaciens* strains (LBA4404, EHA101S and Z707S), and determine effects on transformation

with addition of a plasmid (pCH32) containing additional *virG/virE* sequences (Srivatanakul et al., 2001). The binary plasmid contained *pat* controlled by the ZmUbi1 promoter (tissue selection on 1.5 mg/l PPT) and was introduced into each of the three *A. tumefaciens* strains, then used to inoculate explants. Shoot apices were pre-cultured 2 days on MS-TDZ medium (MS-based containing 0.22 mg/L TDZ), sonicated for 5 sec to micro-wound explants, inoculated with *A. tumefaciens* (pre-treated with 39.24 mg/l acetosyringine 2 hr prior to inoculation), then co-cultured with *A. tumefaciens* for 3 days on MS-TDZ media. Explants were washed in 25 mg/ml Clavamox for 5 min to inhibit *A. tumefaciens* growth, rinsed twice in sterile water, then blotted dry. Explants were then transferred to a selection medium (MS-TDZ media containing 1.5 mg/l PPT and 250 mg/l Clavamox). Strain LBA4404 was determined to yield the greatest number of transgenic tissues. Binary plasmid UBQ-GUS containing a ubiquitin-GUS fusion fragment gene controlled by the CaMV 35S promoter, and *nptIII* (promoter not provided; tissue selection on 100 mg/l kanamycin) was introduced into LBA4404 and compared with LBA4404 that also harbored pCH32 that contained additional *virG/virE* sequences. Post-transformation, a greater number of GUS-positive spots were obtained when pCH32 was included.

Cultivars Dowling and SF459 were successfully transformed by Ruotolo et al. (2011). Explants were mature seeds longitudinally cut in half after surface sterilization. They were placed on a MS-based medium containing 2.2 mg/l TDZ for shoot regeneration. Two *A. tumefaciens* strains, C58C1 and LBA4404, were used in the transformations; each contained binary vector p35SGUS-INT. This plasmid contained *nptIII* controlled by the nos promoter and an intron-containing *gus* gene controlled by the CaMV 35S promoter. *A. tumefaciens* was pre-cultured with 39.24 mg/l acetosyringone for 2 days, then added to a MS-based medium containing AS 2 hr before inoculation. Explants were inoculated with *A. tumefaciens* for 20 min,

blotted dry, transferred to a co-culture medium (MS-based plus 2.2 mg/l TDZ and 39.24 mg/l acetosyringone), and incubated in the dark at 27°C for 48 hr. Co-cultured explants were placed on a selective medium containing 2.2 mg/l TDZ, 300 mg/l kanamycin and 500 mg/l cefotaxime for 12 days, then transferred to a medium containing one-half the amount of cefotaxime. Histochemical GUS assays and PCR (*gus* primers) were used to confirm transient and stable transformation, respectively. After 3 months, the greatest transformation rate obtained was 1% (cultivar Dowling with strain C58C1).

1.4 Genome Editing Techniques/Technologies - prior to CRISPR

Various strategies have been employed to down-regulate gene expression, including disruption of the gene sequence (knock-out). A few methods not employed in this thesis research will be briefly discussed, but will focus on the employed technology for gene knock-out – CRISPR/Cas9.

1.4.1 RNA-based strategies to reduce gene expression

Early attempts to inhibit gene expression entailed use of antisense RNA, which resulted from expression of a transgene introduced into plant cells “backwards”. Transcribed RNA would be homologous to the normal gene’s sense RNA which enabled generation of double-stranded RNA, thereby decreasing the gene’s expression. Through transcription of antisense RNA in carrot protoplasts, expression of the chloramphenicol acetyltransferase (*cat*) gene was decreased (Ecker and Davis, 1986); this indicated the potential application of antisense RNA to inhibit targeted gene expression.

A phenomenon initially called co-suppression was first identified in plants when the chalcone synthase gene (*chs*) was introduced into *Petunia hybrida* (petunia) already containing

endogenous *chs*, trying to increase expression levels. However, the opposite was found; expression of exogenous *chs* induced down-regulation of both it and endogenous *chs* (Napoli et al., 1990). This was later called RNA interference (RNAi). Formation of double-strand RNA was part of the RNA degradation process (Fire et al., 1998), and shortly after, RNAi started to be used to effectively knock-down targeted gene expression. Small interfering RNA (siRNA) and microRNA (miRNA) have been employed to investigate specific gene functions such as those that confer disease resistance, stress response, secondary metabolism, etc., but the focus will be on down-regulation of gene expression (gene silencing). Host-delivered RNAi (hdRNAi) is produced in plants with introduced hairpin RNA (hpRNA) vectors, also known as host-induced gene silencing (HIGS) (Nowara et al., 2010). This method has been demonstrated to effectively combat virus invasion as well as bacterial and fungal infections (Koch and Kogel, 2004).

A review by Saurabh et al. (2014) concluded that the flexibility and high efficiency of RNAi has proved it can work as an efficient gene silencing tool. RNAi has been widely utilized in improving crops for nutritional improvement, altering phenotypes (such as flowers), imparting stress resistances, etc. (Saurabh et al., 2014).

However, RNAi induced gene silencing does not alter the target gene, so the genotype remains unaltered (Gaj et al., 2013). Off-target gene silencing has also been found, which further impacts its effectiveness (Senthil-Kumar and Mysore, 2011).

1.4.2 Transcription activator-like effector nucleases (TALENs)

Transcription activator-like effector nucleases (TALENs) is a genetic modification tool that has been used widely. TALENs works as protein dimers, one each on opposite DNA strands flanking the target DNA, with a double-strand break (DSB) occurring between the two TALEN monomers. Each monomer is composed of a programmable DNA-binding domain and a

nonspecific FokI nuclease domain (Joung and Sander, 2013). The DNA-binding domain is derived from TALE proteins, which were initially identified in *Xanthomonas* (Joung and Sander, 2013); each DNA-binding domain is composed of several highly conserved 33-35 amino-acid repeats, with each recognizing a single base in the DNA target sequence. Each repeat contains two hyper-variable residues that create this DNA base specificity (Joung and Sander, 2013). Individual DNA binding domains can be joined and customized for each targeted DNA sequence. The FokI endonuclease at the C-terminal end of each TALEN induces a DSB in the target DNA to generate nonhomologous end joining (NHEJ)-mediated mutations, leading to knock-out of the target gene (Joung and Sander, 2013).

Selection of the ideal DNA target site can significantly improve TALEN's specificity for target DNA recognition, binding, and DSB efficiency. A large number of comparative analyses confirmed that TALEN target site selection only needs to follow one principle: the first base of the 5' end of the TALEN target site should be thymine (T) (Grau et al., 2013). TALENs have the potential to target most sites in a genome in a flexible way. The high specificity and low risk of off-target of TALENs is due to the TALE repeat code and the required dimerization of the FokI domain on each TALEN C-terminal end (Joung and Sander, 2013). However, reviewed by Nemudryi et al. (2014), off-target effects can still occur. Nucleotide binding efficiencies vary with the different hyper-variable regions used in the constructed TALEs, with some monomers providing weaker hydrogen bonding. The degeneracy of the amino acid code may also lead to mismatches. Another consideration: the delivery efficiency of TALENs is limited by the large size of the genes needed to be introduced into the plant to produce TALENs, so widespread use of this tool for gene knock-outs might rely on developing a better delivering system (Gaj et al., 2013).

1.5 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) System

1.5.1 History of CRISPR

Clustered regularly interspaced short palindromic repeats (CRISPR) is a DNA fragment containing specific sequences identified in bacteria and archaea that is part of a RNA-based bacterial immunological defense mechanism. It also contains genes called *cas* (CRISPR-associated proteins). This system was named the CRISPR/Cas system, which has recently been adapted for use as a gene modification tool (Barrangou, 2015). CRISPR systems are described below.

A short palindromic repeat DNA fragment was first reported in *E. coli* by Ishino et al. (1987). Later, five different highly conserved 29 base pair (bp) palindromic repeats were identified and were separated by 32 different nucleotide sequences (“spacers”) clustered near the 3’ end of the *iap* gene in *E. coli*. These clustered, spaced repeats were then found in 20 additional species among archaea, bacteria and in mitochondria, although the number of repeats in each cluster and sequence length of the spacers varied among species (Mojica et al., 2000). Jansen et al. (2002) subsequently identified this type of repeated sequence in more than 40 prokaryotic species, but not in eukaryotic species or viruses; they named these areas of clustered repeats CRISPR. These researchers also identified CRISPR-associated (*cas*) genes that might correlate with helicase and nuclease functions, based on their functional motifs.

Mojica et al. (2005) began to reveal the potential of CRISPR for use as a genome editing tool by studying CRISPR spacers from 67 strains of prokaryotic species including archaea microorganisms and bacteria (both Gram-positive and Gram-negative). These nonrepeating spacers were found to be homologous to exogenous genes from organisms such as bacteriophages, plasmids or chromosomal sequences. Independent studies in other bacterial

species including *Streptococcus thermophiles* and *S. vestibularis* (Bolotin et al., 2005), and *Yersinia pestis* (Pourcel et al., 2005) provided similar results. Bolotin et al. (2005) suggested that CRISPR could be an immune system producing antisense RNA to inhibit phage gene expression. Most importantly, this immune system was not a simple RNA interference process, but contained a sequence “memory” of previous pathogenic invasions.

1.5.2 Molecular mechanism and classification of CRISPR systems

Barrangou et al. (2007) first determined that CRISPR and associated *cas* genes provided resistance to bacteriophages due to presence of integrated bacteriophage genomic sequences in the spacer DNA found between the clustered repeat sequences. This mechanism was further analyzed by Brouns et al. (2008) and was later classified as a type I system. It was determined that five Cas proteins (named CasA, CasB, CasC, CasD, and CasE) can form a complex, known as Cascade, that is capable of producing a type of small CRISPR-RNA (crRNA) from precursors of small CRISPR-RNA (pre-crRNA, expressed spacer and repeat RNA); they hypothesized that Cascade and crRNA search for and target sequences in phage genes, providing resistance to the bacteriophage. To identify the phage resistance function of Cascade, synthesized Cascade was designed to target four key genes of phage Lambda. Results indicated that introduction of only Cascade did not provide phage resistance, while the other *E. coli* strain that expressed both Cascade along with Cas3 reduced phage’s ability to infect; both Cascade and Cas3 were required in this process. Cas3 was predicted to express both nuclease and helicase functions. Sinkunas et al. (2011) identified N-terminal histidine-aspartate (HD) phosphohydrolase and C-terminal Superfamily 2 (SF2) helicase domains in Cas3 of *Streptococcus thermophilus*; ATP-independent single-strand DNA nuclease and ATP-dependent helicase activities of Cas3 were then confirmed.

Protospacer adjacent motif (PAM) was later determined to be a key element to locate the target sequence for Cascade (Cas:crRNA complex). It is a short sequence that usually contains at least two guanine bases, and can be recognized by sequence alignment of the complex (Shah et al., 2013). This allows the complex scan for target sequences guided by the crRNA. Once the crRNA is matched to a complementary sequence in the target DNA, the target DNA would be cleaved approximately three bases upstream of the PAM sequence (Jinek et al., 2012).

The entire process is divided into three distinct stages: (1) adaptation - fragment of target DNA (spacer) integrated into CRISPR sequence; (2) expression - pre-crRNA and short crRNA are formed; (3) interference - incoming DNA fragments are targeted and cleaved (Makarova et al., 2011).

Based on the enzymes and RNA involved in the process, identified CRISPR/Cas systems were classified into three major types (I, II, and III) by Makarova et al. (2011). The type I system was described above (Brouns et al., 2008) - a complex is formed by Cascade, crRNA recognizes target sequences, and Cas3 works as the endonuclease and helicase.

By contrast to the type I and type II systems, the type III system does not require a PAM sequence in the adaptation stage. In the second stage, the crRNA is then processed by Cas6 protein, is passed on to a Csm [in Type III-A (subtype is not discussed here), also known as Cas10] or Cmr (in Type III-B) complex. Different subtypes may target either DNA or RNA sequences (Corder et al., 2017).

1.5.3 Type II CRISPR/Cas9 system

The most obvious difference between type II CRISPR/Cas system and the other two is the requirement for only one protein - Cas9. This is the only protein needed to achieve site-specific DSBs. The significant role of Cas9 (also called Csn1) was firstly identified in

Streptococcus thermophilus regarding cleavage of plasmid DNA by this CRISPR/Cas system (Garneau et al., 2010). They confirmed double-strand cleavage occurred 3 bases upstream of PAM. Inactivation of *cas5* gene (now classified as *cas9*), confirmed that Cas9 was responsible for endonuclease activity.

Chylinski et al. (2014) further characterized Cas9 function, providing a more complete list of “components” in the CRISPR/Cas9 system. Cas9 protein is the only essential *cas* gene needed to be functional, although there are more than four *cas* genes included in this type II system. Different from type I and III that form Cascade complexes, the final complex inducing DNA cleavage is Cas9 protein accompanied by CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA).

Process initiation: First, pre-crRNA, tracrRNA and Cas9 are combined. Triggered by tracrRNA, RNase III cleaves pre-crRNA into crRNA at the repeat sequences to form multiple crRNA:tracrRNA:Cas9 complexes (Deltcheva et al., 2011).

The cleavage of each targeted DNA strand is induced by two separate domains of Cas9: the target strand is cleaved by the HNH nuclease domain, and the non-target strand is cleaved by the RuvC-like domain. Impressively, Jinek et al. (2012) developed a single chimeric dual-tracrRNA:crRNA that comprised both the target complementary sequence (crRNA) and the tracrRNA sequence. Functions of both tracrRNA and crRNA remained, including guiding the complex to the target sequence and triggering Cas9 endonuclease activity. Results indicated this chimeric RNA can effectively induce DSBs, which was also the first time a single guide RNA (sgRNA, also known as gRNA) was constructed. All these characteristics above demonstrated the possibility of developing the CRISPR/Cas9 system into a precise RNA-guided genome editing tool.

1.5.4 Plant-specific applications of CRISPR/Cas9 (type II) system for genome editing

The use of CRISPR/Cas9 for genome editing in plants was first demonstrated by Xie and Yang (2013). Three gRNAs were designed to knock-out the *OsMPK5* gene, a negative regulator of disease resistance, in *Oryza sativa* (rice). One gRNA targeted the noncoding strand and the other two targeted the coding strand. Restriction enzyme digestion suppressed PCR (RE-qPCR) analysis of mutants indicated the rate of cleavage using these gRNAs varied. The mutation frequencies ranged from 3.5% to 8.2%, and yielded deletions or insertions (indels) with large size variations. Seven potential off-target positions were found based on genomic sequence search, three of them were selected for further detection. Only one off-target position with a mutation frequency of 1.6% was detected by RE-qPCR. By comparing these three potential off-target positions, a larger distance from the first mismatch (in the off-targets) to PAM might induce a higher likelihood of an off-target effect.

Application of CRISPR/Cas9 system for genome modification in eudicot plants was also demonstrated. Jacobs et al. (2015) selected the *gfp* transgene and a glucosyl-transferase gene in soybean as targets to be knocked out. Two gRNAs designed for *gfp* targeted the 3' and 5' ends. Knock out efficiencies in *A.rhizogenes*-generated hairy roots were 88.2% (5' end) and 18.1% (3' end). Homoeologous genes Glyma01g38150 and Glyma11g07220 encoding glucosyltransferase were targeted individually and simultaneously, one gRNA per gene, and one gRNA targeting a conserved area of both genes. An average 70% indel frequency was detected in transgenic roots containing each individual gRNA, and 21% for conserved gRNA targeting both genes. Jacobs et al. (2015) also generated mutations in the microRNA (*miR*) family of genes, thereby confirming that short non-coding sequences can also be targeted via the CRISPR/Cas9 system. The *miR* genes encode short ~500 bp non-coding microRNAs (miRNAs); *miR1514* and *miR1509* were

selected as targets. Although the mature miRNAs were not mutated, indels and single mismatches were observed in those targeted genes.

Since 2013, the CRISPR/Cas9 system has been used for genome editing in numerous plant species, including a member of the Malvaceae family, *Gossypium hirsutum* (cotton). The cotton MYB-like (*GhMYB25-like*) gene is a transcription factor expressed in early stages of fiber cell differentiation (Walford et al., 2011). Since cotton is an allotetraploid, GhMYB25-like-gRNA1 and GhMYB25-like-gRNA2 were designed to target the two subgenomes, and were constructed in a multiple gRNA vector (Li et al., 2017). These gRNAs targeted two exons bracketing the same intron, with the goal of a 265 bp deletion. By applying two gRNAs, they achieved 100% small nucleotide deletions and up 21.4% of double cleavage (large knock-out) in both subgenome sequences.

DNA knock-in via CRISPR/Cas9 and homology-directed repair (HDR) was also achieved in plants. A sequential transformation method was developed to increase the knock-in efficiency for *Arabidopsis* (Miki et al., 2018). It had shown low efficiency when they used an “all-in-one” construct containing gRNA, *cas9* gene driven by the CaMV 35S promoter and the donor *gfp* fragment. For sequential transformation, parental lines (T₀) were transformed to contain the *cas9* gene driven by a germline-specific DD45 promoter. Plasmid containing donor *gfp* sequence, gRNA, and a Basta resistance gene (*ppt*) was then transformed into explants of these parental lines to regenerate T1 plants selected on media containing Basta. Subsequent T2 seeds were harvested from transgenic T1 plants. Without selection, T2 plants were then screened by PCR, and PCR-positive plants were sequenced and analyzed for gene targeting efficiency which was determined to be 8.3-9.1%. The *gfp* gene was targeted for insertion into endogenous

glycosylase genes: repressor of silencing 1 (*ros1*) and transcriptional activator DEMETER (*dme*). Green fluorescence was observed in two mutant T3 plants.

A CRISPR/Cas9 system-based tool developed by Čermák et al. (2017) generated high mutation rates using both single gRNA and multiple gRNA constructs. Direct cloning vectors and the modular assembly vectors were constructed for single gRNA or multiple gRNAs, respectively. For single gRNA, each constructed vector contained a *cas9* expression cassette and a single gRNA cassette controlled by a Pol III promoter (ATU6, At7SL, etc.). For the modular assembly, multiple gRNA spacers were assembled into Csy-type (CRISPR system *Yersinia*) ribonuclease 4 (Csy4) tRNA or ribozyme arrays. A Pol II promoter was known to drive better expression efficiency for long transcripts, so a polycistronic transcript (multiple gRNAs) was driven by the Pol II promoter from *Cestrum* yellow leaf curling virus (CmYLCV).

1.5.5 Off-target effects

The CRISPR/Cas9 system provides a powerful platform for basic research, enabling efficient and rapid genome editing. However, as the research progressed, an important shortcoming of the technology was noted – off-target effects. These created deletions or insertions in non-targeted areas of the genome.

Chromosome co-immunoprecipitation combined with high-throughput sequencing (ChIP-seq) has been used to analyze possible off-target sites of CRISPR/Cas9, and could detect off-target sites relatively well (Duan et al., 2014). Use of protoplast systems have also been used to sequence populations of edited cells, looking for prevalence of off-types. For example, Jacobs et al. (2015) detected potential off-target sequences by BLAST analysis, searching for the 23-bp gRNA target sequences in the soybean reference genome.

1.6 Phytoene Desaturase (PDS) as a Screenable Marker for Knock-outs

For this section, refer to Table 1.2. Phytoene desaturase (PDS) works as a key enzyme early in the carotenoid biosynthesis pathway in angiosperms. Since carotenoid biosynthesis is regulated by hormones, loss of functional PDS could impact chloroplast biogenesis as well as root, shoot and leaf development (Tian, 2015). The *pds3* mutant of *Arabidopsis* demonstrated changes in expression of several key genes in carotenoid, chlorophyll, abscisic acid (ABA) and gibberellin (GA) biosynthetic pathways (Qin et al., 2007); this functional-disruption mutant displayed albino and dwarf phenotypes. Comparing PDS gene sequences among several plant species, Kaur et al. (2018) identified three conserved domains or motifs: dinucleotide binding motif, putative substrate carrier motif, and carotenoid binding domain.

1.6.1 Genome editing - *pds* gene knock-outs

Since the loss of *pds* gene function might lead to a visible pale green or even albino phenotype (Nishitani et al., 2016), the *pds* gene has been widely used as a visible reporter gene for developing gene silencing or gene knock-out procedures.

Its use as a reporter gene began in studies on virus-induced gene silencing (VIGS), a method based on a RNAi-mediated antiviral mechanism in plants. Kumagai et al. (1995) infected *Nicotiana benthamiana* (tobacco) using a recombinant vector containing *pds* cDNA controlled by TMV-U1 coat protein subgenomic promoter. Inhibition of PDS expression was found in tobacco plants and a white phenotype was observed. The *pds* gene was also used as a target to examine VIGS under different conditions. For instance, to confirm the enhanced silencing efficiency of VIGS under low temperature and low humidity conditions in *Lycopersicon esculentum* (tomato; now *Solanum lycopersicum*) (Fu et al., 2006). This research also

demonstrated that silencing *pds* induced the accumulation of phytoene, thereby reducing expression of chlorophyll a and chlorophyll b.

Genome modification mediated by the CRISPR/Cas9 system in species among both monocots and eudicots often uses *pds* as a marker gene.

In both rice protoplasts and rice whole plants, mutagenesis of rice phytoene desaturase gene *OsPDS* were induced separately by two gRNAs that targeted both strands of exon 12 (Shan et al., 2013). Albino and dwarf phenotype plants were observed in biallelic heterozygous mutants.

Glycine max (soybean) hairy root tissues generated by *A. rhizogenes* transformation used two *pds* genes, *GmPDS11* and *GmPDS18*, as targets (Du et al., 2016). Albino and dwarf phenotypes were caused by functional knock-out mutations in these two genes.

Nishitani et al. (2016) selected three different exons as targets using four gRNAs in *Malus pumila* (apple). All four gRNAs were introduced separately and resulted in albino transformants with different levels of white or pale green shoots.

Vitis vinifera (grape) phytoene desaturase gene (*VvPDS*) was knocked out by Nakajima et al. (2017). The mutation efficiency varied among four gRNAs that targeted four different exons. Those that targeted exons 3 and 7 displayed the highest mutation rates. And lower leaves (in late stage of development; mutations first appeared at earlier developmental stage) displayed higher mutation rates than higher leaves (in early stage of development) in the regenerated plants.

In *Manihot esculenta* (cassava), albino plants were developed by targeting and mutation in exon 13 of the *pds* gene, with an efficiency up to 100% (Odipio et al., 2017).

To knock-out the *Citrullus lanatus* (watermelon) *pds* gene, target sites in different exons were selected, and the two designed gRNAs were introduced on separate vectors for analysis in

protoplasts and on the same vector for analysis in stable watermelon transformants (Tian et al., 2017). Point insertions and multiple base deletions were found.

In *Coffea arabica* (coffee), gRNA target sequences were predicted by a genome screening tool developed by Breitler et al. (2018). Exons 3, 5 and 14 of the coffee phytoene desaturase gene (*CcPDS*) were targeted. Three single gRNAs vectors and a vector containing gRNAs that targeted both exons 3 and 5 were constructed. Both small insertions and small deletions were only found in exon 14-targeted plants with a mutation rate of 30.4%.

In *Musa acuminata* (banana), a gRNA targeted to the most conserved domain in exon 5 of *pds* was designed (Kaur et al., 2018). Both albino phenotypes and slow growth were observed in the mutated regenerated plants.

Parkhi et al. (2018) targeted three exons of the tomato *pds* gene (NCBI X78271) using three single gRNAs; point mutations and three-base mutations were found in two exons. The albino phenotype was also observed in T0 regenerated shoots.

Studies using *pds* as a reporter gene for knock-outs in the Malvaceae family have not been reported. However, to study the temperature effect of geminivirus-mediated gene silencing, Tuttle et al. (2008) selected chelatase subunit I gene (*chlI*) and the *pds* gene as targets using a silencing vector in cotton. The cotton leaf crumple virus (CLCrV) coat protein gene was replaced by a 326-bp *pds* gene fragment controlled by the AR1 promoter. Visible changes were observed in the gene-silenced tissues.

Table 1.2 Summary of *pds* knock-outs among species using CRISPR/Cas9

Species	Single/multiple gRNA	No. target sequences	Target location (exon)	Mutant phenotype characteristics	Mutation types	References
Rice	Single	2	12	Albino and dwarf phenotype	Point insertion and multiple base deletion	Shan et al. (2013)
Soybean	Single	4	2, 4, 5, 6	Albino and dwarf phenotype	Insertion and deletion	Du et al. (2016)
Apple	Single	4	3, 6, 7	Albino or pale green shoots	Insertion and deletion	Nishitani et al. (2016)
Grape	Single	4	1, 3, 7, 10	Albino leaves	Point insertion and multiple base deletion	Nakajima et al. (2017)
Cassava	Single	2	13	Albino plant lines	Minor insertion, deletion, and substitution	Odipto et al. (2017)
Watermelon	Single and multiple	2	1, 3	Mosaic or totally albino shoot	Point insertion and multiple base deletion	Tian et al. (2017)
Coffee	Single and multiple	3	3, 5, 14	Abnormal shoot	Small insertion and small deletion	Breitler et al. (2018)
Banana	Single	1	5	Slow growth and albinism	Insertion, deletion and both	Kaur et al. (2018)
Tomato	Single	3	2, 4, 5	Albino shoot	Point mutation and three base deletion	Parkhi et al. (2018)

1.6.2 Potential use of PDS in kenaf

A *pds* gene has yet to be identified in kenaf for use in knock-out experiments. Since a cotton (Malvaceae family) *pds* gene had been identified (Tuttle et al., 2008), kenaf should also contain at least one. Since the transcriptome sequence of kenaf is available (Chen et al., 2014), a conserved domain search and comparison analyses among species could be used to identify a *pds* gene in kenaf. The *pds* gene would be used as a knock-out reporter gene for development and confirmation that the CRISPR/Cas9 system can be used for genome editing in kenaf. Although this trait is not commercially important, development of a combined procedure that leads to generation of genome-edited kenaf plants will be invaluable to its continued improvement as a biomass crop.

1.7 References

- Baldwin BS, Hollowell JE, Mosley JW, Cossar RD (2006) Registration of 'Whitten' kenaf. *Crop Science* 46:988-990
- Banks SW, Gossett DR, Lucas MC, Millhollon EP, LaCelle MG (1993) *Agrobacterium*-mediated transformation of kenaf (*Hibiscus cannabinus* L.) with the β -glucuronidase (GUS) gene. *Plant Molecular Biology Reporter* 11:101-104
- Barrangou R, Fremaux C, Deveau H, Richards M, Boyaval P, Moineau S, Romero D, A, Horvath P (2007) CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 315:1709-1712
- Barrangou R (2015) The roles of CRISPR–Cas systems in adaptive immunity and beyond. *Current Opinion in Immunology* 32:36-41
- Beyer EM (1976) A potent inhibitor of ethylene action in plants. *Plant Physiology* 58:268-271
- Bolotin A, Quinquis B, Sorokin A, Ehrlich SD (2005) Clustered regularly interspaced short palindromic repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology* 151:2551-2561
- Breitler JC, Dechamp E, Campa C, Rodrigues LAZ, Guyot R, Marraccini P, Etienne H (2018) CRISPR/Cas9-mediated efficient targeted mutagenesis has the potential to accelerate the domestication of *Coffea canephora*. *Plant Cell, Tissue and Organ Culture* 134:383-394
- Brouns SJ, Jore MM, Lundgren M, Westra ER, Slijkhuis RJ, Snijders AP, Dickman MJ, Makarova KS, Koonin EV, van der Oost J (2008) Small CRISPR RNAs guide antiviral defense in prokaryotes. *Science* 321:960-964
- Čermák T, Curtin SJ, Gil-Humanes J, Čegan R, Kono TJ, Konečná E, Belanto JJ, Starker CG, Mathre JW, Voytas DF (2017) A multipurpose toolkit to enable advanced genome engineering in plants. *The Plant Cell* 29:1196-1217
- Chen P, Ran S, Li R, Huang Z, Qian J, Yu M, Zhou R (2014) Transcriptome de novo assembly and differentially expressed genes related to cytoplasmic male sterility in kenaf (*Hibiscus cannabinus* L.). *Molecular Breeding* 34:1879-1891
- Christie PJ, Gordon JE (2014) The *Agrobacterium* Ti plasmids. *Microbiology Spectrum* 2:PLAS-0010-2013. <https://doi.org/10.1128/microbiolspec.PLAS-0010-2013>
- Chylinski K, Makarova KS, Charpentier E, Koonin EV (2014) Classification and evolution of type II CRISPR-Cas systems. *Nucleic Acids Research* 42:6091-6105
- Corder B, Ericsson S, Uhler T (2017) Structure and potential of the CRISPR-Cas 10 system. *Bioscription*. <http://bioscriptionblog.com/2017/12/10/structure-potential-crispr-cas-10/>. Accessed 13 August 2019

- Cormack BP, Valdivia RH, Falkow S (1996) FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* 173:33-38
- Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J, Charpentier E (2011) CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* 471:602-607
- Dempsey JM (1975) *Fiber crops*. University Presses of Florida, Gainesville, FL
- Dewir YH, Naidoo Y, da Silva JAT (2018) Thidiazuron-induced abnormalities in plant tissue cultures. *Plant Cell Reports* 37:1451-1470
- Dixon RA, Gonzales RA (1994) *Plant cell culture: a practical approach*, 2nd edn. Oxford University Press, New York, NY
- Dodds JH, Roberts LW (1987) *Plant tissue culture*. Cambridge University Press, New York, NY
- Du H, Zeng X, Zhao M, Cui X, Wang Q, Yang H, Cheng H, Yu D (2016) Efficient targeted mutagenesis in soybean by TALENs and CRISPR/Cas9. *Journal of Biotechnology* 217:90-97
- Duan J, Lu G, Xie Z, Lou M, Luo J, Guo L, Zhang Y (2014) Genome-wide identification of CRISPR/Cas9 off-targets in human genome. *Cell Research* 24:1009-1012
- Ecker JR, Davis RW (1986) Inhibition of gene expression in plant cells by expression of antisense RNA. *Proceedings of the National Academy of Sciences USA* 83:5372-5376
- Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391:806-811
- Fu DQ, Zhu BZ, Zhu HL, Zhang HX, Xie YH, Jiang WB, Zhao XD, Luo YB (2006) Enhancement of virus-induced gene silencing in tomato by low temperature and low humidity. *Molecules and Cells* 21:153-160
- Gaj T, Gersbach CA, Barbas CF III (2013) ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends in Biotechnology* 31:397-405
- Garneau JE, Dupuis MÈ, Villion M, Romero DA, Barrangou R, Boyaval P, Fremaux C, Horvath P, Magadán AH, Moineau S (2010) The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. *Nature* 468:67-71
- Gaspar T, Kevers C, Penel C, Greppin H, Reid DM, Thorpe TA (1996) Plant hormones and plant growth regulators in plant tissue culture. *In Vitro Cellular & Developmental Biology-Plant* 32:272-289

- Gelvin SB (2003) *Agrobacterium*-mediated plant transformation: the biology behind the “gene-jockeying” tool. *Microbiology and Molecular Biology Reviews* 67:16-37
- Gelvin SB (2012) Traversing the cell: *Agrobacterium* T-DNA’s journey to the host genome. *Frontiers in Plant Science* 3:52. <https://doi.org/10.3389/fpls.2012.00052>
- Goforth CE, Fuller MJ (eds) (1994) A summary of kenaf production and product development research 1989-1993. *Bulletin, Mississippi Agricultural and Forestry Experiment Station, Mississippi State, MS*
- Goldman PR, Northrop DB (1976) Purification and spectrophotometric assay of neomycin phosphotransferase II. *Biochemical and Biophysical Research Communications* 69:230-236
- Grau J, Boch J, Posch S (2013) TALENoffer: genome-wide TALEN off-target prediction. *Bioinformatics* 29:2931-2932
- Guo B, Abbasi BH, Zeb A, Xu LL, Wei YH (2011) Thidiazuron: a multi-dimensional plant growth regulator. *African Journal of Biotechnology* 10:8984-9000
- Herath SP, Suzuki T, Hattori K (2005) Factors influencing *Agrobacterium* mediated genetic transformation of kenaf. *Plant Cell, Tissue and Organ Culture* 82:201-206
- Hooykaas PJJ, Hofker M, den Dulk-Ras H, Schilperoort RA (1984) A comparison of virulence determinants in an octopine Ti plasmid, a nopaline Ti plasmid, and an Ri plasmid by complementation analysis of *Agrobacterium tumefaciens* mutants. *Plasmid* 11:195-205
- Htwe NN, Ling HC, Zaman FQ, Maziah M (2014) Plant genetic transformation efficiency of selected Malaysian rice based on selectable marker gene (*hptII*). *Pakistan Journal of Biological Sciences* 17:472-481
- Husaini AM, Abdin MZ, Parray GA, Sanghera GS, Murtaza I, Alam T, Srivastava DK, Farooqi H, Khan HN (2010) Vehicles and ways for efficient nuclear transformation in plants. *GM Crops* 1:276-287. <https://doi.org/10.4161/gmcr.1.5.14660>
- Huetteman, CA, Preece JE (1993). Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell, Tissue and Organ Culture* 33:105-119
- Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A (1987) Nucleotide sequence of the *iap* gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. *Journal of Bacteriology* 169:5429-5433
- Jacobs TB, LaFayette PR, Schmitz RJ, Parrott WA (2015) Targeted genome modifications in soybean with CRISPR/Cas9. *BMC Biotechnology* 15:16. <https://doi.org/10.1186/s12896-015-0131-2>

- Jansen R, Embden JDV, Gaastra W, Schouls LM (2002) Identification of genes that are associated with DNA repeats in prokaryotes. *Molecular Microbiology* 43:1565-1575
- Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *The EMBO Journal* 6:3901-3907
- Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337:816-821
- Joung JK, Sander JD (2013) TALENs: a widely applicable technology for targeted genome editing. *Nature Reviews Molecular Cell Biology* 14:49-55
- Jung HJG, Samac DA, Sarath G (2012) Modifying crops to increase cell wall digestibility. *Plant Science* 185:65-77
- Kaur N, Alok A, Kaur N, Pandey P, Awasthi P, Tiwari S (2018) CRISPR/Cas9-mediated efficient editing in phytoene desaturase (PDS) demonstrates precise manipulation in banana cultivar Rasthali genome. *Functional & Integrative Genomics* 18:89-99
- Khatun A, Laouar L, Davey MR, Power JB, Mulligan BJ, Lowe KC (1993) Effects of Pluronic F-68 on shoot regeneration from cultured jute cotyledons and on growth of transformed roots. *Plant Cell, Tissue and Organ Culture* 34:133-140
- Khatun A, Zabun N, Shirin M, Chandan KS, Shana B (2003) An efficient protocol for plant regeneration from the cotyledons of kenaf (*Hibiscus cannabinus* L.). *Biotechnology* 2:86-93
- Koch A, Kogel KH (2014) New wind in the sails: improving the agronomic value of crop plants through RNAi-mediated gene silencing. *Plant Biotechnology Journal* 12:821-831
- Kumagai MH, Donson J, Della-Cioppa G, Harvey D, Hanley K, Grill LK (1995) Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. *Proceedings of the National Academy of Sciences USA* 92:1679-1683
- Kumar PP, Lakshmanan P, Thorpe TA (1998) Regulation of morphogenesis in plant tissue culture by ethylene. *In Vitro Cellular & Developmental Biology-Plant* 34:94-103
- Leffel SM, Mabon SA, Stewart CN Jr (1997) Applications of green fluorescent protein in plants. *Biotechniques* 23:912-918
- Li C, Unver T, Zhang B (2017) A high-efficiency CRISPR/Cas9 system for targeted mutagenesis in cotton (*Gossypium hirsutum* L.). *Scientific Reports* 7:43902. <https://doi.org/10.1038/srep43902>

- Lim T-K (1997) Optimization of organogenic regeneration and in vitro selection for *Cristulariella moricola* resistance in kenaf (*Hibiscus cannabinus* L.) Thesis, Mississippi State University
- Liu D (1994) Protoplast isolation, fusion, and in vitro regeneration of kenaf (*Hibiscus cannabinus* L.). Thesis, Mississippi State University
- Lorence A, Verpoorte R (2004) Gene transfer and expression in plants. In: Balbás P, Lorence A (eds) Recombinant gene expression. Humana Press, Totowa, NJ, pp 329-350
- Lu CY (1993) The use of thidiazuron in tissue culture. *In Vitro Cellular & Developmental Biology-Plant* 29:92-96
- Makarova KS, Haft DH, Barrangou R, Brouns SJ, Charpentier E, Horvath P, Moineau S, Mojica FJM, Wolf YI, Yakunin AF, van der Oost J, Koonin EV (2011) Evolution and classification of the CRISPR-Cas systems. *Nature Reviews Microbiology* 9:467-477
- Miki D, Zhang W, Zeng W, Feng Z, Zhu JK (2018) CRISPR/Cas9-mediated gene targeting in *Arabidopsis* using sequential transformation. *Nature Communications* 9:1967. <https://doi.org/10.1038/s41467-018-04416-0>
- Mojica FJM, Díez-Villaseñor C, Soria E, Juez G (2000) Biological significance of a family of regularly spaced repeats in the genomes of Archaea, Bacteria and mitochondria. *Molecular Microbiology* 36:244-246
- Mojica FJM, García-Martínez J, Soria E (2005) Intervening sequences of regularly spaced prokaryotic repeats derive from foreign genetic elements. *Journal of Molecular Evolution* 60:174-182
- Murashige T, Skoog FA (1962) Revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* 15:473-497
- Nakajima I, Ban Y, Azuma A, Onoue N, Moriguchi T, Yamamoto T, Toki S, Endo M (2017) CRISPR/Cas9-mediated targeted mutagenesis in grape. *PLoS ONE* 12:e0177966. <https://doi.org/10.1371/journal.pone.0177966>
- Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *The Plant Cell* 2:279-289
- Nemudryi AA, Valetdinova KR, Medvedev SP, Zakian SM (2014) TALEN and CRISPR/Cas genome editing systems: tools of discovery. *Acta Naturae* 6:19-40
- Nester EW, Kosuge T (1981) Plasmids specifying plant hyperplasias. *Annual Review of Microbiology* 35:531-565

- Nishitani C, Hirai N, Komori S, Wada M, Okada K, Osakabe K, Yamamoto T, Osakabe Y (2016) Efficient genome editing in apple using a CRISPR/Cas9 system. *Scientific Reports* 6:31481. <https://doi.org/10.1038/srep31481>
- Nitsch JP, Nitsch C (1969) Haploid plants from pollen grains. *Science* 163:85-87
- Nowara D, Gay A, Lacomme C, Shaw J, Ridout C, Douchkov D, Hensel G, Kumlehn J, Schweizer P (2010) HIGS: host-induced gene silencing in the obligate biotrophic fungal pathogen *Blumeria graminis*. *The Plant Cell* 22:3130–3141
- Odipio J, Alicai T, Ingelbrecht I, Nusinow DA, Bart R, Taylor NJ (2017) Efficient CRISPR/Cas9 genome editing of phytoene desaturase in cassava. *Frontiers in Plant Science* 8:1780. <https://doi.org/10.3389/fpls.2017.01780>
- Parkhi V, Bhattacharya A, Choudhary S, Pathak R, Gawade V, Palan B, Alamalakala L, Mikkilineni V, Char B (2018) Demonstration of CRISPR-cas9-mediated *pds* gene editing in a tomato hybrid parental line. *Indian Journal of Genetics and Plant Breeding* 78:132-137
- Pérez-Clemente RM, Pérez-Sanjuán A, García-Férriz L, Beltrán JP, Cañas LA (2005) Transgenic peach plants (*Prunus persica* L.) produced by genetic transformation of embryo sections using the green fluorescent protein (GFP) as an in vivo marker. *Molecular Breeding* 14:419-427
- Padilla IMG, Burgos L (2010) Aminoglycoside antibiotics: structure, functions and effects on in vitro plant culture and genetic transformation protocols. *Plant Cell Reports* 29:1203–1213
- Pourcel C, Salvignol G, Vergnaud G (2005) CRISPR elements in *Yersinia pestis* acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. *Microbiology* 151:653-663
- Qin G, Gu H, Ma L, Peng Y, Deng XW, Chen Z, Qu LJ (2007) Disruption of phytoene desaturase gene results in albino and dwarf phenotypes in *Arabidopsis* by impairing chlorophyll, carotenoid, and gibberellin biosynthesis. *Cell Research* 17:471
- Rajasekaran K, Cary JW, Jaynes JM, Cleveland TE (2005) Disease resistance conferred by the expression of a gene encoding a synthetic peptide in transgenic cotton (*Gossypium hirsutum* L.) plants. *Plant Biotechnology Journal* 3:545-554
- Rosellini D (2011) Selectable marker genes from plants: reliability and potential. *In Vitro Cellular & Developmental Biology-Plant* 47:222-233
- Ruotolo G, Del Toro A, Chiaiese P, Filippone E (2011) Multiple buds are an alternative target to genetic manipulation of kenaf (*Hibiscus cannabinus* L.). *Industrial Crops and Products* 34:937-942

- Samanthi PW, Puad AM, Suhaimi N, Kumar SM, Aini ASN (2013) In vitro shoot regeneration from leaf explants of kenaf (*Hibiscus cannabinus* L.). *Sains Malaysiana* 42:1505-1510
- Saurabh S, Vidyarthi AS, Prasad D (2014) RNA interference: concept to reality in crop improvement. *Planta* 239:543-564
- Senthil-Kumar M, Mysore KS (2011) Caveat of RNAi in plants: the off-target effect. In: Kodama H, Komamine A (eds) *RNAi and plant gene function analysis*. Humana Press, Totowa, NJ, pp 13-25
- Shah SA, Erdmann S, Mojica FJ, Garrett RA (2013) Protospacer recognition motifs: mixed identities and functional diversity. *RNA Biology* 10:891-899
- Shamsunnaher MA, Mukul MHR, Biswash MR, Zahan A (2013) Study on the appropriate dose of BAP for regeneration of kenaf (*Hibiscus cannabinus* L.). *Eco-friendly Agriculture Journal* 6:6-9
- Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z, Zhang K, Liu J, Xi JJ, Qiu JL, Gao C (2013) Targeted genome modification of crop plants using a CRISPR-Cas system. *Nature Biotechnology* 31:686-688
- Sinkunas T, Gasiunas G, Fremaux C, Barrangou R, Horvath P, Siksnys V (2011) Cas3 is a single-stranded DNA nuclease and ATP-dependent helicase in the CRISPR/Cas immune system. *The EMBO Journal* 30:1335-1342
- Srivatanakul M, Park SH, Salas MG, Smith RH (2001) Transformation parameters enhancing T-DNA expression in kenaf (*Hibiscus cannabinus*). *Journal of Plant Physiology* 158:255-260
- Sticklen M (2006) Plant genetic engineering to improve biomass characteristics for biofuels. *Current Opinion in Biotechnology* 17:315-319
- Strauch E, Arnold W, Alijah R, Wohlleben W, Puhler A, Eckes P, Donn G, Uhlmann E, Hein F, Wengenmayer F (1994) Phosphinothricin-resistance gene, and its use. U.S. Patent No. 5,276,268. U.S. Patent and Trademark Office, Washington, DC
- Sugiyama M (1999) Organogenesis in vitro. *Current Opinion in Plant Biology* 2:61-64
- Tian L (2015) Recent advances in understanding carotenoid-derived signaling molecules in regulating plant growth and development. *Frontiers in Plant Science* 6:790. <https://doi.org/10.3389/fpls.2015.00790>
- Tian S, Jiang L, Gao Q, Zhang J, Zong M, Zhang H, Ren Y, Guo S, Gong G, Xu Y (2017) Efficient CRISPR/Cas9-based gene knockout in watermelon. *Plant Cell Reports* 36:399-406

- Trigiano RN, Gray DJ (eds) (1999) Plant tissue culture concepts and laboratory exercises. CRC Press, New York, NY
- Tuttle JR, Idris AM, Brown JK, Haigler CH, Robertson D (2008) Geminivirus-mediated gene silencing from cotton leaf crumple virus is enhanced by low temperature in cotton. *Plant Physiology* 148:41-50
- Tzfira T, Citovsky V (2006) *Agrobacterium*-mediated genetic transformation of plants: biology and biotechnology. *Current Opinion in Biotechnology* 17:147-154
- Walford SA, Wu Y, Llewellyn DJ, Dennis ES (2011) GhMYB25-like: a key factor in early cotton fibre development. *The Plant Journal* 65:785-797
- Webber CL III, Bledsoe VK (2002) Kenaf yield components and plant composition. In: Janick J, Whipkey A (eds) Trends in new crops and new uses. ASHS Press, Alexandria, VA, pp 348-357
- Woodward AW, Bartel B (2005) Auxin: regulation, action, and interaction. *Annals of Botany* 95:707-735
- Xie K, Yang Y (2013) RNA-guided genome editing in plants using a CRISPR–Cas system. *Molecular Plant* 6:1975-1983
- Young MM (2000) Genetic transformation of kenaf. Dissertation, Mississippi State University
- Zhao Y (2010) Auxin biosynthesis and its role in plant development. *Annual Review of Plant Biology* 61:49-64

CHAPTER II

METHOD AND MATERIALS

2.1 Plant Regeneration

2.1.1 Plants material preparation and culture conditions

Kenaf cultivar Whitten seeds were provided by Dr. Margaret Young, Elizabeth City State University; E41 seeds were provided by Dr. Brian Baldwin, Mississippi State University, recovered from frozen storage (-20°C). The basal medium (MSO) used for all experiments was composed of Murashige & Skoog (1962; MS) basal salts and vitamins, plus 3% (w/v) sucrose and 0.8% (w/v) plant tissue culture agar (PhytoTechnology Laboratories). All MS-based media were adjusted to a pH of 5.8 and autoclaved for 35 min (121°C at 100 kPa). Any additives to this basal medium will be listed in the appropriate sections. All PGR and stock solutions were diluted in sterile deionized water, filter-sterilized using a 0.22 µm filter, and stored at -20°C; PGRs and silver nitrate (AgNO₃) were added to post-autoclaved media. Antibiotics were treated in the same manner.

All seeds and plant tissues were cultured on media in 100 x 20 mm petri dishes. Seedlings and resulting plants were maintained in MagentaTM boxes (GA-7; 77 x 77 mm x 97 mm tall). All seeds, seedlings and plant tissues were cultured in a growth chamber (Percival CU-36L5) at 25°C, under a 16-hr photoperiod (155 µmol/m²/s light intensity).

2.1.1.1 Seed surface disinfestation and germination

To germinate seeds aseptically, surface sterilization of seeds was conducted using dilute concentrations of commercial bleach (composed of 6% sodium hypochlorite). Since surface disinfestation may decrease the germination rate of kenaf seeds, an experiment was designed to determine the best seed treatment and germination procedures.

E41 seeds were collected from frozen storage and 10 each were placed in 4 layers of 70 x 70 mm cheesecloth; each was formed into a loose pouch using a twist-tie. Pouches were dipped in 70% (v/v) ethanol three times, 60 sec each time, then rinsed with sterile deionized water. Seed pouches were then placed in solutions containing different concentrations of sodium hypochlorite (1.3%, 1.5%, 1.9%) using diluted commercial bleach; each solution also contained 0.5% (v/v) sodium dodecyl sulphate (SDS). Seed pouches were placed in autoclaved flasks and shaken at 120 rpm for the designated time (25 min or 30 min). Then, seed pouches were rinsed with sterile deionized water three times. Pouches were opened in sterile 15 x 100 mm petri dishes, and seeds were placed on MSO medium (15 seeds per plate) for germination. Contamination and germination were observed and recorded after 2 weeks. For Whitten seeds, the same seed surface disinfestation protocol was used.

2.1.1.2 Plant maintenance and manipulation

When the radicle of seeds emerged and grew to a length of 5 to 10 mm, seeds were moved from 20 x 100 mm petri dishes to Magenta™ boxes containing MSO medium for continued plant growth and use as leaf explant donors. In vitro plantlets were maintained and multiplied by cutting and culturing nodal sections, 5 sections per Magenta™ box.

2.1.2 Organogenesis - cultivar Whitten

2.1.2.1 Experiment 1

The combination of TDZ and NAA was selected for use, based on Lim (1997) and Young (2000). This experiment was designed to select the PGR concentrations that yielded organogenic callus and potential shoots. The 3 x 5 media matrix contained the following PGR concentrations: 0.05, 0.10, 0.15 mg/l NAA and 0.5, 1.0, 2.0, 3.0, 4.0 mg/l TDZ (media M1 to M15; Table 2.1). Leaf explants (5 x 5 mm) were harvested from in vitro-maintained Whitten plants (classified as mature leaves). All 15 PGR combinations and MSO as control had two replicates with each replicate plate containing 10 explants. All the explants were moved to fresh media every 4 weeks. Presence, amount and type of calli generated were noted weekly.

Table 2.1 Regeneration media tested using Whitten mature leaves

Medium	Auxin NAA (mg/l)	Cytokinin TDZ (mg/l)
M1	0.05	0.5
M2	0.05	1.0
M3	0.05	2.0
M4	0.05	3.0
M5	0.05	4.0
M6	0.1	0.5
M7	0.1	1.0
M8	0.1	2.0
M9	0.1	3.0
M10	0.1	4.0
M11	0.15	0.5
M12	0.15	1.0
M13	0.15	2.0
M14	0.15	3.0
M15	0.15	4.0

2.1.2.2 Experiment 2

To determine if the ratio between NAA and TDZ contributed to organogenic regeneration efficiency, additional PGR concentrations were used in this experiment. Based on experiment 1 results, two media based on the a/c ratio of M12 (0.15/1; Table 2.1) were compared to M12: M12A contained 0.1125 mg/l NAA and 0.75 mg/l TDZ, M12B contained 0.225 mg/l NAA and 1.5 mg/l TDZ. Leaf explants (5 x 5 mm) were placed on the three media with three replicates of 10 leaf explants per medium. Presence and amount of organogenic calli were recorded every 4 weeks.

2.1.2.3 Experiment 3

Explants of mature Whitten leaves (5 x 5 mm) and petioles (5 mm) were used in this experiment to compare responses in culture and to determine effects of silver nitrate (AgNO_3). Based on previous experimental results, 9 shoot regeneration media were used. Media TNA1 to TNA9 contained 0.1 mg/l NAA, 10.2 mg/l AgNO_3 , and 9 concentrations of TDZ (0.25, 0.3, 0.35, 0.79, 0.95, 1.1, 2.5, 3.0, 3.5 mg/l). For each explant type, two replicates of 12 explants were cultured on each medium. Organogenic calli presence and amount for both tissues were recorded at 2 weeks and 6 weeks after placement on media.

2.1.2.4 Experiment 4

Since Whitten seeds were in limited supply, this was the only experiment designed to analyze seedling tissues for responses on a medium identified by Dr. Margaret Young in separate kenaf experiments (personal communication). Cotyledon (5 x 5 mm), hypocotyl (5 mm) and petiole (5 mm) sections were harvested from 10 to 14 day-old Whitten seedlings. Medium S5 (MSO supplemented with 0.1 mg/l NAA, 2.5 mg/l TDZ and 10.2 mg/l AgNO_3) was used.

(Medium S5 was the same medium formulation as TNA7 listed above). Six replicates of 4 petiole explants, 8 cotyledon explants and 8 hypocotyl explants were placed on this medium. Results were observed after 4 weeks in culture.

2.1.3 Organogenesis - cultivar E41

2.1.3.1 Experiment 1

Cotyledon (5 x 5 mm) and cotyledonary petiole (5 mm) sections were excised from 10 to 14 day-old E41 seedlings. In addition, cotyledonary nodal sections (cotnodes) were split longitudinally and set as the positive control. Media reported by others to induce shoots on various kenaf explants noted in Table 1.1 were compared. These media are listed in Table 2.2 and include: S1 (Khatun et al., 2003); S2 (Shamsunnaher et al., 2013), S3 (Sultana et al., 2016; this medium was reported to produce abundant callus only), S4 (Young, 2000; this medium was used to successfully regenerate E41 shoots from mature leaf explants, and was the same medium formulation as TNA3 listed above), and S5 (used in Whitten experiment 4). There were two replicates of 8 explants for seedling cotyledon tissues, and two replicates of 3 explants for seedling petiole and cotnode tissues. All explants were moved to fresh media every 4 weeks. Calli and shoot initiation results were recorded every 2 weeks.

Table 2.2 Regeneration media tested using E41 seedling tissues

Medium	-----Auxin-----		----Cytokinin----		Additional component	
	PGR	mg/l	PGR	mg/l	Chemical	mg/l
S1	IAA	0.5	BAP	5.0	-	-
S2	IAA	0.5	BAP	3.0	-	-
S3	NAA	2	BAP	7.5	-	-
S4	NAA	0.1	TDZ	0.35	AgNO ₃	10.2
S5	NAA	0.1	TDZ	2.5	AgNO ₃	10.2

2.1.3.2 Experiment 2

An additional experiment using medium S5 was designed for E41 seedling tissues. Cotyledon (5 x 5 mm), hypocotyl (5 mm) and cotyledonary petiole (5 mm) sections were harvested from 10 to 14 day-old E41 seedlings. Three replicates of 4 petiole explants, 8 cotyledon and 8 hypocotyl explants were placed on S5. To determine if the regeneration potential differed within the cotyledon and petiole organs, the positions of each explant was tracked throughout the experiment. All explants were moved to fresh S5 every 4 weeks. Calli and shoot initiation results were recorded every 2 weeks.

2.2 Transformation

2.2.1 Dose-response curves for two antibiotics

2.2.1.1 Experiment 1

Explants including cotyledon (5 x 5 mm), hypocotyl (5 mm), petiole (5 mm) and cotnode sections were harvested from 10 to 14 day-old E41 seedlings. To test the selection efficiency of hygromycin for transgenic cells/tissues, a dose-response curve was generated, looking for the minimum inhibitory concentration (MIC) that halted wild-type cell growth. Explants were placed

on S5 medium (MS-O supplemented with 0.1 mg/l NAA, 2.5 mg/l TDZ and 10.2 mg/l AgNO₃) containing varying concentrations of hygromycin (2.5, 5, 7.5 and 10 mg/l). S5 without antibiotics was set as the control. For each medium, there were three replicates of 10 cotyledon, 10 hypocotyl, 4 petiole and 4 cotnode explants. Results were recorded weekly.

2.2.1.2 Experiment 2

A dose-response curve was also generated for geneticin. Cotyledon (5 x 5 mm), hypocotyl (5 mm), petiole (5 mm) and cotnode sections were used. S5 containing geneticin concentrations (2.5, 5, 7.5, 10, 12.5 and 15 mg/l) were used to test the sensitivity (selective efficiency) of seedling explants to geneticin. S5 medium without antibiotic was set as the control. There were three replicates of 10 cotyledon, 10 hypocotyl, 4 petiole and 4 cotnode explants. Results were recorded every two weeks.

2.2.2 Plasmid and *A. tumefaciens* preparation

Plasmid pGH00.0126 was used in the following experiments. Plasmid pGH00.0126 was a gift from Mark Gultinan and Siela Maximova (Addgene plasmid #64257; <http://n2t.net/addgene:64257>; RRID:Addgene 64257; Maximova et al., 2003). In this binary T-DNA vector, both neomycin phosphotransferase II (*nptII*) and enhanced green fluorescence protein (*egfp*) genes were controlled by the strong constitutive promoter E12-Ω. As described by Maximova et al. (2003), it was composed of the CaMV 35S promoter, a duplicated part of its upstream sequence, and the 5' untranslated region of the tobacco mosaic virus. *E. coli* containing pGH00.0126 was maintained on Luria-Bertani, Lennon agar (LB: 10 g/l tryptone, 5 g/l yeast extract, 5 g/l sodium chloride, 15 g/l Bacto-agar, pH 7.5) containing 50 mg/l kanamycin for selection.

Plasmid pGH00.0126 was transferred to EHA105 by tri-parental mating. Cultures of *E. coli* harboring pGH00.0126 and pRK2013 were separately inoculated in 10 ml LB broth containing 50 mg/l kanamycin and cultured overnight on a shaking incubator (150 rpm) at 37°C. *A. tumefaciens* harboring EHA105 was inoculated in 10 ml LB broth containing 25 mg/l chloramphenicol and cultured overnight (up to 20 hr) on a shaker (150 rpm) at room temperature. Using a pipettor, 10 µl of EHA105, 5 µl of *E. coli* with pGH00.0126 and 5 µl of pRK2013 were overlaid onto each other on LB agar and co-cultured for 2 days at 28°C in the dark. One-half of the resulting growth was resuspended in 1 ml LB broth. A 100X serial dilution of the mixed culture was inoculated on LB agar containing 50 mg/l kanamycin and 25 mg/l chloramphenicol, and incubated at 28°C. After 2 to 3 days, a single colony was picked and restreaked on the same selective medium (LB plate with 50 mg/l kanamycin and 25 mg/l chloramphenicol). This step was repeated twice to ensure generation of a single pure colony of *A. tumefaciens* containing EHA105 and pGH00.0126; this strain was labeled EHApGH.

2.2.3 Transformation procedure

2.2.3.1 Experiment 1

This transformation procedure was based on procedures described by other researchers (Herath et al., 2005; Ruotolo et al., 2011; Withanage et al., 2015). Cotyledon (5 x 5 mm), petiole (5 mm) and cotnode (split longitudinally) explants were excised from 10 to 14 day-old E41 and Whitten seedlings. All explants were pre-cultured on S5 medium 2 days before co-culture. A single colony of EHApGH was picked and inoculated in 40 ml LB broth containing 50 mg/l kanamycin and 25 mg/l chloramphenicol, and grown overnight on a shaking incubator (150 rpm) at 26°C.

EHApGH was centrifuged and resuspended in S5 medium plus 39.24 mg/l acetosyringone, but without AgNO₃. The OD₆₀₀ value was determined, and diluted to OD₆₀₀ = 0.6-0.8 in the same medium. Explants were inoculated by immersion in EHApGH for 30 min in 100 x 20 mm petri dishes. Inoculated explants were blotted dry using sterile Whatman™ filter paper, and transferred to S5 containing 39.24 mg/l acetosyringone, but without AgNO₃. Explants were co-cultured in a growth chamber for 2 days.

After co-culturing, explants were rinsed in a solution containing 500 mg/l cefotaxime, blotted dry and placed on selective medium. Based on dose-response curve results, petiole and cotnode sections were placed on selection medium S5 containing 7.5 mg/l geneticin and 500 mg/l cefotaxime; cotyledon sections were placed on selection medium S5 containing 10 mg/l geneticin and 500 mg/l cefotaxime. There were 8 explants per plate, yielding 4 cotnode plates, 10 cotyledon plates and 8 petiole plates. Explants were transferred to fresh selection media every 4 weeks. Expression of EGFP was screened in a dark room via microscopy using attached blue light filter (blue light excitation at a wavelength of 440–480 nm and green light emission peak at 509 nm).

2.2.3.2 Experiment 2

Another transformation procedure was based on Young (2000). Cotyledon (5 x 5 mm) and petiole (5 mm) explants were harvested from 10 to 14 day-old E41 seedlings, and pre-cultured on S5 for 2 days. A single colony of EHApGH was inoculated in 40 ml LB broth containing 50 mg/l kanamycin, 25 mg/l chloramphenicol and 3.92 mg/l acetosyringone, and cultured overnight on shaking incubator (150 rpm) at 26°C. The OD₆₀₀ value was measured, and the culture was diluted with LB broth until OD₆₀₀ = 0.6-0.8. Explants were inoculated as previously described, but only for 10 min, and inoculation plates were swirled occasionally.

Inoculated explants were blotted dry and placed on S5 medium containing 19.62 mg/l acetosyringone, but without AgNO₃. Co-cultured explants were placed in a growth chamber for 2 days.

After co-culturing, explants were rinsed and blotted dry as described in experiment 1. Petiole explants were then placed on selection medium (S5 medium plus 7.5 mg/l geneticin and 500 mg/l cefotaxime), and cotyledon explants were placed on the different selection medium (S5 medium plus 10 mg/l geneticin and 500 mg/l cefotaxime). There were 8 explants per plate, and 5 plates per tissue/explant type. Explants were transferred to fresh selection media every 4 weeks. Expression of EGFP was screened via microscopy as previously described.

2.3 Identification of *pds* Gene in Kenaf

2.3.1 Searching for kenaf *pds* gene in transcriptome

As the literature review described, a kenaf *pds* gene has not been identified or discussed, to the date. To identify *pds* sequences in kenaf, a multiple sequence comparison among species was conducted by nucleotide Basic Local Alignment Search Tool (BLAST).

The kenaf transcriptome sequences (Li et al., 2016) were downloaded from NCBI nucleotide database. The *pds* mRNA sequences from cotton (*Gossypium raimondii*) were used for alignment with the kenaf transcriptome to search for potential *pds* genes in kenaf. The discontinuous megablast program was used which detects highly similar sequences allowing small mismatches.

The transcribed kenaf RNA sequence that shared the greatest identity/homology with cotton *pds* mRNA sequences was selected. A reverse comparison to the nucleotide collection database confirmed the reliability of the selected mRNA sequence. BLASTn was used, set for somewhat similar sequences and without a limitation of organisms.

The Conserved Domain search (CD-search) tool was used for the selected kenaf mRNA sequence to identify putative functional domains (Marchler-Bauer et al., 2014). Functional domain amino acid sequences were confirmed using protein BLAST.

The motif scan function of MyHits (<https://myhits.isb-sib.ch>) was used for the putative codon translation sequence of kenaf GEED01047592.1.

2.3.2 Kenaf genomic DNA extraction

Kenaf genomic DNA was extracted using Thermo Fisher Scientific GeneJET Plant Genomic DNA Purification Mini Kit. Kenaf genomic DNA was extracted from E41 mature leaf explants. Although the kit protocol set the amount of tissue needed at 100 mg, about 150 to 200 mg kenaf leaf tissue was used to account for losses during transfer of ground tissue into collection tubes. Leaf tissues were placed in a mortar, immersed in liquid nitrogen and ground to a fine powder with a pestle. The powder was transferred into a 1.5 ml microcentrifuge tube. Extraction procedures were provided by the kit, through lysis, separation, precipitation, DNA binding, washing and elution. Extracted DNA was dissolved in elution buffer and stored at -20°C for downstream experiments.

2.3.3 Polymerase chain reaction (PCR) and gel analysis

To confirm existence of the putative kenaf *pds* gene in genomic DNA, PCR and gel analysis were used. Primers were designed to align with, and amplify, putative kenaf *pds* sequences using the NCBI primer-designing tool. The forward primer was 5'-TTCTTTCCGTTCTGCTCCCC -3' and the reverse primer was 5'-TGCTTTACTCTGGTCCGCAG -3'. Primers for all experiments were purchased from Eurofins Genomics. AccuPrime™ GC-rich DNA polymerase and buffer were used for PCR. A 25 µl-

reaction included 4.5 μ l (25 to 100 ng) genomic DNA, 0.5 μ l forward primer, 0.5 μ l reverse primer, 5 μ l Buffer A, 0.5 μ l AccuPrime GC-rich DNA polymerase and 14 μ l DEPC-treated water. The PCR reaction: (1) initial denaturation at 95°C for 3 min; (2) 30 cycles (30 sec at 95°C, 30 sec at 55°C, and 75 sec at 72°C); (3) final extension at 72°C for 10 min. Products were maintained at 4°C after cycling and used for gel electrophoresis. Gels were composed of 50 ml 1% agarose plus 3 μ l SYBR safe gel stain, and 5 μ l TrackIt™ 1 Kb Plus DNA Ladder was used for sizing resulting DNA fragment(s).

2.4 Gene Knockouts

2.4.1 GUS assays on previously transformed plants

When first looking for target genes to knock-out, the β -glucuronidase (*gus*) was first discussed. There was access to transgenic kenaf seeds (T₁) that contained *gus*. This experiment was designed to determine the strength of *gus* expression (amount of blue color present in assays) to see if it was strong enough to detect color decline in re-transformed knock-outs.

Transformed E41 kenaf seeds containing an inserted *gus* gene (Young, 2000) were screened for GUS activity via a GUS assay. The histochemical reagent X-Gluc solution was made: a 100 ml reaction mix included 10 ml 50 nM potassium ferricyanide, 10 ml 50 nM potassium ferrocyanide, 10 ml 10% Triton X-100, 50 ml 200 mM sodium phosphate buffer (pH 7.0), 0.2 ml 0.5 M sodium EDTA and 7.8 ml H₂O, then 12 ml 25 mg/ml X-Gluc (5-bromo-4-chloro-3-indoyl-beta-D-glucuronide) in dimethylformamide was added for a final concentration of 3 mg/ml X-Gluc. Four transgenic seed lines (A-3-2, A-3-7, B-2-2, B-2-9) were disinfested and germinated as described previously. Leaves harvested from seedlings were incubated at 37°C with 3 mg/ml X-Gluc solution in petri dishes. Two different incubation times were compared: 4 hr and 16 hr (overnight). Incubated leaves were transferred to new petri dishes and rinsed twice

in 70% ethanol for 5 min. Wild-type E41 was the control, treated as described above. Results were observed under a dissecting microscope.

2.4.2 CRISPR *pds* target selection and gRNA designation

The gRNA designed to target *pds* in other plant species appeared to be randomly dispersed throughout the gene (targeted 11 of 14 exons; Table 1.2), and no “standard” guiding principles seemed to be followed. Therefore, this approach was to target areas in kenaf *pds* that were, most likely, conserved domains.

CD-search was used to identify known conserved areas or predicted functional domains (Marchler-Bauer et al., 2014). The selected kenaf transcribed RNA (Unigene18524) was searched against the Conserved Domain Database (CDD v3.17-52910 PSSMs). In addition, the conserved domains in banana *pds* (Kaur et al., 2018) were used as the template for comparison to Unigene18524.

Two gRNAs were designed using the online tool CRISPRdirect, (<https://crispr.dbcls.jp/>). Unigene18524 was set as the input sequence and the specificity check was against cotton (*Gossypium raimondii*) genome to predict potential off targets, since the kenaf genome has yet to be sequenced.

The final gRNA selection strategy was based on three criteria: (1) basic requirement of gRNA location (upstream of PAM sequence NGG); (2) CD-search results and conserved sequences in banana *pds*; (3) specificity based on cotton genome.

2.4.3 Construct gRNAs into plasmids

DNA that corresponded to individual gRNA were cloned directly into a vector, based on the Golden Gate method as described by Čermák et al. (2017).

2.4.3.1 Plasmid used for gRNA insertion

Plasmid pDIRECT_22A (Addgene plasmid #91133; <http://n2t.net/addgene:91133>; RRID:Addgene_91133) was a gift from Daniel Voytas. Plasmid pDIRECT_22A was selected to receive each separate gRNA; it contained *cas9* controlled by the CaMV 35S promoter, *nptII* controlled by an enhanced CaMV 35S promoter (selectable marker gene), and a site for specialty gRNA to be inserted between *Arabidopsis thaliana* U6 promoter and a gRNA scaffold sequence.

2.4.3.2 Preparation of plasmid and gRNA oligonucleotides

Plasmid pDIRECT_22A was extracted using Invitrogen (Thermo Fisher Scientific) Purelink Quick Plasmid Miniprep Kit. A single colony of *E. coli* harboring pDIRECT_22A was inoculated in 40 ml LB broth containing 50 mg/l kanamycin and cultured overnight in a shaking incubator (150 rpm) set at 37°C. Procedures that included cell harvesting, resuspending, lysing, precipitating, binding, washing and eluting followed the provided protocol. Concentration of the plasmid was measured using a Nanodrop spectrophotometer. The extracted plasmid was stored at -20°C for downstream experiments.

Both strands of each designed gRNA were obtained from Eurofins Genomics (24-mers, Table 2.3). Annealed phosphorylated gRNA oligonucleotides were prepared for downstream experiments. To construct each gRNA, a phosphorylated sense oligonucleotide and a phosphorylated antisense oligonucleotide were designed; a “GATT” (5’ to 3’) overhang was required at the 5’ end of the sense oligonucleotide and a “AAAC” (5’ to 3’) overhang was needed for the antisense oligonucleotide. A 30 µl reaction was set up for each gRNA that included 3 µl 100 µM phosphorylated sense oligonucleotide, 3 µl 100 µM phosphorylated antisense oligonucleotide and 24 µl DMSO-treated water. Reaction was incubated at 37°C for 1 h, then boiled in a water bath for 2 min, then gradually cooled down to room temperature (25°C).

2.4.3.3 Golden Gate reaction

A Golden Gate reaction was run for each gRNA individually. A 20 μ l Golden Gate reaction included 50 ng pDIRECT_22A, 1 μ l 25X diluted annealed gRNA oligonucleotides, 0.4 μ l *AarI* oligonucleotide (to enhance DNA cleavage), 0.5 μ l *AarI* (restriction endonuclease), 2 μ l 10X T4 DNA ligase buffer, 1 μ l T4 DNA ligase, and DEPC-treated water to bring up to 20 μ l. The Golden Gate reaction consisted of only 1 cycle: 5 min at 37°C, 10 min at 16°C, 15 min at 37°C and 5 min at 80°C.

Invitrogen (Thermo Fisher Scientific) One Shot™ TOP10 chemically competent *E. coli* was used to receive the modified plasmid. The Golden Gate reaction product (gRNA-inserted pDIRECT_22A; 5 μ l) was added to a vial of competent cells and mixed by inverting the vial 2 to 3 times. After incubating on ice for 30 min, cells were heat-shocked at 42°C for 30 sec, then moved back to ice for 2 min. S.O.C. medium (Invitrogen; 250 μ l) was added to each vial, and incubated for 1 hr on a shaking incubator (225 rpm) at 37°C. The transformed *E. coli* was plated on LB agar containing 50 mg/l kanamycin and 32 mg/l X-gal. Plates were incubated at 37°C overnight. Since the initial plasmid contained a *lacZ* gene that could be replaced by a gRNA sequence, a blue-white colony screen was used to select for the gRNA-inserted plasmids; white colonies were picked and maintained on fresh plates for downstream experiments.

2.4.3.4 Molecular confirmation

Confirmation of gRNA-inserted plasmids (called 22AgRNA1 and 22AgRNA2) were identified by sequencing. Primers used were the sense gRNA oligonucleotides and NB463 (Čermák et al., 2017) as the reverse primer. Sanger sequencing was conducted by DNASU Sequencing Core of Arizona State University (Tempe, AZ).

2.4.4 Transformation

E. coli containing 22AgRNA1 or 22AgRNA2 were transformed into to *A. tumefaciens* strain EHA105 via tri-parental matings yielding EHAgRNA1 and EHAgRNA2. They were maintained on LB agar containing 50 mg/l kanamycin and 25 mg/l chloramphenicol. Cotyledon (5 x 5 mm), hypocotyl (5 mm) and petiole (5 mm) explants were harvested from 10 to 14 day-old E41 seedlings for use. Aside from using *A. tumefaciens* EHAgRNA1 and EHAgRNA2, there were two modifications to the procedure described in transformation experiment 1. (1) During incubation of EHAgRNA1 and EHAgRNA2 with explants, instead of stationary incubation (at 26°C for 30 min), they were shaken at 150 rpm (in sterile flask). (2) After the 2-day co-culture, explants were rinsed in cefotaxime 3 times instead of once, and were shaken at 150 rpm (room temp.) for 3 min in sterile flasks (vs. stationary rinse). Petiole explants were placed on the same selection medium as previously noted (S5 medium plus 7.5 mg/l geneticin and 500 mg/l cefotaxime); cotyledon and hypocotyl explants were placed on the same selection medium as previously noted (S5 medium plus 10 mg/l geneticin and 500 mg/l cefotaxime). Explants were moved to fresh selection medium every 4 weeks. In total: 572 cotyledon explants, 216 hypocotyl explants and 104 petiole explants were transformed with EHAgRNA1; 592 cotyledon explants, 116 hypocotyl explants and 84 petiole explants were transformed with EHAgRNA2.

2.4.5 Screen for transgenic knock-outs

Explants surviving selection were observed for regeneration and tissue albinism 10 weeks and 20 weeks after placement on media. Tissue displaying visually abnormal phenotypes were selected for genomic DNA extraction for further analysis.

The DNA extraction method needed to be adjusted to accommodate for the limited tissue able to be sacrificed. From tissues in culture, 100 mg fresh cut tissue plus fine glass powder

(about 50 mg) were added to a 1.5 ml microcentrifuge tube. The tube was partially immersed in liquid nitrogen, and tissues were ground to a fine powder using a plastic microcentrifuge pestle. Genomic DNA was extracted according to Thermo Fisher Scientific GeneJET Plant Genomic DNA Purification Mini Kit with the following exception: the microcentrifuge tube containing ground tissue and glass powder was vortexed for 60 sec after adding the lysis buffer and RNase A. Extracted genomic DNA was stored at -20°C for downstream work.

2.4.5.1 *Cas9* and *nptII* identification

Primers were designed for *cas9* and *nptII* genes separately (Table 2.3). AccuPrime™ *Taq* DNA polymerase was used in PCR analyses. A 25 µl reaction included 18 to 21 µl genomic DNA (amount depended on the concentration of genomic DNA), 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM), 2.5 µl 10X AccuPrime™ PCR Buffer II, 0.5 µl AccuPrime™ *Taq* DNA polymerase and 0 to 3 µl DEPC-treated water. Both positive (pDIRECT_22A) and negative (non-transformed E41 genomic DNA) controls were also included.

The PCR reaction for *cas9* amplification: (1) initial denaturation at 94°C for 2 min; (2) 35 cycles (30 sec at 94°C, 30 sec at 57.4°C, and 55 sec at 68°C). The PCR reaction for *nptII*: (1) initial denaturation at 94°C for 2 min; (2) 35 cycles (30 sec at 94°C, 30 sec at 56.4°C, and 40 sec at 68°C).

PCR products were maintained at 4°C after cycling and used for agarose gel electrophoresis. Gels were composed of 100 ml 1% agarose gel including 8 µl SYBR safe gel stain, and 5 µl TrackIt™ 1 Kb Plus DNA Ladder was used for sizing resulting DNA fragment(s).

2.4.5.2 Mutation detection

To estimate the correct size of PCR products we might obtain for sequence analysis, exon and intron lengths needed to be considered. In the absence of kenaf genome data, the cotton *pds* DNA sequence (provided by Phytozome V.12.1) was set as the template to align with the kenaf *pds* mRNA sequence. Both NCBI BLAST tool and Molecular Evolutionary Genetics Analysis (MEGA 7.0) were used. Primers were designed to amplify across putative indel sequences and were based on the exon position in cotton *pds*. Primer sequences were listed in Table 2.3 and primers were purchased from Eurofins Genomics. Two pairs of primers were designed for each gRNA target: a pair of primers throughout adjacent exons of the target, another pair within the targeted exon.

Invitrogen (Thermo Fisher Scientific) PlatinumTM SuperFiTM PCR Master Mix was used. A 50 μ l reaction included 2 to 5 μ l genomic DNA (amount depended on the concentration of genomic DNA), 2.5 μ l forward primer (10 μ M), 2.5 μ l reverse primer (10 μ M), 25 μ l 2X PlatinumTM SuperFiTM PCR Master Mix, and 15 to 18 μ l nuclease-free water. The PCR reaction: (1) initial denaturation at 98°C for 30 sec; (2) 35 cycles (6 sec at 98°C, 10 sec at specific anneal temperature, and 15 to 30 sec at 72°C); (3) final extension at 72°C for 5 min. PCR products were sent to DNASU Sequencing Core of Arizona State University (Tempe, AZ) for sequencing.

Table 2.3 Oligonucleotides and primers used in molecular experiments

Primer	5' to 3'
gRNA 1 sense	GATTGAAATCTCTCTCCAGTCTTC
gRNA 1 antisense	AAACGAAGACTGGAGAGAGATTTC
gRNA 2 sense	GATTGCAAAGTATCTAGCGGATGC
gRNA 2 antisense	AAACGCATCCGCTAGATACTTTGC
<i>cas9</i> forward	TTCGATCTCGCTGAGGATGC
<i>cas9</i> reverse	AAGCAGGCTTCCTCATTCCC
<i>nptII</i> forward	CGTTGGCTACCCGTGATATT
<i>nptII</i> reverse	GCCCAGTCATAGCCGAATAG
NB463	CGAACGGATAAACCTTTTCACG
Mutation g1F1	GGCAGTACAATTGAAGGAGATGC
Mutation g1R1	ACTGGAACTCCAACCTAATTCTC
Mutation g1F2	TTGATATCTTCAAGTTACTTTTGCC
Mutation g1R2	TGTGAACGTTGATAACTGGAAC
Mutation g2F1	CCGCTTCTTTCCGTTCTGCT
Mutation g2R1	CACCTTTCCACCTAGAACATCTCT
Mutation g2F2	GTTTGGCTGGTTTGTCAACTG
Mutation g2F2	CACCTTTCCACCTAGAACAT

2.5 References

- Čermák T, Curtin SJ, Gil-Humanes J, Čegan R, Kono TJ, Konečná E, Belanto JJ, Starker CG, Mathre JW, Greenstein RL, Voytas DF (2017) A multipurpose toolkit to enable advanced genome engineering in plants. *The Plant Cell* 29:1196-1217
- Herath SP, Suzuki T, Hattori K (2005) Factors influencing Agrobacterium mediated genetic transformation of kenaf. *Plant Cell, Tissue and Organ Culture* 82:201-206
- Kaur N, Alok A, Kaur N, Pandey P, Awasthi P, Tiwari S (2018) CRISPR/Cas9-mediated efficient editing in *phytoene desaturase* (*PDS*) demonstrates precise manipulation in banana cv. Rasthali genome. *Functional & Integrative Genomics* 18:89-99
- Khatun A, Zabun N, Shirin M, Chandan KS, Shana B (2003) An efficient protocol for plant regeneration from the cotyledons of kenaf (*Hibiscus cannabinus* L.). *Biotechnology* 2:86-93
- Li H, Li D, Chen A, Tang H, Li J, Huang S (2016) Characterization of the kenaf (*Hibiscus cannabinus*) global transcriptome using illumina paired-end sequencing and development of EST-SSR markers. *PLoS ONE* 11:e0150548. <https://doi.org/10.1371/journal.pone.0150548>
- Lim T-K (1997) Optimization of organogenic regeneration and in vitro selection for *Cristulariella moricola* resistance in kenaf (*Hibiscus cannabinus* L.) Thesis, Mississippi State University
- Marchler-Bauer A, Derbyshire MK, Gonzales NR, Lu S, Chitsaz F, Geer LY, Geer RC, He J, Gwadz M, Hurwitz DI, Lanczycki CJ, Lu F, Marchler GH, Song JS, Thanki N, Wang Z, Yamashita RA, Zhang D, Zheng C, Bryant SH (2014) CDD: NCBI's conserved domain database. *Nucleic Acids Research* 43:D222-D226. <https://doi.org/10.1093/nar/gku1221>
- Maximova S, Miller C, De Mayolo GA, Pishak S, Young A, Gultinan MJ (2003) Stable transformation of *Theobroma cacao* L. and influence of matrix attachment regions on GFP expression. *Plant Cell Reports* 21:872-883
- Murashige T, Skoog FA (1962) Revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* 15:473-497
- Ruotolo G, Del Toro A, Chiaiese P, Filippone E (2011) Multiple buds are an alternative target to genetic manipulation of kenaf (*Hibiscus cannabinus* L.). *Industrial Crops and Products* 34:937-942
- Shamsunaher MA, Mukul MHR, Biswash MR, Zahan A (2013) Study on the appropriate dose of BAP for regeneration of kenaf (*Hibiscus cannabinus* L.). *Eco-friendly Agriculture Journal* 6:6-9

Sultana R, Quader AKML, Haque M, Mazumder S, Paul SK (2016) In vitro studies on shoot proliferation of kenaf (*Hibiscus cannabinus* L.). World Journal of Agricultural Sciences 12:25-30

Withanage SP, Hossain MA, Roslan HAB, Abdullah MP, Napis SB, Shukor NAA (2015) Overexpression of *Arabidopsis thaliana* gibberellic acid 20 oxidase (AtGA20ox) gene enhance the vegetative growth and fiber quality in kenaf (*Hibiscus cannabinus* L.) plants. Breeding Science 65:177-191

Young MM (2000) Genetic transformation of kenaf. Dissertation, Mississippi State University

CHAPTER III
RESULTS AND DISCUSSION

3.1 Plant Regeneration

3.1.1 Plants material preparation and culture conditions

3.1.1.1 Seed surface disinfestation and germination

Germination and contamination rates were recorded 2 weeks after placing E41 seeds on MSO medium. At this time point, treatment with 1.5% sodium hypochlorite for 25 min presented the highest germination rate (26.7%) and the lowest contamination rate (1.3%). At 4 weeks after placement, treatment with 1.3% sodium hypochlorite for 25 min yielded the highest germination rate (42.7%) with a contamination rate of 9.3%; 1.5% sodium hypochlorite for 25 min presented 36% germination rate and 8% contamination rate; 1.9% sodium hypochlorite for 30 min presented 18.6% germination rate and 2.7% contamination rate. Based on higher germination rate, 1.3% sodium hypochlorite (plus SDS) for 25 min was chosen as the standard treatment for seed surface disinfestation. Seed materials used in this experiment were E41 seeds recovered from frozen storage (-20°C). This might have led to the relatively low germination rates, and it was found that seeds took up to 3 weeks to respond.

3.1.2 Organogenesis - cultivar Whitten

3.1.2.1 Experiments 1 and 2

Mature leaf explants placed on the 3 X 5 media matrix (M1-M15; Table 2.1) expanded and began to curve during in the first week on all media. After 1 to 2 weeks in culture, most of

the calli appeared at the cut surfaces of explants on all media, only explants on media M1 and M5 produced calli from the leaf midrib. Three primary types of calli were observed on all media after 2 – 3 weeks in culture: (1) white, compact, unorganized shaped calli, (2) translucent friable calli, and (3) dark/bright green, smooth surface, round shaped calli. The green round-shaped-smooth calli was classified as organogenic calli by Young (2000), because shoots would only arise from this type of calli.

Explants continually expanded and enlarged to 10-30 mm in diameter (started as 5 x 5 mm sections) after 6-7 weeks in culture, but no adventitious shoots were noted. Since curved explants had less access to the media, they were gently pushed down into the media to ensure adequate contact. Media M1, M5, M8, M12 and M13 produced greater amounts of calli than all the other media. Root organogenesis was observed on medium M6.

All calli types described above were represented after 3 months (Table 3.1). Although the limited number of replicates did not allow statistical analysis for significance, observed trends are discussed. The amount of organogenic calli increased on most regeneration media, but no adventitious shoots were observed. In fact, all organogenic calli remained undifferentiated, although some enlarged in size. M12 yielded the greatest number of responding explants (65%) that produced organogenic calli (Table 3.1). In general, explants on all media produced greater percentages of the calli types compared to organogenic. Translucent friable calli grew to sizes of 15 to 20 mm. Media M6, M7, M11 and M13 produced roots; explants (20%) on M11 yielded the most roots (1.5 root/ responding explant) of varying lengths (5 to 50 mm).

Since M12 yielded the greatest response for generation of organogenic calli in experiment 1, the a/c ratio of M12 (0.15/1) was maintained in two additional media tested. We wanted to identify if this a/c ratio specifically improved the percentage of organogenic calli generated.

Organogenic calli were first observed on M12 during the third week in culture. After 8 weeks, compared to M12A (0.1125 mg/l NAA and 0.75 mg/l TDZ) and M12B (0.225 mg/l NAA and 1.5 mg/l TDZ), M12 still yielded the greatest percentage of organogenic calli (46.7%). M12B yielded the highest percentage of white compact calli (85%) and M12 had the greatest number of explants that generated roots (13.3%; 1 root/explant). M12A yielded a lower percentage (20%) of organogenic calli than M12, but a greater percentage (60%) of white compact calli.

Reviewed by Duclercq et al. (2011), the process of in vitro organogenesis from explants was influenced by factors including type and age of explant and exogenous PGR(s) added to the media. They also indicated that a lower a/c ratio induced shoot regeneration, while a higher a/c ratio induced to callus formation or root development. Results among these kenaf media did not show similar organogenic calli response rates nor any shoot regeneration, so it appears that this exogenous a/c ratio (0.15/1) was not a specific key factor in the kenaf shoot regeneration process.

Table 3.1 Calli arising from mature leaf explants of Whitten on 15 media after 3 months in culture

Medium	Auxin		Cytokinin		Organogenic	White compact	Translucent
	NAA (mg/l)	TDZ (mg/l)	A/C ratio	calli (%) ^x	calli (%)	friable calli (%)	
M1	0.05	0.5	0.1	10	90	80	
M2	0.05	1	0.05	30	80	55	
M3	0.05	2	0.025	25	85	60	
M4	0.05	3	0.017	30	95	80	
M5	0.05	4	0.013	45	95	90	
M6	0.1	0.5	0.2	50	75	60	
M7	0.1	1	0.1	30	75	50	
M8	0.1	2	0.05	45	95	80	
M9	0.1	3	0.033	40	80	55	
M10	0.1	4	0.025	35	100	80	
M11	0.15	0.5	0.3	40	70	35	
M12	0.15	1	0.15	65	95	55	
M13	0.15	2	0.075	45	65	45	
M14	0.15	3	0.05	25	85	65	
M15	0.15	4	0.038	35	100	60	

x: Percentage of explants that generated each type of calli

3.1.2.2 Experiment 3

The PGR combinations used in this experiment were based on optimal media reported by Lim (1997) and Young (2000). In their research on different cultivars, the optimized NAA (auxin) concentration remained the same (0.1 mg/l), but TDZ concentrations ranged from 0.35 to 3.5 mg/l. However, silver nitrate (AgNO_3) was also added; the concentration of AgNO_3 used followed results of Young (2000; 10.2 mg/l). This addition to media was expected to yield adventitious shoots from Whitten explants, since it was an ethylene inhibitor, and ethylene can inhibit shoot organogenesis (Kumar et al., 1998).

For mature leaf petioles, no adventitious shoots or organogenic calli developed. Only translucent friable callus appeared on the cut surfaces of most petiole explants after 2 to 3 weeks. For leaf explants, the earliest organogenic calli response appeared on TNA6 (additives described above plus 1.1 mg/l TDZ), TNA7 (2.5 mg/l TDZ) and TNA8 (3.0 mg/l TDZ) after 2 weeks in culture (Table 3.2). Organogenic calli were visually similar to those noted in experiments 1 and 2. Medium TNA7 (same as S5 in the following experiments) had been identified by Dr. Margaret Young to initiate organogenic calli on Whitten mature leaf explants (personal communication). No organogenic calli arose from explants on media TNA1, TNA2 or TNA9 (0.25, 0.3, 3.5 mg/l TDZ, respectively) throughout the entire culture period of 3 months. The number of leaf explants producing organogenic calli on TNA7 increased over time (33.3% to 50%), whereas that number stayed the same or decreased on all other media (Table 3.2). It was observed that translucent friable calli increased in both number and size, and could actually cover the organogenic calli, hindering the ability to accurately count them, and also probably hindered their continued development. No organogenic calli generated shoots on any media after 3 months.

Table 3.2 Calli arising from mature leaf explants of Whitten on 9 media after culture for 2 and 6 weeks

Media ^x	Cytokinin TDZ (mg/l)	Organogenic calli (%) ^y	
		2 weeks	6 weeks
TNA1	0.25	0 a	0 a
TNA2	0.3	0 a	0 a
TNA3	0.35	25 abc	0 a
TNA4	0.79	16.7 ab	0 a
TNA5	0.95	16.7 ab	8.3 a
TNA6	1.1	41.7 bc	33.3 b
TNA7	2.5	33.3 bc	50 b
TNA8	3.0	50 c	50 b
TNA9	3.5	0 a	0 a

Means followed by different letters within columns are significantly different at $P < 0.05$ by Duncan's Multiple Range Test (DMRT)

x: All media were MSO supplemented with 0.1 mg/l NAA and 10.2 mg/l AgNO₃; TDZ concentrations are listed in 2nd column

y: Percentage of mature leaf explants that generated organogenic calli

3.1.2.3 Experiment 4

Whitten seedling tissues were used in this experiment to explore their potential for shoot organogenesis using different seedling tissues. Seedling tissue explants included cotyledon, cotyledonary petiole and hypocotyl, and were expected to have a better expression of totipotency compared to mature leaves. Medium S5 (MSO supplemented with 0.1 mg/l NAA, 2.5 mg/l TDZ and 10.2 mg/l AgNO₃; same as TNA7 in experiment 3) was used in this experiment, since it yielded one of the greatest organogenic calli responses for leaf explants after 2 weeks in culture and increased over time (Table 3.2).

One week after culture initiation, only about 50% of petiole explants remained viable, probably as a result of their small size, with the smallest displaying lower viabilities. Some cotyledonary petioles had relative smaller diameters, so petiole explants were a smaller overall in size compared to hypocotyl explants, even though they were the same length (5 mm). However, 5 out of the 6 cotyledonary petiole explants that survived produced organogenic calli within a month. For cotyledon and hypocotyl explants, the earliest organogenic calli were observed 2 to 3 weeks after culture. Two months after placement, 20.8% petiole explants, 25% hypocotyl explants and 62.5% cotyledon explants produced organogenic calli. No roots or adventitious shoots were observed on any seedling tissue explants.

Although the generation of organogenic calli (rudimentary shoot primordia) and roots were induced, no adventitious shoots were generated from any Whitten seedling or mature tissues. A successful outcome was expected since both parental cultivars (E41 and G45) of Whitten were successfully regenerated in vitro. Two SCLs of E41 were able to regenerate adventitious shoots from mature leaf explants by Lim (1997), and one E41 SCL regenerated shoots on a different medium by Young (2000); one of seven G45 SCLs yielded adventitious shoots from mature leaf explants (Lim, 1997). Comparing these results to that of Lim (1997) and Young (2000), it can be concluded that kenaf shoot regeneration efficiency is highly dependent on cultivar/genotype as well as the PGR (and additive) combinations and concentrations used.

Based on our results, key factors are yet to be discovered to convert organogenic calli generated on Whitten explant tissues into adventitious shoots. Young (2000) indicated that a noted limitation to kenaf shoot regeneration was the failure of rudimentary shoot primordia to continue their development into shoots.

3.1.3 Organogenesis - E41 cultivar

3.1.3.1 Experiment 1

After placement on 5 media (S1-S5, Table 2.2), seedling explant responses were noted. Media S4 and S5 were the same media formulations as TNA3 and TNA7, respectively, discussed above for Whitten; Young (2000) successfully generated shoots on S4 using E41 mature leaf explants. On all media, the size of cotyledon and cotyledonary petiole explants enlarged during the first 2 weeks after culture, although some of the smaller petiole explants did not survive beyond 2 weeks. Greater amounts of translucent friable calli were observed on petiole compared to cotyledon tissues, but white compact calli were seldom found on petiole tissues. Organogenic calli were first observed on both tissues after 2 weeks in culture. Roots also developed on both tissues on media S1 and S2 about 3 weeks after culture. Regenerating shoots were observed on petiole explants on medium S5 after just 4 weeks in culture, the fastest regeneration observed in all experiments. The initial shoot regenerated from the edge of a petiole explant. Cotnode tissues used as controls (pre-formed axillary bud present) yielded elongated shoots after 4 weeks in culture. Results recorded after 6 weeks and 3 months in culture are listed in Table 3.3. Since limited explants were available for culture, no statistical analysis was conducted, but results seemed to be fairly clear-cut.

After 6 weeks in culture, surviving explants continued to grow in size. Cotyledon explants enlarged to 15-20 mm in diameter, whereas petiole explants enlarged up to 10 mm. All cotyledon explants generated organogenic calli (100%) on media S4 (MSO supplemented with 0.1 mg/l NAA, 0.35 mg/l TDZ and 10.2 mg/l AgNO₃) and S5 (MSO supplemented with 0.1 mg/l NAA, 2.5 mg/l TDZ and 10.2 mg/l AgNO₃), as did petiole explants on medium S5 (Table 3.3). Adventitious shoots were generated on cotyledon tissues cultured on S1 (6.3%) and S5 (6.3%),

and petiole explants on S5 (66.7%) – the highest percentage observed. Roots were observed on both explant types on all media except S5. Medium S2 yielded the highest root regeneration rate (80% in cotyledon and 100% in petiole).

After 3 months in culture, additional explants generated adventitious shoots on both explant types on media S4 and S5; petiole explants on S5 maintained the same percentage (Table 3.3). All shoots generated on petiole explants arose from the explant edges. Shoots arising from cotyledons were either located on edges or midribs of the explants.

Table 3.3 Organogenic calli and adventitious shoot responses on E41 seedling tissues after culture on media for 6 weeks and 3 months

Media ^x	Cotyledon explants			Petiole explants		
	Organogenic calli (%) ^y	Adventitious shoots (%)		Organogenic calli (%) ^y	Adventitious shoots (%)	
	at 6 weeks	at 6 weeks	at 3 months	at 6 weeks	at 6 weeks	at 3 months
S1	62.5 a	6.3	6.3 ab	75	0	0 a
S2	62.5 a	0	0 a	83.3	0	0 a
S3	81.3 ab	0	0 a	50	0	0 a
S4	100 b	0	18.8 bc	83.3	0	16.7 a
S5	100 b	6.3	31.3 c	100	66.7	66.7 b

Means followed by different letters within columns are significantly different at $P < 0.05$ by Duncan's Multiple Range Test (DMRT)

Columns without letters are not significantly different with $P > 0.05$ by DMRT

x: PGRs and additional components followed media reported by others (details in Table 4)

y: Percentage of explants that responded

3.1.3.2 Experiment 2

Seedling tissues (cotyledon, hypocotyl, and cotyledonary petiole) were placed on medium S5, determined the best for adventitious shoot regeneration across explant types. Shortly after being placed in culture (during the first week), hypocotyl explants expanded. Translucent friable calli developed on all hypocotyl explants during the second week, with great expansion (over 20 mm in diameter). Organogenic calli were observed on hypocotyl tissue after 3 weeks; after 3 months, 33.3% of hypocotyl explants generated organogenic calli. White compact calli was seldom observed on hypocotyl tissues. No adventitious shoots or roots developed from hypocotyl tissues on S5.

Cotyledon and petiole explants exhibited similar responses as experiment 1. After 6 weeks in culture, the percentages of explants generating organogenic calli were 88.9% for cotyledon and 75% for petiole. At least one adventitious shoot was observed on cotyledon explants as early as 5 weeks. After 6 weeks, the percentages of explants generating adventitious shoots were 7% for cotyledon and 8.3% for petiole; the shoot initiation percentage for cotyledon explants increased to 13.9% 3 months after culture.

Upon culturing these tissues, both explant types were tracked based on what side of the organ they were cut from. Cotyledon explants were divided into two groups: basal (near the petiole), and non-basal which represented the rest of cotyledon (included explants near the tip or in the middle of the cotyledon). Petiole explant groups: basal (near the hypocotyl), and apical (near the cotyledon). Overall, no differences were observed in responses between the two positional groups for either explant, although differences were expected. Sharma and Rajam (1995) observed positional effects on the regeneration of adventitious shoots on hypocotyl explants of eggplant (*Solanum melongena* L.) using seedling tissues; the basal segment of

hypocotyl presented the greatest number of adventitious shoots. Lim (1997) observed greater number of shoots initiated from the mature leaf basal region than the leaf tip region; the basal region contained the most leaf veins.

Therefore, successful shoot organogenesis from E41 seedling tissues was obtained by culturing cotyledon explants and cotyledonary petiole explants on media S4 and S5. Each part of the successful explant types yielded shoots (no positional effects noted).

The earliest regenerated shoot was observed on E41 cotyledonary petiole explants during week 4 on medium S5. Young (2000) observed adventitious shoots arising from E41 mature leaf explants after 2 months. In Lim's (1997) research, the first adventitious shoot appeared from a E41 SCL mature leaf explant after 3 weeks on medium NH10 (Nitsch and Nitsch basal medium plus 0.1 mg/l NAA, 2.2 mg/l TDZ and 20 mg/l AgNO₃), although most shoots generally appeared after 6 to 8 weeks in culture.

3.2 Transformation

3.2.1 Dose-response curves for two antibiotics

3.2.1.1 Experiments 1 and 2

Dose-response curves using hygromycin and geneticin were established for four different seedling tissues (cotnodes, cotyledon, hypocotyl and petiole) in these experiments, since it was initially unknown what transformation vector(s) would be used. Most control (S5 medium without antibiotic) tissues remained green and kept growing in size, and organogenic calli were observed on all tissue types.

For hygromycin, each type of seedling tissue expressed different levels of sensitivity to the antibiotic (2.5, 5, 7.5, 10 mg/l). After two weeks, about one-half of the cotnode tissues on S5 with 5 mg/l hygromycin turned white (bleached), the remaining tissues were yellow in color and

most were covered with translucent friable calli. Cotnodes on S5 with 7.5 mg/l hygromycin were totally bleached. For cotyledons, 75% explants on S5 with 7.5 mg/l hygromycin were bleached. For hypocotyls, there were 33.3% yellow explants and 66.6% bleached explants on S5 with 7.5 mg/l hygromycin. For petiole explants, S5 with 5 mg/l hygromycin led to 100% tissue bleaching, while the explants on 2.5 mg/l hygromycin remained green and produced limited amounts of calli.

After 4 weeks in culture on selection media, the percentages of surviving explants are presented in Figure 3.1. At the concentration of 2.5 mg/l, 83.3% petiole explants remained green and kept arising callus, while all the petiole explants were bleached at the concentration of 5 mg/l. At the concentration of 5 mg/l, 33.3% cotnode explants remained viable. At the concentration of 7.5 mg/l, 16.7% cotyledon and 25% hypocotyl kept growing slowly. Therefore, the suggested inhibitory concentrations of hygromycin chosen for selection of transgenic tissues were 5 mg/l for cotnode, 7.5 mg/l for cotyledon, 7.5 mg/l for hypocotyl and 5 mg/l for petiole. It was noted that the hygromycin could provide efficient growth inhibition of kenaf explants. Higher concentrations of hygromycin than noted for selection could lead to tissue bleaching within one week.

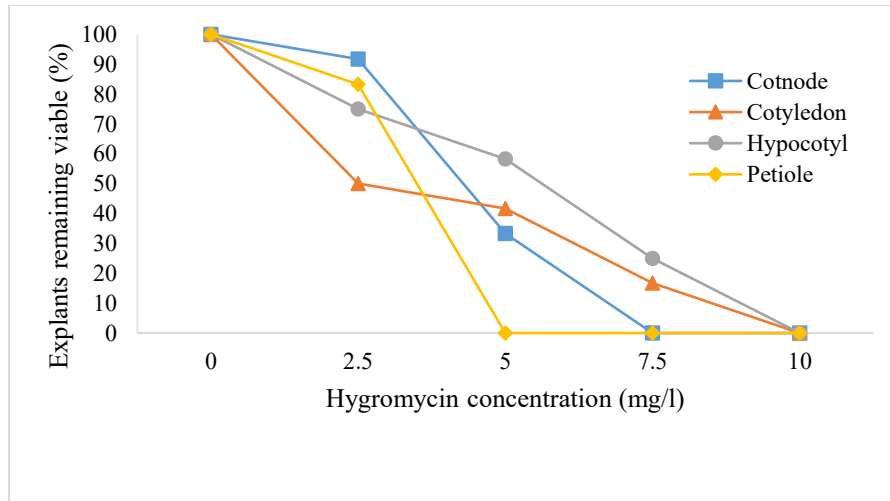


Figure 3.1 Dose-response curves for E41 seedling tissues on S5 medium containing hygromycin.

Results from culturing on S5 (0.1 mg/l NAA, 2.5 mg/l TDZ and 10.2 mg/l AgNO₃) containing 0, 2.5, 5, 7.5, 10 mg/l hygromycin for 4 weeks; viable explants included those displaying any growth.

For geneticin, based on the selection concentration used by Young (2000) for mature leaf explants (10 mg/l), and the relatively smaller size of seedling explants, the concentrations tested ranged from 2.5 to 15 mg/l geneticin. After 6 weeks in culture on selection media, the percentages of surviving explants are presented in Figure 3.2. At the concentration of 7.5 mg/l, 66.7% cotyledon, 41.7% hypocotyl and 16.7% petiole explants remained viable and kept growing slowly, with the remainder turning white or did not generate any calli. At the concentration of 10 mg/l, 16.7% cotyledon and 8.3% of hypocotyl explants still produced organogenic calli, while none of the petiole explants generated any calli. At the concentration of 12.5 mg/l, all explant types bleached, except 5 cotyledon explants on one plate. In a repeat experiment, 12.5 mg/l geneticin resulted in all explants bleaching, so this specific plate was discarded and not utilized in the dose-response curve. Therefore, 10 mg/l geneticin was used for

selection of transgenic E41 cotyledon and hypocotyl tissues, while a concentration of 7.5 mg/l was used for the cotnode and petiole tissues.

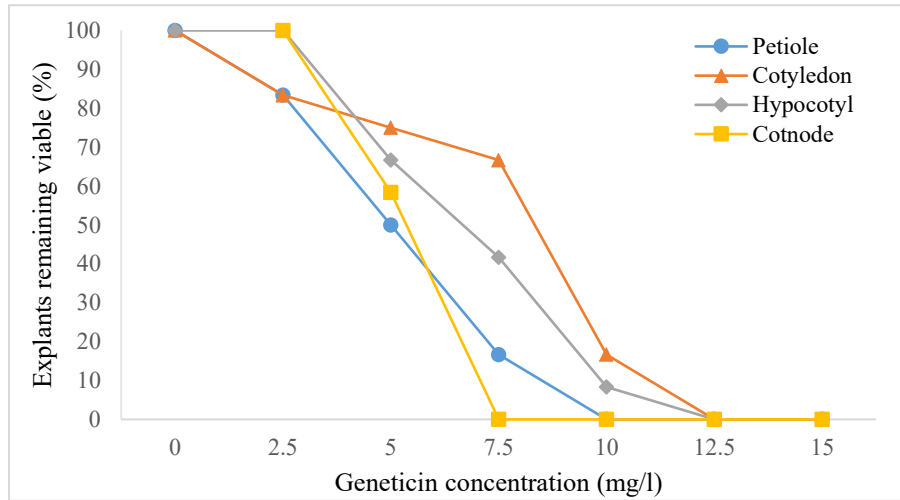


Figure 3.2 Dose-response curves for E41 seedling tissues on S5 medium containing geneticin.

Results from culturing on S5 (0.1 mg/l NAA, 2.5 mg/l TDZ and 10.2 mg/l AgNO₃) containing 0, 2.5, 5, 7.5, 10, 12.5, 15 mg/l geneticin for 6 weeks; viable explants included those displaying any growth.

3.2.2 Transformation procedure

3.2.2.1 Experiments 1 and 2

Transformation procedures can influence the overall transformation efficiency in several key steps, such as pre-culture, inoculation and co-culture, as well as application of acetosyringone, etc. This experiment did not focus on the influence of each factor (discussed by Young, 2000), but rather combined successful transformation protocols generated by others.

Experiment 1 combined procedures described by Herath et al. (2005), Ruotolo et al. (2011) and Withanage et al. (2015). Some key steps shared common treatments among these

reports: time of pre-culture of explants set at 2 days; a re-suspension step of *A. tumefaciens* before co-culture with explants; a relatively high concentration of acetosyringone (up to 39.24 mg/l) was used in the co-culture procedure.

After 2 days on pre-culture medium (S5), no visible changes were observed. Co-culture medium (S5) contained *A. tumefaciens* harboring EHApGH, and acetosyringone, but not AgNO₃ due to its bactericidal activity (Pandian et al., 2010). A few tissues were lost so, in total, 28 cotnode explants, 80 cotyledon explants and 62 petiole explants were cultured on selection media (S5 plus geneticin). After culturing on selection medium for 15 days, some explants generated translucent friable calli, but most cotnode and petiole explants bleached without generating any organogenic calli. No EGFP expression (green fluorescence) was observed on cotnode or petiole explants.

After 15 days on selection medium, 32.5% cotyledon explants kept growing, but only 10% out of all explants generated organogenic calli. Browning due to phenolics was later observed on cotyledon explants; either the explant itself or translucent friable calli expressed phenolics to differing degrees. The first EGFP expression was observed in a callus cluster 3 weeks after culture on selection medium; it was friable, translucent callus located on the edge of a cotyledon explant. Overall, 5% of the cotyledon explants expressed EGFP 4 weeks after culture. All green fluorescence was noted in cell clusters of friable translucent calli which were incapable of generating transformed shoots. Green fluorescence continued to be observed, but intensity gradually decreased in these transformed calli over the following 2 months.

For experiment 2, the co-culture procedure was based on Young (2000). This protocol shared the same pre-culture and co-culture times, but there was no re-suspension step of *A. tumefaciens*, and there was a shorter inoculation time. Then, a lower concentration of

acetosyringone was included in co-culture medium. Forty-five cotyledon explants and 38 petiole explants were used in this experiment. Cotyledon explants survived (13.3%) after 4 weeks on selection medium, but no adventitious shoots were observed 2 months after culture. No petiole explant survived 2 weeks after placement on selection medium. The transformed explants in this experiment were not maintained long enough to generate adventitious shoots. Regenerated adventitious shoots from *A. tumefaciens* transformants were observed after 8-10 months in culture by Young (2000).

Based on results, the following transformation procedure was used: explants were pre-cultured for 2 days on S5. *A. tumefaciens* was centrifuged and resuspended in S5 medium plus 39.24 mg/l acetosyringone, but without AgNO₃. The OD₆₀₀ value was determined, and bacteria diluted to OD₆₀₀ = 0.6-0.8 in the same medium. Explants were inoculated by immersion in *A. tumefaciens* for 30 min in sterile flasks on a shaking incubator (150 rpm). Inoculated explants were blotted dry using sterile WhatmanTM filter paper, and transferred to S5 containing 39.24 mg/l acetosyringone, but without AgNO₃. Explants were co-cultured in a growth chamber for 2 days. After the 2-day co-culture, explants were rinsed in 500 mg/l cefotaxime 3 times on a shaking incubator for 3 min at 150 rpm (room temp.) in sterile flasks, and were then placed on selection medium.

3.3 Identification of *pds* Gene in Kenaf

3.3.1 Searching for kenaf *pds* gene in transcriptome

BLAST-alignment (Altschul et al., 1997) results showed the similarity between the input sequence (query sequence) and database(s) it was compared to. There were two numbers used for measuring the similarity: query cover and identity. Query cover was the percentage that described how much of the target sequence covered the query sequence, indicating the length of

similarity between query sequence and target sequence. Identity was the percentage of identical nucleotides between the query sequence and the target sequence, indicating the nucleotide sequence similarity.

Cotton *pds* transcript sequence (Gorai.011G149900.1) was downloaded from Phytozome V.12.1. This 2192 bp transcript sequence included a 1689 bp coding sequence (CDS) that started at bp 272; 77% was CDS, with the rest comprised of 5' untranslated region (5' UTR) or 3' untranslated region (3' UTR). Gorai.011G149900.1 was set as the query sequence and aligned with the kenaf transcriptome (as the database; Li et al., 2016) to search for potential *pds* sequences. In our BLAST-alignment result, an identified kenaf transcribed sequence showed 90% query cover and 91% identity with Gorai.011G149900.1; it was RNA sequence Unigene18524 (GEED01047592.1). The area of each that shared the highest identity: 8 to 1921 bp in GEED01047592.1 and 115 to 2050 bp in Gorai.011G149900.1 (Figure 3.3). Within these areas, 147 mismatches and 6 gaps were found. The largest two gaps (11 bp) were located at 185 to 229 bp in cotton *pds* transcript, which were in the 5' UTR.

To eliminate the influence of lower similarities between the two transcripts in 5' UTR and 3' UTR areas, a modified alignment compared cotton *pds* CDS area of the transcript to GEED01047592.1. An identity of 93.5% was found between the whole CDS area (~1689 bp length) in the cotton *pds* Gorai.011G149900.1 transcript and 144 to 1829 bp (~1685 bp length) in the kenaf GEED01047592.1 transcript, with a 3 bp gap and 106 mismatches found in this alignment.

Based on these alignments, kenaf transcribed RNA sequence Unigene18524 (GEED01047592.1) was identified to have highest identity (91%) to the cotton *pds* transcript (Gorai.011G149900.1), which indicated GEED01047592.1 was a potential *pds* gene in kenaf.

To confirm and further analyze this result, kenaf GEED01047592.1 was set as the query sequence to search the database nucleotide collection (nr/nt) using the blastn algorithm (looked for somewhat similar sequences). Output results included 100 sequences that had query cover percentages ranging from 56% to 99%. The identities to GEED01047592.1 ranged from 81.5% to 93.5%; these sequences represented *pds* mRNA or CDS from 50 organisms. The greatest query (99%) was found in *Abelmoschus esculentus*; the greatest identity (93.5%) was found in *Gossypium barbadense*. Among these 100 sequences, 22 were from 8 plant species in the Malvaceae family, including genera like *Gossypium*, *Theobroma* and *Abelmoschus*; 14 of the 100 sequences had identities greater than 90%, and all of these were in the Malvaceae family.

Putative codon translation sequences of GEED01047592.1 were provided in these alignment results; this putative CDS was comprised of about 560 amino acids (aa), beginning around 140 bp and ending around 1830 bp (~1690 bp length). As indicated previously, this kenaf putative CDS area corresponded to, and aligned with, the cotton *pds* CDS area of the transcript; the highest identity area in kenaf was found to be located at 144 to 1829 bp (~1685 bp length).

NCBI's CD-search tool was utilized to search for potential conserved domains in GEED01047592.1. Two result display options could be chosen: standard result and full result. In the standard result, four main domain hits were provided: PLN02612, COG3349, phytoene_desat, and Amino_oxidase, described below.

- PLN02612 is described as phytoene desaturase in the PLN02487 superfamily (Marchler-Bauer et al., 2016). Thirteen representative genes were provided in PLN02612, including phytoene desaturase of *Arabidopsis thaliana*, predicted 15-cis-phytoene desaturase of *Vitis vinifera*, *Populus trichocarpa* and other plant species.

All showed similar gene function and shared a highly conserved amino acid sequence.

- COG3349 is an uncharacterized domain family that contained a NAD-binding domain (Marchler-Bauer et al., 2016). The representative genes in this family are mostly phytoene desaturase, or Zeta-carotene desaturase in cyanobacteria (Du and Fang, 2018).
- Phytoene_desat is also known as a domain family whose members are phytoene desaturase from cyanobacteria (Marchler-Bauer et al., 2016).
- The most conserved sequence in Amino_oxidase family is a flavin binding site, and the primary members are maize polyamine oxidase and monoamine oxidases (Marchler-Bauer et al., 2016). However, some phytoene desaturases from bacteria were included in this family, such as phytoene desaturase in *Streptomyces* and *Flavobacterium*.

In the full CD-search result, domain hits, 51 in total, were identified via the CD-search tool search. Among them, three main interval regions were noted (Figure 3.4).

- Intervals (placements on the input sequence) from 147 to 1823 bp accounted for 31.4% (16) of the domain hits. Most domain hits in this area represented desaturases or oxidases, such as zeta-carotene desaturase and 9,9'-di-cis-zeta-carotene desaturase, the other two conserved domain families in PLN02487 superfamily.
- Intervals from 312 to 896 bp accounted for 60.8% (31) of the domain hits. Among these domain hits, several domains families contained a dinucleotide binding domain in this area. To confirm this, an additional analysis was conducted using the motif scan function of MyHits (<https://myhits.isb-sib.ch>); the putative codon translation

sequence of kenaf GEED01047592.1 was used as the input for motif scanning. The most conserved fragments (two) were identified at 112 to 143 aa which matched with a NAD binding site, and 111 to 146 aa which matched with a FAD-dependent oxidoreductase.

- The remaining 4 domain hits from the CD-search, located at 1071 to 1154 bp, had low bit scores (identity), and therefore, were not discussed.

```

Query 8      AAAAAATAGAAGCACAACTAACTTATATCCTCCACCCTTCCCTTGGATCTGCCCAACC 67
Sbjct 115    AAAAAATACAAGTACACGATTAACCTTATCACCTCCGCCACTTCTTGTATCTGCCCAACC 174
Query 68     CACTTTCCAT-----TCTTAATCTTAAG-----CATCTGCAGTTGAAT 105
Sbjct 175    CACTTTCCATATCCCACCTTCTTTTCAACTCAAGTGCTTTACTCACCTTTGCTTTGAAT 233
Query 106    CGGGTCATCTGAGCTTTGGGATTTTGGTTTTGAAGAAAATGAGTCTCTCTGGGAGTGTT 165
Sbjct 234    TCGGTCATCCGAGCTTTGGGATTTTGGTCTTGAAGAAAATGAGTCTCTGTGGGAGTGTT 293
Query 166    CTGCTTTGCACCTAACTTCCAAGCAACAGGATGCGCATGGGAAATGCCTTGGCTTTTA 225
Sbjct 294    CTGCCCTGTACTTAACTTACAAGCAGCAAGATAAGCATGGGAAGTGCTTAGCTTTTA 353
Query 226    AAAGTGGTGAATCCATGGGAAATACCTTGAGAGTTCCATTAAGAAAGAGGTCAAGTAAGG 285
Sbjct 354    GAAGTGGTGAATCCATGGGAAATACCTTGAGAATTCCCTTTAAAAAGAGGTCAAGTAAGG 413
Query 286    GTGCT---TGTCCTTTGCAGGTAGTTTGCATAGATTATCCAAGGCCAGAGCTTGAGAATA 342
Sbjct 414    GTGCTGCTTGTCCTTTGCAGGTAGTTTGCATAGATTATCCAAGGCCAGAGCTAGAGAATA 473
Query 343    CCGTTAATTTTTGGAGGCTGCTTCTCTATCCGCTTCTTTCCGTTCTGCTCCCCGTCAG 402
Sbjct 474    CTGTTAATTTTTGGAGGCTGCTTCTCTATCTGCTTCTTTTCGTTCTGCTCCCCGTCAG 533
Query 403    CTAAGCCATTGCAAATCATAGTTGCTGGTGCAGGTTTGGCTGGTTTTGTCAACTGCAAAGT 462
Sbjct 534    CTAACCATTGAAAGTCATAATTGCTGGTGCAGGTTTGGCTGGTTTTGTCAACTGCAAAGT 593
...
Query 1663  CGATACAGGGGTTCTATCTAGCAGGTGACTACACAAAGCAAAGTATTTAGCTTCAATGG 1722
Sbjct 1794  CAATACAGGGGTTCTATCTAGCAGGTGATTACACAAAGCAAAGTATTTAGCTTCGATGG 1853
Query 1723  AAGGTGCTGCTCTCAGGGAAGCTTTGTGCTCAGTCTATTGTACAGGATTATGAGTTGC 1782
Sbjct 1854  AAGGGGCTGTGCTCTCAGGGAAGCTTTGTGCACAGTCTATTGTACAGGATTATGAGTTGC 1913
Query 1783  TTTGTAGTATGGGACAAAGAAAGTTGACTGGAGCAAGCATTTCGCTGATGTTCTTTAAGTT 1842
Sbjct 1914  TTCGTACTTTGGGACAAAGAAAGTTGACAGGAGCAAGCATTCACTGATGTCCTTTGAATT 1973
Query 1843  GAAAGTAAGCGATTTACGTGTTACAAGGATCATCTGCAAATCAATTGTTTAAGGCCAAT 1902
Sbjct 1974  GAAAGTAAACAGGTCACAAGTTATCGAGGATCTTCTGCTAATAGATTGTTTAAGGCCACT 2032
Query 1903  AGGTGAGTGTGGTCTTTTT 1921
Sbjct 2033  AGGTTAGTGTTCTTTTT 2050

```

Figure 3.3 The highest identity area of alignment between transcripts GEED01047592.1 and Gorai.011G149900.1.

Query (GEED01047592.1; putative kenaf *pds* transcript) ranged from 8 to 1921 bp; Sbjct (Gorai.011G149900.1; cotton *pds* transcript) ranged from 115 to 2050 bp. Shaded areas identified gaps; CDS of cotton *pds* was underlined. No gaps were present in the sequences not included (“...”; 463-1662 bp of GEED01047592.1).

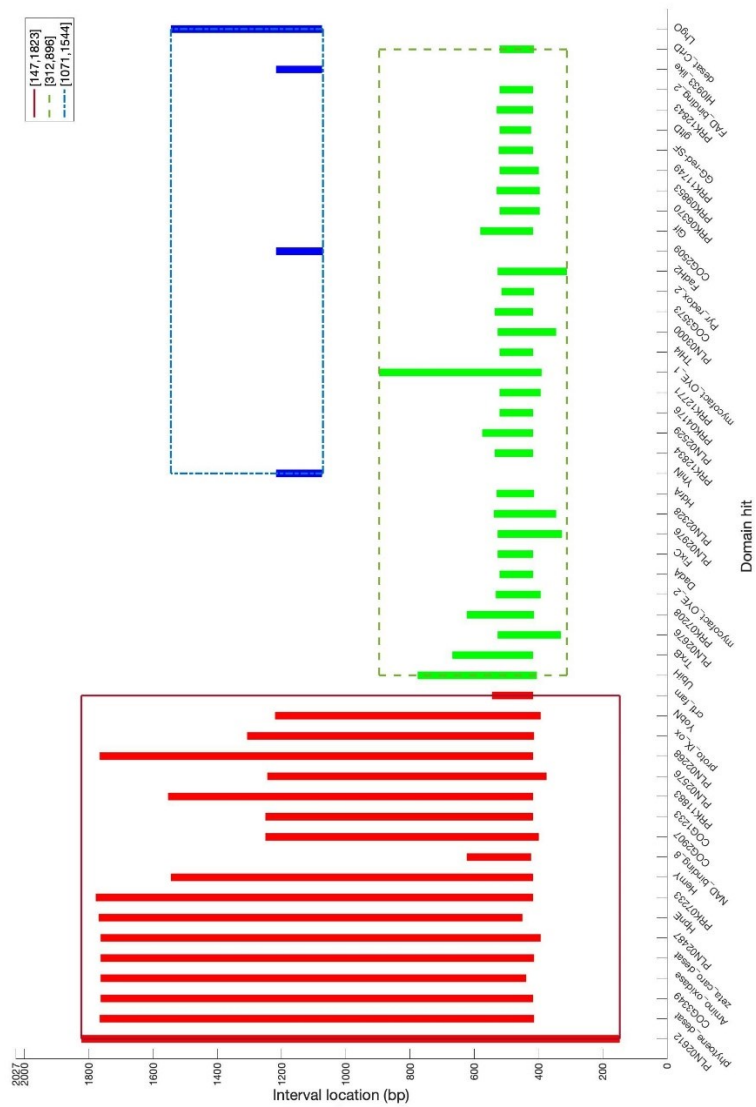


Figure 3.4 Domain hits and their locations in GEED01047592.1 using Conserved Domain search (CD-search) tool.

All 51 domain hits were included. Domain names were listed on horizontal axis in the order provided by CD-search. Vertical axis indicated their length and location (number of bp each spans). Three identified groups (in boxes) represented different ranges of locations: left box ranged from 147 to 1823 bp; right bottom box ranged from 312 to 896 bp; right top box ranged from 1071 to 1544 bp.

3.3.2 Polymerase chain reaction (PCR) and gel analysis

E41 kenaf genomic DNA was extracted from mature leaf tissues three times; the DNA concentrations ranged from 3.4 to 7.6 ng/ μ l (measured by Nanodrop spectrophotometer).

Analyses characterized the genomic counterpart of transcript GEED01047592.1.

The first pair of primers was not designed to yield a certain length genomic DNA fragment, since only the transcriptome sequence was available which did not contain intron sequences. The forward primer (*pds* forward) was located at 377 bp, and the reverse primer (*pds* reverse) was located at 1552 bp of the transcript sequence. Post-PCR, DNA was analyzed via gel electrophoresis; all three genomic DNA samples yielded the same size fragment, about 400 bp in length. In a repeat experiment with increased extension time, the same size band was obtained. Therefore, to confirm the specificity of the primer for kenaf GEED01047592.1 (*pds*), an alignment between primers and the whole kenaf transcriptome was conducted using BLAST-alignment. The forward primer had 6 potential targeted sequences in the transcriptome containing 16/20 matches to the primer, and the reverse primer had 9 potential targeted sequences in the transcriptome containing 13/20 matches to the primer. All potential targets for forward and reverse primers were in different transcript sequences, so no amplification of off-target genes was expected. However, if there were unexpected annealing of primer(s) to intron sequences, a PCR product with a different size could have been amplified.

In another experiment, when the full-length cotton *pds* gene was used to predict possible kenaf *pds* gene intron positions, use of two additional pairs of primers further confirmed the sequence being analyzed was a *pds* gene in kenaf. The first pair of primers (forward primer Mutation g1F1 and reverse primer Mutation g1R1; Table 2.3) targeted 1179 to 1284 bp (105 bp) of the kenaf transcript. Comparing this sequence (transcript) to cotton *pds* genome sequence, the

expected size of this fragment in kenaf was about 200 bp (spanned a short intron). On a 4% agarose gel, an actual size about 200 bp band was confirmed to be present in each kenaf DNA sample analyzed. The second pair of primers (forward primer Mutation g2F1 and reverse primer Mutation g2R1; Table 2.3) targeted 373 to 504 bp (131 bp) of the kenaf transcriptome sequence. Based on the cotton *pds* genome sequence, the expected size of this fragment in kenaf was about 850 bp. The actual band amplified in kenaf DNA was between 400 bp and 500 bp; since kenaf DNA was successfully amplified, kenaf's intron might be shorter than cotton's intron at that location. Subsequent Sanger sequencing results (will be discussed in the gene knock-out section) also confirmed the successful amplification of *pds* in kenaf.

Therefore, although the kenaf genome sequence was not available, we could identify a kenaf transcribed RNA sequence Unigene18524 (GEED01047592.1) that appears to be a putative kenaf *pds* gene. It is, most likely, comprised of 1690 bp CDS encoding ~560 aa sequence; a putative dinucleotide binding domain (most probably, a NAD binding domain) was potentially located at 312 to 896 bp. This is the first phytoene desaturase gene identified in kenaf. As a key gene early in the carotenoid biosynthesis pathway, the knock-out of *pds* could lead to visual phenotypic changes, such as an albino phenotype. It has the potential to be a knock-out reporter gene for developing procedures using CRISPR or other genome-editing tools for kenaf.

3.4 Gene Knockouts

3.4.1 GUS assay on previously transformed plants

Transgenic E41 seeds (T1) that contained β -glucuronidase (*gus*) were from 4 SCLs (A-3-2, A-3-7, B-2-2, B-2-9), generated by Dr. Margaret Young (Young, 2000). Seeds were germinated and leaves tested for GUS activity. After incubation in X-gluc solution, no areas of blue color were observed on any mature leaves after either incubation time (4 hr or 16 hr). The

failure to identify GUS expression in transgenic kenaf tissues did not allow that gene to be targeted for knock-out. That led us to focus on the *pds* gene. The goal was to develop the CRISPR genome-editing method for kenaf by targeting the kenaf *pds* gene for knock-out.

3.4.2 CRISPR *pds* target selection and gRNA designation

The goal was to design gRNAs to target potential functional domains in *pds* for greater knock-out or knock-down efficiency. Therefore, results of Unigene18524 (GEED01047592.1) against the Conserved Domain Database using CD-search, and the alignments between banana *pds* conserved motifs and GEED01047592.1 were important criteria used in determining target locations of gRNAs.

As analyzed and described in the previous section, domain hits of CD-search results ranged from 147 to 1823 bp of GEED01047592.1 (Figure 3.4, left box). A dinucleotide binding domain was expected to be located in the area spanning 312 to 896 bp (Figure 3.4, right bottom box).

Three motifs were identified in the banana *pds* gene *RAS-PDS1* by Kaur et al. (2018): (1) dinucleotide binding motif with a length of a 31 aa, (2) putative substrate carrier motif with a length of 11 aa, and (3) carotenoid binding domain with a length of 22 aa. Alignments between these motifs and GEED01047592.1 showed 100% identities for the dinucleotide binding motif and the carotenoid binding domain, located at 429-521 bp and 1683-1748 bp, respectively, in GEED01047592.1. The putative substrate carrier motif was located at 1218-1250 bp in GEED01047592.1, and presented an identity of 73%. Combining these results with the CD-search results, the goal was to design gRNAs that could target these areas that contained functional domains to, potentially, increase the chance of successful knock-out (or knock-down) after genome editing.

Potential gRNA targets were generated by the gRNA design tool CRISPRdirect, which listed all the potential gRNA target sites in the input nucleotide sequence. All the gRNAs in this list ended with a PAM sequence (NGG) downstream.

GEED01047592.1 was set as the input sequence, and for the specificity check, cotton genome (*Gossypium raimondii*, v2.1) was chosen as the comparison genome. The CRISPRdirect output identified 189 candidate 20-bp kenaf gRNAs; 32 of 189 represented unique target sites in the cotton genome. The rest (167 potential gRNAs) showed a “0 target site” to cotton genome, which could have resulted from imperfect identity between kenaf *pds* mRNA sequence and the cotton genome, or target sites were split by introns.

Three additional criteria were also considered in gRNA selection, including “12mer+PAM” hits, “TTTT” and “GC%”. The 12mer+PAM analysis searched each gRNA’s 12 bp sequence (adjacent to PAM) against the cotton genome. Such a region was considered to contain key residues for target-specificity by CRISPRdirect; a lower number of “12mer+PAM” hits indicated higher specificity. “TTTT” sequences in gRNA were also suggested to be avoided in gRNA vectors by CRISPRdirect. A moderate GC content was also considered to increase gRNA stability.

Among those 32 gRNAs that had a unique target site, we finally selected 2 gRNAs. Both contained a GC content from 45% to 50%, did not include “TTTT” in their sequences, and only had one or two “12mer+PAM” hits. The first gRNA (named “gRNA1”) bound to the antisense strand at 1245-1267 bp, which partially overlapped the *pds* putative substrate carrier motif. The second gRNA (named “gRNA2”) bound to the sense strand at 456-478 bp, which was located in the *pds* putative dinucleotide binding motif (Figure 3.5).

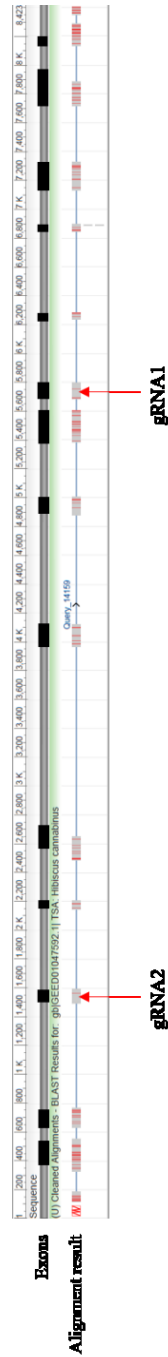


Figure 3.5 BLAST-alignment between cotton *pds* genome sequence and kenaf GEED01047592.1 transcript.

In the “Exons” line, exon sequences were labeled with black boxes; in the “Alignment result” line, high identity areas were showed in gray boxes; mismatches or gaps were indicated with darker lines; arrows pointed to the selected gRNA target sites.

3.4.3 Construct gRNAs into plasmids

Both gRNAs were individually cloned into plasmid pDIRECT_22A. After blue-white colony screening, 14 positive colonies that contained 22AgRNA1 and 43 positive colonies that contained 22AgRNA2 were grown on selection medium (LB agar containing 50 mg/l kanamycin and 32 mg/l X-gal). Four samples (two 22AgRNA1 plasmids and two 22AgRNA2 plasmids) were sent to the DNASU Sequencing Core for Sanger sequencing; 3 contained correctly cloned gRNA. These cloned plasmids were extracted and one representative of each gRNA was introduced into *A. tumefaciens* strain EHA105. Resulting *A. tumefaciens* contained one of the gRNAs: EHAgRNA1 and EHAgRNA2.

3.4.4 Screen for transgenic knock-outs

In the transformation experiment, 572 cotyledon explants, 216 hypocotyl explants and 104 petiole explants were transformed with EHAgRNA1; 592 cotyledon explants, 116 hypocotyl explants and 84 petiole explants were transformed with EHAgRNA2.

After 2 weeks on selection media containing geneticin, expanded explants as well as bleached explants were both observed on all the three tissue types, regardless of which gRNA was introduced (EHAgRNA1 or EHAgRNA2). Surviving cotyledon explants produced organogenic calli, whereas surviving petiole explants and hypocotyl explants only generated translucent friable calli. Browning due to phenolics was noted and seemed to initiate where explants were in direct contact with selection media. Since the plasmid used to introduce gRNA and *cas9* did not contain any reporter gene, early transformation efficiencies could not be determined.

After 10 weeks on selection media, non-viable (bleached or dark brown) explants were observed in differing percentages: 73.8% petiole explants, 62% hypocotyl explants and 54%

cotyledon explants. Cotyledon explants continued to yield organogenic calli (38.3%; percentage of cotyledon explants that yielded organogenic calli among all cultured cotyledon explants). Other viable explants started to generate organogenic calli, albeit in lower percentages: petiole explants (12.8%) and hypocotyl explants (7.5%). White/pale green (chlorotic) organogenic calli were first observed on cotyledon explants transformed with either gRNA construct, but not hypocotyl or petiole explants. These organogenic calli presented the same smooth surface and round shape; however, they were not bright green, but rather white or pale green in color (Figure 3.6).

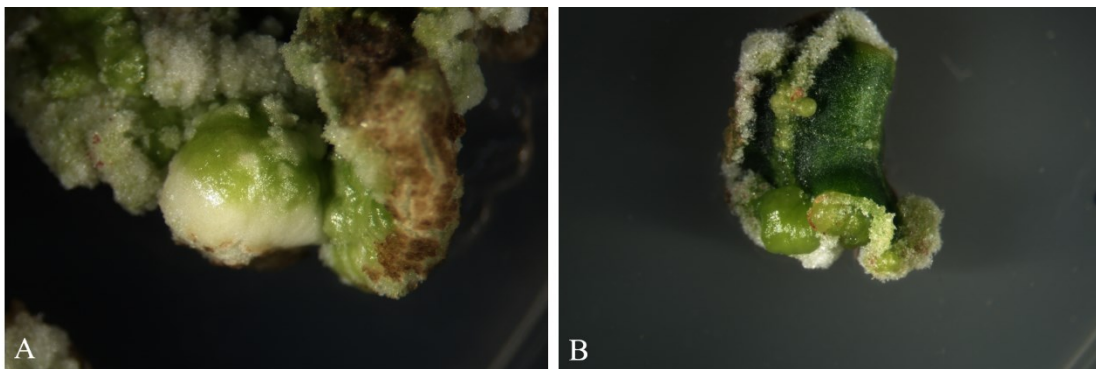


Figure 3.6 Putative transformed calli and control.

(A) Partially chlorotic organogenic calli arising from cotyledon explant transformed with EHAgRNA1; picture was taken at 2 months on S5 (0.1 mg/l NAA, 2.5 mg/l TDZ and 10.2 mg/l AgNO_3) with 10 mg/l geneticin. (B) Wild-type E41 cotyledon explant that yielded normal organogenic calli; picture was taken at 2 months in culture on medium S5.

Adventitious shoots were also observed arising from both cotyledon and petiole explants after 10 weeks on selection media. Cotyledon explants yielded the greatest percentage (3.4%) of regenerating shoots, with petiole explants at 1.6%. Two pale green shoots were observed on cotyledon explants transformed with EHAgRNA1. No white/pale green shoots were generated

from any petiole or hypocotyl explants, nor cotyledon explants transformed with EHAgRNA2. The pale green shoots contained newly arising leaves that were partially white.

At 16 weeks, cotyledon explants transformed with each gRNA construct displayed albino/chlorotic calli and diminutive shoots. To begin to molecularly characterize these phenotypically abnormal tissues, we selected 6 white/pale green shoots/organogenic calli from explants transformed with EHAgRNA1, and 4 white/pale green shoots/organogenic calli transformed with EHAgRNA2 for analyses described below (Figure 3.7).

Genomic DNA was extracted from each white/pale green tissue (arose from cotyledon explants) and a wild-type adventitious shoot (arose from a cotyledon explant, the negative control); DNA concentrations ranged from 6.5 to 31.7 ng/ μ l (measured by Nanodrop spectrophotometer). PCR products were amplified with *cas9* forward and *cas9* reverse primers (listed in Table 2.3), and analyzed via gel electrophoresis. The *cas9* gene was successfully amplified in 6/10 DNA samples isolated from putative transgenic tissues (Figure 3.7A); tissues 8A, 8B, 9A, 9B, 9D and 22A presented the same size band (850 bp) as the positive control (pDIRECT_22A). Among these tissues, 8A, 8B, 9A, 9B and 9D were transformed with EHAgRNA1, and 22A was transformed with EHAgRNA2. Morphologies of the tissues subjected to DNA analysis are shown in Figure 3.7B-D.

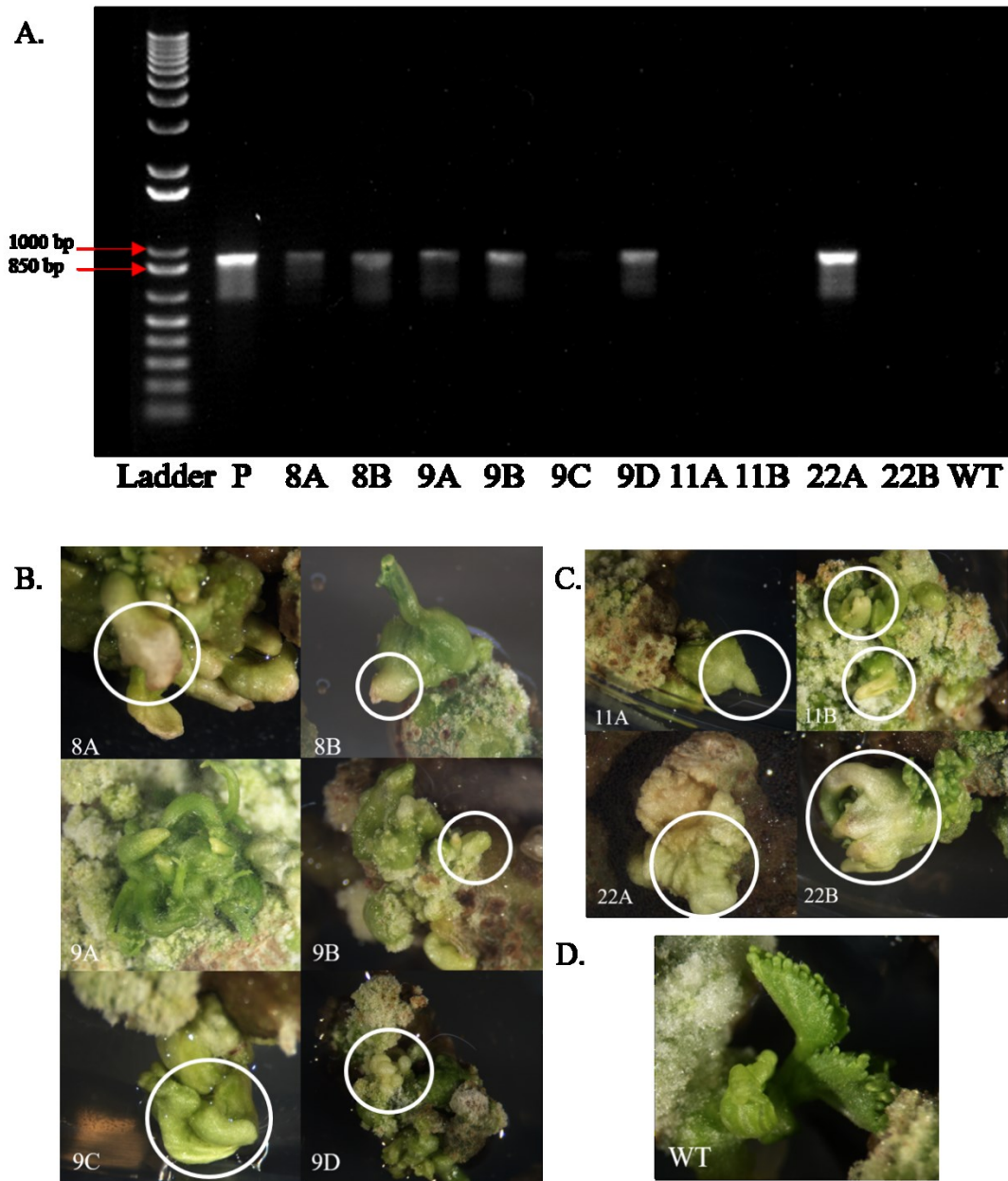


Figure 3.7 Gel analysis of *cas9* in putative transformed tissues from cotyledon explants, and their observed phenotypes in culture.

(A) Agarose gel electrophoresis of amplified *cas9* DNA. PCR-amplification using “mutation” primers (listed in Table 5); P is DNA isolated from plasmid pDIRECT_22A, as positive control; 8A, 8B, 9A, 9B, 9C, 9D were DNA isolated from individual explant tissues transformed with EHAgrRNA1; 11A, 11B, 22A, 22B were explants that transformed with EHAgrRNA2; WT DNA was isolated from a wild-type E41 as negative control. (B) Individual tissues transformed with EHAgrRNA1; tissues in white circles were albino/chlorotic. (C) Individual tissues transformed with EHAgrRNA2; tissues in white circles were albino/chlorotic. (D) Wild-type E41 explant with a regenerating adventitious shoot. All the explant photos were taken at 4 months in culture.

Primers (listed in Table 2.3) were also used to amplify the introduced *nptII* gene, but no specific bands were correctly amplified, including the positive control (plasmid pDIRECT_22A); this potentially indicated problems with initial primer design. Although the *nptII* gene fragment was not amplified correctly, the amplified *cas9* fragment in putative transgenic tissues confirmed the successful transformation of these tissues.

As described in the *pds* identification section, *pds* fragments that spanned each gRNA target site were amplified using “mutation” primers (Table 2.3). In the absence of kenaf genome data, the cotton genome was used to evaluate the size of each resulting amplified fragment (including the intron sequence). Finally, a ~200 bp fragment that spanned the gRNA1 target and a ~500 bp fragment that spanned the gRNA2 target were PCR-amplified using template DNA from transformed E41 samples and a wild-type E41 sample. Amplified DNA fragments spanning gRNA1 and gRNA2 target sites were sent for Sanger sequencing. Sequencing results indicated that the DNA sequence for all transformed samples (8A, 8B, 9A, 9D, 11A, 11B, 22A, 22B) were identical to the wild-type sample. No indels were observed in any sample. Such a result did not support our assumption and observation of albino/abnormal phenotype tissues, and the reason was unclear. Odipio et al. (2017) used amplicons to molecularly analyze mutations induced by CRISPR in the *pds* gene (*MePDS*) of *Manihot esculenta*. PCR products were cloned and introduced into *E. coli*. They then selected 5-10 single colonies for each putative transgenic plant line; DNA from each was sent for Sanger sequencing. Results showed that most transgenic plants (89.5% and 78.9% in two cultivars) were heterozygous for *pds* knock-out. Nakajima et al. (2017), Nishitani et al. (2016) and Kaur et al. (2018) used a similar method of amplicon sequencing to detect mutations induced by CRISPR. In our experiment, genomic DNA was extracted from compact calli or newly arising leaves, which, most likely, led to a mixture of

transgenic and non-transgenic tissues. Lower percentages of genome-edited cells in the tissues might have reduced the chance for mutation detection by sequencing. In hindsight, amplicons should have been generated and individually sequenced, although tissue sample sizes were extremely limited due to the noted diminutive/dwarf-like growth of these tissues.

After 20 weeks on selection media, white/pale green adventitious shoots continued to regenerate from cotyledon explants. At this time point, 7 white/pale green shoots regenerated from cotyledon explants transformed with EHA_gRNA1, and 6 from cotyledon explants transformed with EHA_gRNA2. In addition to chlorotic phenotype, clusters of small shoots arose but failed to elongate. An albino small shoot-like cluster was also observed on a hypocotyl explant transformed with EHA_gRNA2. Neither chlorotic/albino phenotype or the diminutive shoot-like clusters were ever previously noted in regenerated adventitious shoots, either wild-type or transgenic. These types of shoots were continually generated beyond 20 weeks in culture, although all remained diminutive/dwarf-like.

Via construction of CRISPR/Cas9 plasmids containing designed gRNAs, we regenerated organogenic calli and adventitious shoots from E41 seedling tissues that presented albino/abnormal phenotypes. These phenotypes were never observed in previous research. Although, with the current lack of molecular evidence, we could not confirm successful genome editing through this protocol. However, successful plasmid construction, transformation and regeneration were confirmed in these experiments. Considering the possibility of low percentages of indels that could not be detected by sequencing, future studies could focus on methods such as amplicon generation/sequencing and T7 endonuclease1(T7E1) assay. A T7E1 assay was used for mutation detection in *pds* of tomato (Parkhi et al., 2018); through the formation of a heteroduplex structure, T7 endonuclease1 digested the mismatches and detected

the mutations in *pds* DNA. These putative kenaf transgenic (knock-out) tissues are being maintained (Figure 3.8); future studies on these tissues may confirm successful genome-editing of kenaf.

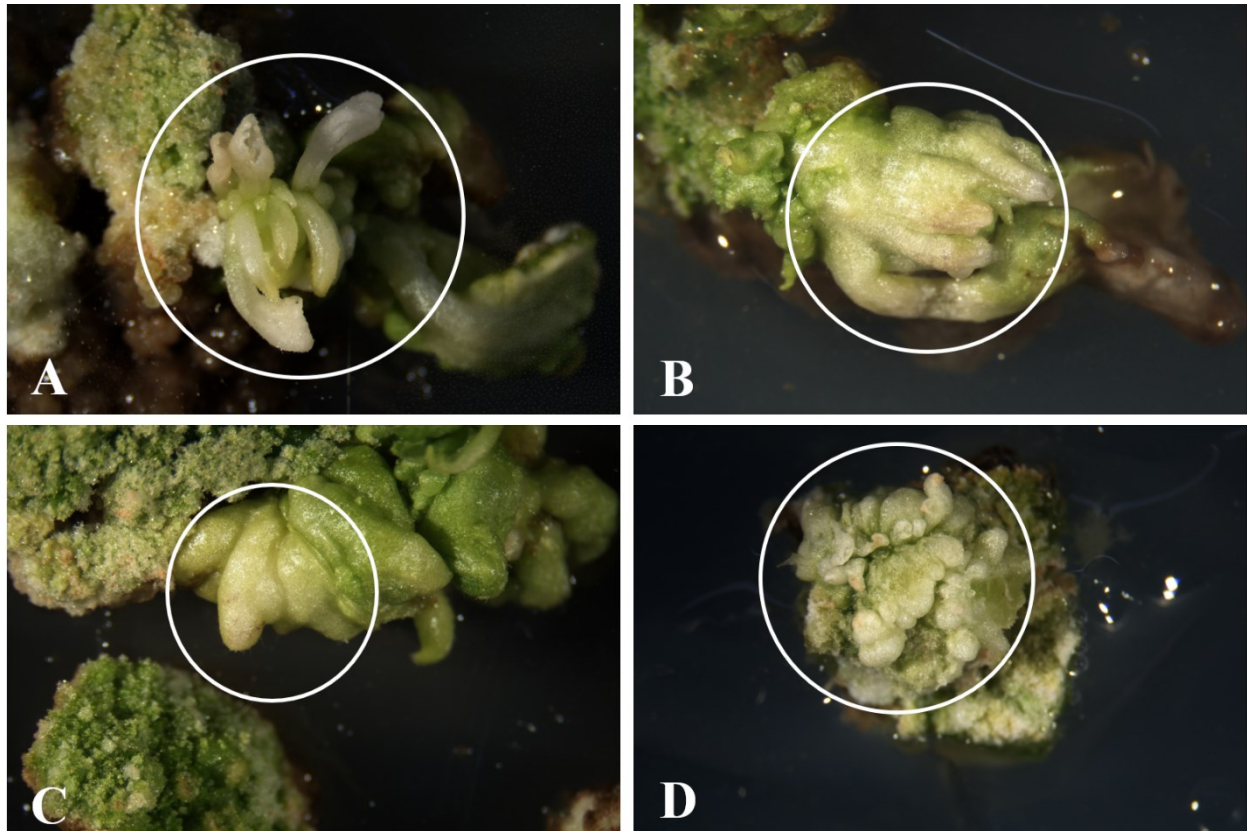


Figure 3.8 Putative transgenic adventitious shoots after 6 months in culture.

All four pictures were taken at 6 months after placing on selection media (S5 supplied with geneticin). (A) Albino shoots arising from cotyledon explant transformed with EHAgRNA1. (B) Chlorotic shoots arising from cotyledon explant transformed with EHAgRNA2. (C) Chlorotic shoots arising from cotyledon explant transformed with EHAgRNA1. (D) Clustered albino shoot-like structure arising from hypocotyl explant transformed with EHAgRNA2.

3.5 References

- Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25:3389-3402
- Du R, Fang Z (2018) Statistical correction for functional metagenomic profiling of a microbial community with short NGS reads. *Journal of Applied Statistics* 45:2521-2535
- Duclercq J, Sangwan-Norreel B, Catterou M, Sangwan RS (2011) De novo shoot organogenesis: from art to science. *Trends in Plant Science* 16:597-606
- Herath SP, Suzuki T, Hattori K (2005) Factors influencing *Agrobacterium* mediated genetic transformation of kenaf. *Plant Cell, Tissue and Organ Culture* 82:201-206
- Kaur N, Alok A, Kaur N, Pandey P, Awasthi P, Tiwari S (2018) CRISPR/Cas9-mediated efficient editing in phytoene desaturase (PDS) demonstrates precise manipulation in banana cv. Rasthali genome. *Functional & Integrative Genomics* 18:89-99
- Kumar PP, Lakshmanan P, Thorpe TA (1998) Regulation of morphogenesis in plant tissue culture by ethylene. *In Vitro Cellular & Developmental Biology-Plant* 34:94-103
- Lim T-K (1997) Optimization of organogenic regeneration and in vitro selection for *Cristulariella moricola* resistance in kenaf (*Hibiscus cannabinus* L.) Thesis, Mississippi State University
- Marchler-Bauer A, Bo Y, Han L, He J, Lanczycki CJ, Lu S, Chitsaz F, Derbyshire MK, Geer RC, Gonzales NR, Gwadz M, Hurwitz DI, Lu F, Marchler GH, Song JS, Thanki N, Wang Z, Yamashita RA, Zhang D, Zheng C, Geer LY, Bryant SH (2016) CDD/SPARCLE: functional classification of proteins via subfamily domain architectures. *Nucleic Acids Research* 45:D200-D203
- Nakajima I, Ban Y, Azuma A, Onoue N, Moriguchi T, Yamamoto T, Toki S, Endo M (2017) CRISPR/Cas9-mediated targeted mutagenesis in grape. *PLoS ONE* 12:e0177966. <https://doi.org/10.1371/journal.pone.0177966>
- Nishitani C, Hirai N, Komori S, Wada M, Okada K, Osakabe K, Yamamoto T, Osakabe Y (2016) Efficient genome editing in apple using a CRISPR/Cas9 system. *Scientific Reports* 6:31481. <https://doi: 10.1038/srep31481>
- Odipto J, Alicai T, Ingelbrecht I, Nusinow DA, Bart R, Taylor NJ (2017) Efficient CRISPR/Cas9 genome editing of phytoene desaturase in cassava. *Frontiers in Plant Science* 8:1780. <https://doi.org/10.3389/fpls.2017.01780>
- Pandian SRK, Deepak V, Kalishwaralal K, Viswanathan P, Gurunathan S (2010) Mechanism of bactericidal activity of Silver Nitrate-a concentration dependent bi-functional molecule. *Brazilian Journal of Microbiology* 41:805-809

- Parkhi V, Bhattacharya A, Choudhary S, Pathak R, Gawade V, Palan B, Alamalakala L, Mikkilineni V, Char B (2018) Demonstration of CRISPR-*cas9*-mediated *pds* gene editing in a tomato hybrid parental line. *Indian Journal of Genetics and Plant Breeding* 78:132-137
- Ruotolo G, Del Toro A, Chiaiese P, Filippone E (2011) Multiple buds are an alternative target to genetic manipulation of kenaf (*Hibiscus cannabinus* L.). *Industrial Crops and Products* 34:937-942
- Sharma P, Rajam MV (1995) Genotype, explant and position effects on organogenesis and somatic embryogenesis in eggplant (*Solanum melongena* L.). *Journal of Experimental Botany* 46:135-141
- Withanage SP, Hossain MA, Roslan HAB, Abdullah MP, Napis SB, Shukor NAA (2015) Overexpression of *Arabidopsis thaliana* gibberellic acid 20 oxidase (AtGA20ox) gene enhance the vegetative growth and fiber quality in kenaf (*Hibiscus cannabinus* L.) plants. *Breeding Science* 65:177-191
- Young MM (2000) Genetic transformation of kenaf. Dissertation, Mississippi State University

CHAPTER IV

SUMMARY

In regeneration experiments, there were successes and failures. Although adventitious shoots were not generated on any tissues of kenaf cultivar Whitten, organogenic calli were produced on several media. Adventitious shoot organogenesis from both cotyledon and cotyledonary petiole tissues of cultivar E41 was successfully demonstrated. The greatest generation of organogenic calli and adventitious shoots were observed on media S4 (MSO supplemented with 0.1 mg/l NAA, 0.35 mg/l TDZ and 10.2 mg/l AgNO₃) and S5 (MSO supplemented with 0.1 mg/l NAA, 2.5 mg/l TDZ and 10.2 mg/l AgNO₃). Through green fluorescence observation (EGFP) in transformed E41 seedling tissues, modified transformation procedures were developed. This confirmed transformation protocol enabled further research of genome editing using the CRISPR/Cas 9 system.

A kenaf *pds* gene (GEED01047592.1) was identified from the kenaf transcriptome and molecularly confirmed, the first time this transcript was analyzed and confirmed to encode a *pds* gene. The comparison between the kenaf transcriptome and *pds* genes in other species was conducted. Via bioinformatic tools including BLAST-alignment, CD-search tool and MyHits, putative conserved areas in kenaf *pds* were identified. These results provided potential target locations for CRISPR/Cas 9 editing. This protocol for searching and identifying genes could also be used to target other functional genes in kenaf. This protocol also included an efficient way for selecting CRISPR targets in kenaf genes.

The gRNAs were designed and screened using CRISPRdirect. Two selected gRNAs were individually cloned into the chosen vector using the Golden Gate assembly method.

Post-transformation, *cas9* DNA was detected in DNA of adventitious shoots that survived selection, which indicated the vector was introduced into plant tissues successfully. After 20 weeks on selection media, in total, 13 seedling tissue explants yielded albino/abnormal organogenic calli or adventitious shoots that remained diminutive/dwarf-like; these morphologies had never been noted in culture before. Future research efforts could focus on molecularly confirming knock-out of the *pds* gene in kenaf.

Our experiments set an example for the processes of gene identification, gRNA selection, vector construction, transformation and shoot organogenesis. The combined protocol provided a pathway for the potential use of genome editing on other functional genes to genetically modify kenaf plants in the future.