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An Approach to Genetic Silencing of Ricin in Castor (Ricinus Communis L.)

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An approach to genetic silencing of ricin in castor (*Ricinus communis* L.)

By

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Castor (*Ricinus communis* L.) is a high-yielding oilseed crop native to tropical Africa. The seed contains ~60% oil by weight, yielding approximately 1,200 kg of oil per hectare. The oil is composed of ~90% ricinoleic acid, a unique hydroxyl-fatty acid. Its unique composition provides castor oil with distinctive characteristics important for industrial use. Unfortunately, this valuable oilseed has not been widely cultivated in the United States since 1972, due in part to the presence of ricin in the seed. Ricin is a highly toxic lectin found in the endosperm of mature castor seed. This project sought to silence ricin production through the introduction of an RNAi element into the castor genome. The RNAi vector (pC1-RKO) containing a segment of ricin mRNA and its inverted repeat separated by a chalcone synthase A intron from pFGC5941 enclosed in a pCambia1301 backbone was created, verified via sequencing, and transformed into *Agrobacterium tumefaciens* for castor transformation. Fungal contamination was a serious concern; successful disinfestation used a 10-minute wash with 0.1% mercuric chloride (w/v). Media supplemented with 6-benzylaminopurine generated healthier shoots from embryo axes dissected from mature seed compared to thidiazuron-treated

mesocotyls dissected from mature seed. Short treatments of thidiazuron on 6-benzylaminopurine initiated shoot cultures showed greater shoot proliferation on embryo axes dissected from mature seed. Rooting occurred with incubation on half-strength medium containing naphthaleneacetic acid or indole-3-butyric acid; however, naphthaleneacetic acid produced hardier roots which better survived acclimatization. Inoculation of embryo axis explants after 2 days pre-culture improved survivability. Likewise, transformations using *A. tumefaciens* cultures of 0.5 $O.D._{600}$ and lower did not lead to downstream bacterial contamination. The pCambia1304 vector was used as a test plasmid for refinement of the transformation protocol. Of the 870 pCambia1304 inoculation explants, 2 survived hygromycin screening and showed *gusA* activity. Of the 2,500 pC1-RKO inoculated explants, 6 survived hygromycin selection and rooted. Further analysis via PCR, end-point RT-PCR, and Western and dot-blotting showed these to be non-transformed and ricin content unaffected.

DEDICATION

I would like to dedicate this work to my wonderful family, especially my loving wife, Alicia, and amazing son, Ennis, who have had to carry on with their lives in my limited presence as I finish my studies.

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NOMENCLATURE

- BA 6-Benzylaminopurine
- ER Endoplasmic Reticulum
- ERAD Endoplasmic-Reticulum-Associated Protein Degradation
- IAA Indole-3-acetic acid
- IBA Indole-3-butyric acid
- MES 2-(N-Morpholino)ethanesulfonic acid
- MS Murashige & Skoog medium (Murashige and Skoog 1962)
- NAA Naphthaleneacetic Acid
- PCR Polymerase Chain Reaction
- PGR Plant Growth Regulator
- RNAi RNA interference
- TDZ Thidiazuron
- UPR Unfolded Protein Response
- X-gluc 5-bromo-4-chloro-3-indolyl glucuronide

CHAPTER I

LITERATURE REVIEW

The castor plant (*Ricinus communis* L.) has been cultivated by mankind for thousands of years for the oil present within its seed. Castor seed can be comprised of as much as 60% oil by weight and approximately 90% of this oil is in the form of ricinoleic acid. The unique properties of this oil convey a wide range of industrial applications. However, the endosperm of the castor seed also contains high concentrations of a potent toxin known as ricin (RCA_{60}) and a hemagglutinin, *Ricinus communis* agglutinin (RCA_{120}), as well as a class of proteins known to cause severe allergic reactions in sensitive individuals, 2S albumins. Ricin poisoning is rarely fatal in modern times, and most fatalities involving the handling of castor seed were the result of anaphylaxis caused by allergic reaction to the 2S albumins (Chen et al. 2004). However, perception of ricin as a security risk has contributed to a limited domestic castor crop. A genetic knockout of the ricin gene from a domestic castor cultivar would, hopefully, generate new interest in castor oil production in the United States. An appropriate tissue culture mediated transformation protocol is needed to generate this knockout and facilitate the re-commercialization of this crop in the United States.

Castor is a diploid (2n=2x=20) in the *Ricinus* genus, a monotypic genus of the Euphorbiaceae family (spurges). However, the castor plant presents a wide diversity of phenotypes within the *R. communis* species. The castor plant is typically believed to have

originated in tropical regions of Africa, where it behaves as a perennial. However, in subtropical and temperate climes, it is treated as an annual, producing winter-hardy seed that volunteer in the spring (Brigham 1993). Castor is normally characterized as a short-day plant; however, it is capable of flowering in a large range of day-lengths with variation in seed yield (Weiss 2000; Baldwin and Cossar 2009). Castor racemes generally contain both male and female flowers, and, in most varieties, the female flowers are distal on the raceme whereas the male flowers are proximal. However, owing to the phenotypic diversity of the species, some genotypes have distal male flowers or sexes segregated among racemes. Female flowers typically are pollinated and ripen sequentially from proximal to distal (White 1918). This is counter to most domestic crop species which typically flower and ripen synchronously enabling the seed to be harvested at once. The genus name *Ricinus* indicates the resemblance of the castor seed to a tick; 'ricinus' is the Latin word for 'tick'. The common name 'castor' was possibly contributed by English traders who mistook castor for *Vitex agnus-castus*, or due to castor oil's use as a substitute for castoreum, a substance expressed from the glands of both North American and European beavers (genus *Castor*) used in some perfumes, medicines, and food products (Weiss 2000).

Some wild varieties of castor can grow as large as 12 meters tall; however, mankind has bred castor varieties to allow for contemporary cultivation. Castor is phenotypically similar to cross-pollinated crops; however, it suffers from no inbreeding depression allowing breeders to screen and select single plants with desirable traits and generate new cultivars relatively quickly. This has led to a wealth of phenotypically separate castor cultivars (White 1918; Weiss 2000). Modern cultivars have shortened

internodes to allow for mechanized harvest (Brigham 1993). In addition, most wild castor varieties exhibit dehiscence (shattering) where the ripened seed are dropped to the ground. Most modern cultivars have been bred to retain their seed after ripening since shattered seed cannot be mechanically harvested (Weiss 2000). This is of special importance in castor since the seed ripen sequentially. Resistance to diseases and fungi such as *Alternaria ricini* and *Xanthomonas ricinicola* has also been bred into many modern industrial cultivars (Brigham 1993). With all of these improvements, the yield from a modern castor cultivar can be upwards of 2,250 kg/ha of seed providing as much as 1,200 kg/ha of oil (Domingo and Crooks 1945; Baldwin and Cossar 2009).

Archeologists have discovered that the use of castor oil dates back as far as 6,000 years ago when Egyptians used it as lamp oil (Brigham 1993; Weiss 2000). Since ancient times, mankind has found many more uses for castor oil. It has been used as a laxative for centuries, and modern uses range from pharmaceuticals to cosmetics and from industrial lubricants to hydraulic fluids. In fact, the lubricants company Castrol derived its name from the words 'castor oil' due to its early use of castor oil in its lubricants (BP 2014). The primary reason castor oil has so many uses is the presence of a unique hydroxyl-fatty acid, ricinoleic acid (Zimmerman 1958). Castor oil is approximately 90% ricinoliec acid by weight, and is the only commercially significant source for hydroxyl-fatty acids (James et al. 1965; Broun and Somerville 1997).

Ricinoleic acid's unusual chemistry provides several unique properties to castor oil, making castor oil one of the most industrially important plant oils. Most notable is the fact that ricinoleic acid is easily miscible in polar alcohols such as methanol and ethanol. This is of particular importance in the transesterification of castor oil into biodiesel. In

this process, filtered castor oil is mixed with a polar alcohol and a base catalyst and heated to produce ethyl or methyl esters from the triacyl and diacyl glycerides, generating crude biodiesel (Conceição et al. 2007). Ricinoleic acid also reduces the gelling temperature of castor oil. This allows castor oil to remain fluid at lower temperatures compared to other vegetable oils; permitting its use in cold-weather applications (Zimmerman 1958). Additionally, ricinoleic acid does not rancify unless heated, allowing it to be stored for long periods of time without spoiling (Ogunniyi 2006).

Cultivation of castor within the United States started as early as the mid-1850's and lasted until the 1970's. In fact, castor oil was used by the United States military during World Wars I & II since it was a principle ingredient in many hydraulic fluids, greases, and military grade lubricants of the day. In 1984, the Agricultural Materials Act (P.L. 98-284) passed by the United States Congress listed castor as a strategic material for national defense, and Public Law 81-774 required stockpiles be maintained for all classified strategic materials in the event of war. In 1972, low prices, competition from higher value crops, oil price disagreements, and elimination of government price support ended the widespread cultivation of castor in the United States (Brigham 1993). In 2011, the United States imported 49,297 tonnes of castor oil at $2,340 per tonne. That equated to $115.4 million, not including shipping costs, spent by domestic industries on foreign castor oil that could have been produced domestically (FAOSTAT 2014). Returning castor cultivation to the United States would return this revenue back to the United States and help stimulate its use in other domestic markets, such as biofuels and chemurgics.

The castor oil collects within the endosperm of the mature castor seed. In order to extract the oil, the seed is generally mechanically pressed. However, this pressing only

removes 45-50% of the oil, requiring the addition of a solvent extraction process in order to obtain the remaining oil (Ogunniyi 2006). In solvent extraction, the mechanically-pressed seed meal, also known as pomace, is mixed with an organic solvent such as hexane. The filtered solvent is recovered by distillation, separating the oil from the solvent (Gardner et al. 1960). The final pomace contains the entirety of the seed minus only the oil, resulting in a meal that is high in nitrogen and has potential for use as fertilizer (Spies et al. 1962). An added benefit for its use as fertilizer is that it has an apparent nematocidal effect when used with plants susceptible to nematode infection (Akhtar 1997). Unfortunately, the removal of oil leaves ricin, a potent cytotoxin, within the meal. Previous work has shown that a heated press at 275°C is capable of denaturing ricin during extraction. Alternatively, it has been shown that boiling or autoclaving seed meal for 10 minutes also denatured ricin (Barnes et al. 2009a). The detoxification of ricin within the seed meal could produce a product similar to soybean meal. Soybean meal currently sells for approximately $500 per tonne (IndexMundi 2014). A 2,000 kg/ha average harvest of castor seed at an average of 60% oil by weight would generate approximately 800 kg/ha of seed meal. That translates to an estimated potential $400 per hectare in seed meal value, or around 14% of the value of the oil. Unfortunately treating seed meal would add cost to the milling process, potentially offsetting any gains from its use as a by-product. Therefore, it would be far more attractive to remove the ricin altogether.

When castor seed is milled and the oil extracted, the concentration of ricin within the meal is effectively doubled. Ricin is a water-soluble protein found concentrated in the endosperm of the mature castor seed. Older literature has stated that ricin is actually

found in all parts of the plant and that all parts of the castor plant are toxic (Knight 1979; Weiss 2000). However, more current work has shown that detectable levels of ricin are only found in the endosperm between 28 days post-pollination and 6 days post-germination (Barnes et al. 2009b). Ricin is not the only toxic material present in the castor plant. The endosperm also contains a relatively weak hemagglutinin, RCA_{120}, as well as potent allergens, 2-S albumins (Chen et al. 2004). The presence of these additional compounds means that castor pomace must be treated in some manner prior to handling for use as fertilizer or feed for livestock (Bris and Algeo 1970; Vilhjalmsdottir and Fisher 1971). Therefore, the removal or modification of these would be of particular interest in future research.

Ricin is perceived as the most potentially harmful component of castor seed in particular due to its extreme toxicity. The LD_{50} (lethal dose required to kill 50% of test subjects) in mice is 30 mg/kg when ingested and 3-5 µg/kg when inhaled or injected. Assuming a similar LD_{50} for humans, the lethal dose for a 90 kg (198 lb) person would only be 270-450 µg injected or inhaled. Ricin is potentially lethal to anyone, and there is no known antidote, resulting in widespread concerns over its potential use in bioterrorism (Audi et al. 2005). However, more deaths occur from anaphylaxis due to allergic reactions to the 2S albumins found in the seed meal as opposed to ricin exposure (Knight 1979; Chen et al. 2004).

Ricin and RCA_{120} are both lectins, a family of carbohydrate-binding proteins found in plants and thought to be responsible for defense responses. Ricin is a type II ribosome-inactivating protein (RIP), meaning that it is heterodimeric consisting of a toxic A-chain domain and a glycosylated B-chain domain. The A-chain, when free and active

within a cell, functions by enzymatically depurinating an adenine residue in the sarcin/ricin loop of the 28S ribosomal subunit (Endo et al. 1987). The effect is the irreversible deactivation of the elongation factor binding site of the ribosome, rendering the ribosome inactive. Since the A-chain functions enzymatically, its effect can be devastating to the cell. A single active A-chain is capable of deactivating as many as 1,500 ribosomes per minute *in vitro* (Pinkerton et al. 1999). The cell eventually loses the ability to produce new proteins and dies.

The B-chain is responsible for getting the A-chain into the cell. Without the B-chain, the A-chain is essentially inactive since it cannot enter the cell (Harley and Beevers 1982; Lord et al. 2003). The B-chain recognizes and binds β-1, 4-linked galactose residues on the cell surface, which are present in large numbers on the typical mammalian cell (typically on the order of 107 potential binding sites per cell). The bound ricin then enters the cell via an endocytic pathway, either clathrin-dependant or independent, depending on what surface component it has bound to. Once in an early endosome (EE), the ricin molecule follows one of several paths. It can be taken to the lysosome where the ricin molecule is degraded and nullified. Alternatively, it could be taken to a recycling endosome and merely trafficked back to the extracellular matrix by endosome delivery to the plasma membrane. However, a fraction of the introduced ricin will travel with the EE to the trans-Golgi network where an unknown process conducts the ricin molecule retrograde into the endoplasmic reticulum (ER) via the Golgi complex (Lord and Spooner 2011). Once in the ER, the endogenous protein disulphide isomerase cleaves the A-chain from the B-chain revealing a hydrophobic segment of the A-chain which then interacts with the ER membrane. This interaction stimulates the endoplasmic-

reticulum-associated protein degradation (ERAD) pathway. Typically a protein that triggers the ERAD pathway would be ubiquinated and marked for degradation; however, the ricin A-chain does not possess adequate lysine residues to be ubiquinated and can, thus, migrate out of the ER via the Sec61 channel into the cytosol without being degraded. At this point, the A-chain interacts with a chaperone protein, Hsc70, to stabilize the exposed hydrophobic segment. Once stable, the A-chain is enzymatically active and can begin depurinating ribosomes (Olsnes and Pihl 1972; Endo et al. 1987; Lord and Spooner 2011). It has also been shown that the processing of ricin by the ER and the induction of the ERAD pathway induces the unfolded protein response (UPR) within the cell. The UPR then stimulates several apoptotic pathways within the cell as a result of stress in the ER (Horrix et al. 2011). Thus, it has been theorized that the inhibition of translation by the A-chain may not be the sole cause of cell death. It may indeed be a combination of the inability to translate and the activation of apoptotic pathways.

Ricin is the product of a single gene. However, a recent draft of the castor genome (350 Mbp at 4.5x coverage) has identified 28 members of the ricin gene family (Chan et al. 2010). Of these, seven appear to be full genes and all of these copies show RIP activity (Leshin et al. 2010). This indicates the ricin gene could be present in seven separate loci within the genome, possibly more. This information suggests a mutation approach would be ineffective since all active copies of the gene would need to be mutated at once. Therefore, an RNAi approach is more desirable since only one copy of the RNAi gene needs to be active to silence all seven copies of the ricin gene.

The specific scenario for ricin silencing is described. The RNAi gene is comprised of a short hairpin RNA transcript (shRNA) preceded by a promoter and followed by a 3` UTR contained within the vector. The shRNA segment itself is composed of sense-strand and antisense segments flanking a chalcone synthase intron (Kerschen et al. 2004). This construct is designed in such a way that a single-stranded RNA that is transcribed from the inserted DNA within the castor genome will fold back upon itself via the included intron, creating a section of the transcript that is effectively double-stranded RNA. This double-stranded RNA segment will be identical to a sequence segment somewhere within the ricin mRNA. Dicer, an endogenous plant cell enzyme, recognizes the double-stranded RNA and cleaves it into a 22 base-pair segment with phosphorylated 5` ends (Hannon 2002). The two strands of this segment will then bind to RISC (RNA-induced silencing complex) which will unwind the two strands leaving a single RNA strand bound to the complex. The bound strand will then complementarily attach to the ricin mRNA, eliciting endonucleolytic cleavage of the mRNA by RISC, preventing translation of the mRNA into the ricin protein. Every time the mRNA is cleaved, new RNA segments are produced that bind to more ricin mRNA, shutting down ricin translation (Hannon 2002).

There are several current techniques that would achieve the same result as effective RNAi knockout. Each of these techniques has its own advantages and drawbacks. Gene targeting uses the phenomenon of homologous recombination to permanently edit a specific locus on the host genome. This process works well in prokaryotes and lower eukaryotes; however, in plants, it has a low efficiency as illegitimate recombination is the predominant form of DNA integration (Puchta 2002).

Some methods have been developed to increase the efficiency of this process in plants, but for this project, since ricin is expressed by multiple loci, requiring that each loci be altered in order to completely knockout ricin production, gene targeting would not be efficient (Hanin et al. 2001; Puchta 2002). Several newer techniques for altering the host genome involve the use of designed nucleases to cleave the genome at a specific locus. Zinc finger nucleases (ZFN) are a class of endonucleases that can be designed to recognize a very specific DNA sequence for cleavage. These nucleases take advantage of a class of eukaryotic transcription factors known as zinc finger proteins (ZFP). These proteins possess a DNA-binding domain that recognizes a specific 3 bp sequence of DNA. When multiple engineered ZFP domains are combined with the nuclease domain of FokI, a zinc finger nuclease is created. The sequence for the designed ZFN can then be transformed into the cell, and expression of the ZFN within the cell will lead to targeted cleavage of the host genome (Urnov et al. 2010; Hauschild-Quintern et al. 2013). Recently, ZFNs have been replaced by transcription activator-like effector nucleases (TALENs) due to their increased specificity and wider range of target sequences. These nucleases take advantage of the DNA binding capacity of a series of proteins generated by the plant pathogen *Xanthomonas*, known as transcription activator-like effectors (TALEs). When the *Xanthomonas* bacterium infects a cell, it typically releases TALEs which then bind and activate host cell promoters, activating genes to help the bacterium infect the cell. Much like ZFNs, TALEs can be engineered to recognize specific DNA sequences, and when bound to the nuclease domain of FokI, can induce site-specific DNA double-strand breaks (Chen and Gao 2013). More recently, a new system that utilizes an acquired immune response present in many bacteria and archae known as the

CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated) system has been used to generate site-specific double-strand breaks in plant genomes. The CRISPR/Cas system utilizes the sequence specificity of a single-guide RNA (sgRNA) and the nuclease activity presented by Cas9, an endonuclease. A vector containing the gene for Cas9 and a specifically designed sgRNA sequence would be capable of detecting a 14 bp specific sequence and cleaving the DNA in a site-specific manner (Larson et al. 2013; Zhang and Zhou 2014). There has been recent news regarding a company, Nova Synthetix, and their intent to develop two ricin-free castor lines that would not be classified as genetically modified organisms (GMOs) in order to avoid the regulatory requirements imposed by that classification (Burns 2013). Novo Synthetix is reportedly working with Precision Biosciences to generate this ricin-free castor. It stands to reason based on the developments established by this company, that they will be attempting to utilize a recently developed technique involving homing endonucleases. These homing endonucleases are large, engineered nucleases capable of cleaving DNA at a site-specific locus of 18-40 bp in length (Gao et al. 2010). These nucleases must be experimentally engineered, but even transient expression within the host cell would lead to site-specific DNA double-strand breaks. Regardless of the nuclease mechanism used, the resulting double-stranded break in the genome is repaired by the host cell mechanism. This repair can happen either through non-homologous end-joining (NHEJ) or homology-direct repair (HDR). Non-homology end-joining is error-prone, inserting or deleting base-pairs during correction of the double-strand break. These errors can lead to frame-shifting of the targeted gene, resulting in disruption of the gene. Homology-direct repair uses regions of the sister chromatid to assist in repairing the

damaged section of DNA. If a sequence homologous to the sites bordering the directed double-strand break is provided during transformation, the host-cell mechanism will incorporate this sequence in a site-specific manner. This can be useful for inducing gene disruption as well as introducing new sequence into the genome at a given locus (Hauschild-Quintern et al. 2013). The benefits of nucleases are that they can be directed to a specific locus and only require transient expression to accomplish gene disruption. However, they can be complicated to design and can exhibit non-specific activity within the genome, resulting in unwanted effects. Therefore, RNAi was the mechanism chosen for this project.

Castor tissue culture began in 1944 when La Rue was able to regenerate roots from endosperm culture derived from mature castor seed (La Rue 1944). Subsequent research ventures led to the establishment and proliferation of castor endosperm cultures from mature seed using a medium containing 2,4-dichlorophenoxyacetic acid (2,4-D), kinetin, and yeast extract (Mohan-Ram and Satsangi 1963; Satsangi and Mohan Ram 1965; Brown et al. 1970). Bahadur et al. (1991) established callus from cotyledon explants which then exhibited what the authors referred to as 'xylogenesis' when placed on a medium containing 10.7 µM 1-naphthaleneacetic acid (NAA) and 2.2 µM 6-benzylaminopurine (BA) (Bahadur et al. 1991). More recently, Rahman and Bari (2012) demonstrated the induction of callus from hypocotyl tissue from germinated castor seed using Murashige & Skoog (MS) medium supplemented with 8.9 µM BA and 2.7 µM NAA. This callus was then used to initiate cell cultures in liquid MS medium supplemented with 8.9 µM BA and 1 µM NAA (Murashige and Skoog 1962; Rahman and Bari 2012).

The principal issue surrounding castor culture is its recalcitrance to re-differentiation following callus formation. For this reason, the most successful and reproducible protocols have utilized direct organogenesis. Athma and Reddy (1983) successfully generated new shoots from shoot explants on a medium containing 2.2-8.9 μM BA followed by rooting on a medium containing 2.7 μM NAA. However, they were not able to promote organogenesis in callus derived from shoot, root, or cotyledon (Athma and Reddy 1983). Reddy et al. (1987) established shoot buds from hypocotyl and shoot tip explants as well as shoot tip callus generated on MS medium with Gamborg's B5 vitamins (Gamborg et al. 1968) supplemented with 9.3 μM kinetin and 5.4 μM NAA. This study also showed increased root formation in explants on 10.7 μM NAA-supplemented MS medium compared to MS medium supplemented with the same concentration of indole-3-acetic acid (IAA) (Reddy et al. 1987). Reddy and Bahadur (1989) later showed adventitious bud formation from 12-day-old castor leaf tissue placed on MS medium supplemented with 9.3 μM kinetin and 5.7 μM IAA. These shoots subsequently rooted on half-strength MS medium supplemented with 2.3 μM kinetin and 10.7 μM NAA (Reddy and Bhadur 1989). Sanguduen et al. (1987) showed multiple shoot proliferation from embryo explants cultured on MS basal medium supplemented with 15% coconut milk and 17.7 μM BA (Sangduen et al. 1987). Molina and Schobert (1995) described a protocol in which apical and axillary bud explants were cultured on a modified MS basal medium with NH_4N0_3 reduced to 450 mg/L and supplemented with 1 μM BA. This generated 3.6 shoots per explant in 79% of the explants (Molina and Schobert 1995). Sujatha and Reddy (1998) later tested embryo axes and shoot tips on MS supplemented with various levels of adenine, BA, kinetin, TDZ, and zeatin separately and

found that embryo axes on 22.7 μM TDZ generated on average 81.7 shoots while embryo axes on 2.2 μM BA generated on average 6.3 shoots. These shoots were elongated on MS supplemented with 2.2 μM BA and rooted on half-strength MS medium supplemented with 4.9 μM indole-3-butyric acid (IBA). The addition of 0.3 μM gibberellic acid (GA_3) to the elongation medium was shown to enhance the elongation of the shoots; however, it drastically reduced proliferation (Sujatha and Reddy 1998). Ahn et al. (2007) compared shoot proliferation of mesocotyl explants on MS medium supplemented with 1 μM TDZ or 20 μM BA, showing 24.2 shoots per explant on TDZ-supplemented MS medium compared to 6.8 on MS medium fortified with BA. This study also showed an 82% increase in shoot regeneration in explants cultured in dark conditions for the first 7 days. Rooting was achieved using 5 μM IBA or NAA; however, IBA-rooted plants showed a significantly higher rate of establishment (93.5%) versus NAA-rooted plants (39.5%) (Ahn et al. 2007). Ahn and Chen (2008) later described a protocol producing 25 adventitious shoots per explant when cotyledon explants dissected from mature castor seed were placed on MS medium supplemented with 5 μM TDZ and incubated in the dark for the first 7 days of culture. These shoots were then successfully rooted on 5 μM IBA-supplemented MS medium (Ahn and Chen 2008).

In addition to castor, there are several other industrially important members of the *Euphorbiaceae* family whose tissue culture protocols offer potential insights for further development of castor culture. Included among these are *Jatropha curcas* L., *Manihot esculenta* Crantz (cassava), and *Hevea brasiliensis* Müll.Arg. (rubber tree). *Jatropha* produces seed that, much like castor, is highly prized for its oil content. Micropropagation studies of *Jatropha* have generated several useful protocols. Jha et al.

(2007) produced embryogenic callus from leaf explants placed on MS medium supplemented with 9.3 μM kinetin. Somatic embryogenesis was then induced in 58% of the cultures by placing the cultures on MS meda supplemented with 2.3 μM kinetin and 1 μM IBA. The mature somatic embryos were then cultured on half-strength MS medium to stimulate development of plantlets (Jha et al. 2007). Cai et al. (2011) also induced somatic embryogenesis in embryo explants on a medium containing MS salts and Gamborg's B5 vitamins supplemented with 22-44 μM 2,4-D followed by a shift to hormone-free medium containing glutamine and asparagine (Cai et al. 2011). Deore and Johnson (2008) achieved direct shoot regeneration from leaf-disc explants cultured on MS supplemented with 2.27 μM TDZ, 2.2 μM BA, and 0.49 μM IBA. The shoots were then elongated on MS medium containing 4.4 μM BA, 2.3 μM kinetin, 1.43 μM IAA, and 0.72 μM GA_3 (Deore and Johnson 2008). Likewise, Kumar et al. (2010) successfully regenerated shoots from cotyledon explants on MS medium containing 9 μM TDZ (Kumar et al. 2010). Misra et al. (2010) showed direct shoot regeneration from mature *Jatropha* leaf explants cultured on MS medium containing 2.2 μM BA and 2.5 μM IBA (Misra et al. 2010). Sharma et al. (2011) showed direct shoot induction on hypocotyl explants from mature *Jatropha* seed when the explants were placed on MS medium supplemented with 2.2 μM TDZ. These shoots were then elongated on MS medium containing 9.3 μM kinetin and 4.5 μM BA. Rooting of the elongated shoots was performed on half-strength MS medium containing 14.8 μM IBA, 5.7 μM IAA, and 5.4 μM NAA (Sharma et al. 2011). Singh et al. (2010) described a protocol for generating *Jatropha* shoots from stem explants placed on MS medium containing 4.4 μM BA and

4.6 µM kinetin. Elongated shoots were then rooted on MS medium containing 0.5 µM IBA (Singh et al. 2010).

Cassava (*Manihot esculenta* Crantz) is an important starch crop in many developing countries. Cassava culture mainly takes place via somatic embryogenesis initiated from immature leaf lobes or shoot tips cultured on MS medium containing 2 µM $CuSO_4$, and 27.1 µM 2,4-D. Cotyledons from the somatic embryos were then transferred to organogenesis medium comprised of MS medium containing 4.4 µM BA, and 2.5 µM IBA. The shoots are then rooted on MS medium containing 0.5 µM NAA (Li et al. 1996; Bull et al. 2009). Rubber tree (*Hevea brasiliensis* Müll.Arg.) is also typically propagated via somatic embryogenesis. This process involves the culture of the internal seed coating on MH medium as defined by Carron and Enjalric (1985) to generate friable callus that can then be sub-cultured long-term (Carron and Enjalric 1985). The callus can then be cultured in embryogenic medium to stimulate embryogenesis. The immature embryos are then placed on hormone-free medium to develop into plantlets (Cailloux et al. 1996; Etienne et al. 1997; Blanc et al. 1999; Kumari Jayasree et al. 1999).

Agrobacterium tumefaciens is a soil bacterium capable of transmitting sections of DNA into a host plant cell. Originally, this introduced DNA was a section of a large, naturally-occurring plasmid that caused tumors to form in the infected tissue. This plasmid is referred to as the Ti-plasmid (Tumor inducing) and the tumor formation is known as Crown gall disease (Chilton et al. 1977; Păcurar et al. 2011). The creation of disarmed Ti-plasmids possessing the ability to transmit DNA but not induce tumor growth allowed scientists a new tool for plant genetic modification (Zambryski et al. 1983). The gene of interest is generally inserted between the left and right border regions

on a T-DNA (Transfer DNA) vector. This T-DNA may be provided by a second vector transformed into the *Agrobacterium* host, known as the binary vector system, or inserted into the *Agrobacterium* Ti plasmid via a recombination event facilitated by a helper plasmid (Mozo and Hooykaas 1992). When incubated with wounded plant host cells, the host cells produce acetosyringone, which stimulates the transcription of the *vir* (virulence) genes on the disarmed Ti-plasmid. The *vir* genes initiate the transfer of single-stranded T-DNA associated with the border region(s) into the host cell by first nicking the T-DNA at the right border 25 base-pair repeat sequence. The VirD2 protein, which performs this nicking, then covalently binds to the single-stranded T-DNA. Often the left border repeat sequence is also nicked; however, this is not always the case, resulting in conduction of segments of the vector backbone (non-T-DNA) into the host genome (Gelvin 2003). The single-stranded T-DNA is then conducted through a pillus, a proteinaceous conduit linking the bacterium to the host cell, through the plant cell wall into the cytoplasm. At this point, the T-DNA has been coated with proteins that guide it through the host cell cytoplasm and into the nucleus. After localization in the nucleus, the T-DNA is integrated into the host genome through the participation of a mixture of bacterial and host proteins (Tzfira and Citovsky 2006; Lacroix et al. 2010; Păcurar et al. 2011). This undirected integration creates random integration patterns and potentially numerous copy-numbers of the inserted T-DNA (Gelvin 2003; Oltmanns et al. 2010). Since integration of the T-DNA is dependent on host cell machinery, the integration usually takes place at transcriptionally active sites as these sites are open and available to the repair mechanism (Gelvin 2003; Stewart 2008). This can cause issues as it may lead to insertion of the T-DNA into a vital gene, leading to cell morbidity or mortality.

There are three main factors that contribute to the success of *A. tumefaciens*-mediated transformation: introduction of the bacteria, activation of the *vir* genes, and suppression of the target cell defense response (Tzfira and Citovsky 2006; Stewart 2008; Lacroix et al. 2010). The bacteria must be able to access the host plant cells in the sample. In some instances, these cells can be buried under layers of other cells. So, some procedures require precise wounding of the plant tissue prior to incubation and co-cultivation with the bacteria (Potrykus 1991; Trigiano and Gray 1996). It is important to select tissues that are capable of handling this wounding process and also exhibit the ability to form new mature plants in culture. Additionally, the *vir* genes that are stimulated by the presence of acetosyringone must be activated (Lacroix et al. 2010; Barampuram and Zhang 2011). The wounding process discussed above can cause increased release of acetosyringone; however, some plants or tissue types might not produce adequate amounts of acetosyringone (Potrykus 1991; Stewart 2008). In this case, addition of acetosyringone is required. Lastly, due to the wounding process and the incubation with the bacteria, the plant tissue defense response may cause browning of the tissue and cell death due to the up-regulation of oxidizing compounds. The addition of reducing agents to the medium prior to co-cultivation may help increase explants survival by inactivating these oxidizing compounds (Stewart 2008).

Few studies have reported successful *A. tumefaciens*-mediated transformation of castor. A study by Malathi et al. (2006) showed 0.42% efficiency in transforming castor embryo axes with the *cry1Ab* gene (Malathi et al. 2006). In addition, one study by Sujatha and Sailaja (2005) showed 0.08% efficiency in meristem transformation using embryo axes dissected from seed and *A. tumefaciens* harboring the pCambia1304 binary

vector (Sujatha and Sailaja 2005). In 2009, the researchers were also able to insert the *cry1EC* gene into castor using the same procedure, reporting an efficiency of 0.82% (Sujatha et al. 2009). It was this procedure that was primarily utilized for this project. However, the results were not completely reproducible and multiple experiments were necessary to adjust the protocol.

The primary goal of this project was to create a ricin-free castor line via genetic modification with an RNAi element specific to ricin. This was to be accomplished through the completion of three objectives: The creation of the RNAi vector, screening of tissue culture protocols, and optimization of a genetic transformation protocol.

CHAPTER II

VECTOR DESIGN AND CONSTRUCTION

Introduction

The goal of this project relies on the construction of a vector containing an RNAi gene construct capable of silencing ricin production. This is accomplished by constructing a gene that, when transcribed, will fold back upon itself to produce a double-stranded RNA molecule that is complementary to the ricin mRNA sequence. The double-stranded RNA in this case is produced by two reverse complement segments of DNA derived from the ricin mRNA sequence flanking an intron segment provided by the pFGC5941 vector (Genbank accession number AY310901; ABRC stock number CD3-447). This vector contains two cloning sites flanking a chalcone synthase intron (chsA) from *Petunia hybrida* (Figure 2.1a) (Kerschen et al. 2004). After transcription, the flanking segments bind complementarily on either side of the included intron, creating double-stranded RNA. This double-stranded RNA is recognized by Dicer which then cleaves the RNA into 22 base-pair fragments. These fragments are incorporated into the RISC complex which seeks out complementary single-stranded RNA sequences (i.e. ricin mRNA) and cleaves them, destroying the message (Hannon 2002).

In order to identify transformed plants, the vector must also contain at least one marker gene, such as one coding for resistance to a specific selective agent or one which results in some visible signal. For this purpose, pCambia1304 (Figure 2.2a; Genbank

accession number AF234300.1) was chosen for a test vector, and pCambia1301 (Figure 2.2b; Genbank accession number AF234297.1) was chosen as the base vector for insertion of the RNAi gene. Both pCambia vectors were developed by the Center for Application of Molecular Biology to International Agriculture (CAMBIA; http://www.cambia.org/daisy/cambia/585). These vectors both contain the *hptII* and *gusA* genes. The *hptII* gene encodes the hygromycin phosphotransferase enzyme which confers resistance to the antibiotic hygromycin B. The *gusA* gene codes for the enzyme β-glucuronidase which converts the substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) into glucuronic acid and ClBr-indigo. The ClBr-indigo dimerizes in the presence of oxidizers to form dichloro-dibromoindigo, a blue, insoluble precipitate, at the site of the reaction. This gene is, therefore, useful in identifying transformed tissues since only tissues containing the product of the gene will be stained blue in the presence of the X-gluc reagent. In addition, the pCambia1304 vector contains the *gfp* gene fused to the *gusA* gene. The *gfp* gene product is green fluorescent protein, a protein that emits green light when excited by a UV light source. The *gusA* gene within the pCambia1301 vector also contains an intron which prevents the gene from being translated by the bacterial host which is meant to prevent false positives produced by presence of *A. tumefaciens* in putatively transformed samples (Ohta et al. 1990).

Additionally, these genes were controlled by the constitutively expressed cauliflower mosaic virus (CaMV) 35S promoter. Marker and reporter genes need to be constitutively expressed so that they are present in all of the tissues at all times. Otherwise the transformed individual may not be resistant to the selective agent or will not show positive expression of the reporter gene. The RNAi gene, however, could be

controlled by one of many different promoters. In this case, the CaMV 35S promoter was used to ensure expression throughout the plant.

Materials and Methods

Escherichia coli culture and transformation

Competent *E. coli* DH5α cultures were prepared by first streaking freezer-stored cells onto Luria-Bertani (LB) agar plates (Qbiogene; Quebec, Canada) and incubating at 37°C overnight. Single colonies from the plates were inoculated into 50 mL of liquid Super-Comp medium (Qbiogene; Quebec, Canada) in a 125 mL Erlenmeyer flask and incubated at 37°C overnight with constant 250 rpm agitation. Next, 0.5 mL of the overnight culture were inoculated into 50 mL of liquid Super-Comp medium (Qbiogene; Quebec, Canada) in a 125 mL Erlenmeyer flask and incubated at 37°C with 250 rpm agitation until the absorbance at 600nm reached 0.4 as measured in a cuvette of 1 cm pathlength in an 8452A diode array spectrophotometer (Hewlett-Packard; Palo Alto, CA). The cells were transferred to a sterile ice-cold centrifuge tube and incubated on ice for 10 minutes. The cells were pelleted by centrifugation at 1,600 g in a swinging-bucket rotor at 4°C for 7 minutes. The supernatant was removed, and the cells were resuspended in 10 mL of ice-cold $RuCl_2$ transformation salts (Qbiogene; Quebec, Canada). The cells were recentrifuged at 1,100 g at 4°C for 5 minutes, and the supernatant was again discarded. The cells were resuspended once more in 2 mL of of ice-cold $RuCl_2$ transformation salts. The resulting competent cells were stored on ice for use within 48 hours or aliquoted into pre-chilled tubes and stored at -80°C.

Competent *E. coli* DH5α cells to be transformed were removed from freezer storage and rapidly thawed or freshly prepared competent cells were used. In either case,

100 µL of competent cells were added to a test-tube containing 10 ng of plasmid DNA, swirled gently to mix, and incubated on ice for 10 minutes. The cells were heat-shocked by gently swirling the tubes in a water bath at 42°C for 2 minutes. After heat-shocking, 1 mL of liquid LB medium (Qbiogene; Quebec, Canada) was added to the cells and incubated at 37°C for 1 hour on a rotating mixer at 60 rpm. After incubation, several dilutions of the transformed cells were plated on LB agar plates supplemented with 50 µg/mL kanamycin for selection of bacteria containing the plasmid. The plates were incubated at 37°C overnight. The resulting colonies were individually inoculated into 30 mL test tubes containing 10mL liquid LB medium supplemented with 50 µg/mL kanamycin and incubated at 37°C overnight with constant 250 rpm agitation. Cultures were aliquoted into storage tubes containing 20% sterile glycerol (v/v) and stored at -80°C.

Plasmid isolation

E. coli DH5α cultures transformed with an experimental plasmid were grown overnight at 37°C in 50 mL of liquid Circlegrow (Qbiogene; Quebec, Canada) medium supplemented with 50 µg/mL kanamycin with constant 250 rpm agitation in a 125 mL Erlenmeyer flask. The culture was transferred to a 50 mL centrifuge tube and centrifuged at 3,000 g for 10 minutes in a swinging bucket rotor. The supernatant was removed via aspiration, and the pellet resuspended in 4 mL of ice-cold GTE buffer [50 mM glucose, 25 mM Tris, 10 mM EDTA (pH 8.0)]. Next, 400 µL 20 mg/mL RNase A (Invitrogen; Carlsbad, CA) was added, and the sample was incubated at 25°C for 5 minutes. Next, 8 mL of freshly prepared lysis buffer [0.2 N NaOH, 1% sodium dodecyl sulfate (w/v)] was added to each sample and homogenized by flicking the tube several times followed by a 5

minute incubation on ice. Six milliliters of 5 M potassium acetate was added to the sample and mixed by inverting before incubation on ice for 5 minutes. The sample was centrifuged at 15,000 g for 10 minutes in a fixed-angle rotor at 4°C to pellet cell debris. The resulting supernatant was transferred to a fresh centrifuge tube, and 60 µL of 100% isopropanol were added per 100 µL of supernatant recovered. The sample was incubated on ice for 20 minutes prior to centrifugation at 15,000 g for 10 minutes at 4°C to pellet DNA. The supernatant was removed, and the pellet was washed with 4 mL of 70% ethanol (v/v). The sample was centrifuged again at 15,000 g for 3 minutes at 4°C. Ethanol was removed, and the pellet was allowed to dry under vacuum. The resulting DNA pellet was resuspended in 500 µL TE buffer [10 mM Tris, 1 mM EDTA (pH 8)]. The concentration and relative purity of the sample were analyzed via a Nanodrop 2000c (ThermoScientific; Waltham, MA).

Plant DNA isolation

A modified version of the CTAB extraction method described by Keb-Llanes (2002) was used to extract plant genomic DNA (Keb-Llanes et al. 2002). The procedure was performed as follows. Castor leaf tissue was frozen in liquid nitrogen and ground using a mortar and pestle, and a 0.1g sample of the ground material was weighed into a 1.5mL Eppendorf tube. To this tube, 100 µL of extraction buffer A [2% hexadecyltrimethylammonium bromide (CTAB) (w/v), 100 mM Tris-HCl (pH 8), 20 mM EDTA, 1.4 M NaCl, 4% polyvinylpyrrolidone (PVP-40) (w/v), 0.1% ascorbic acid (w/v), and 10 mM β-mercaptoethanol (BME)], 300 µL of extraction buffer B [100 mM Tris-HCl (pH 8), 50 mM EDTA, 100 mM NaCl, and 10 mM BME], and 33 µL of 20% sodium dodecyl sulfate (SDS) (w/v) were added. The sample was vortexed until

homogenized and incubated in a 65ºC water bath for 10 minutes. The sample was placed on ice, and 137 µL of cold 5 M potassium acetate was added, and the tube was inverted several times to mix. The tube was incubated on ice for 3 minutes. The sample was centrifuged at 16,000 g for 15 minutes to pellet cell debris, and the supernatant was transferred to a fresh tube taking care to measure the volume of the supernatant. For every 100 µL of supernatant, 60 µL of ice-cold 100% isopropanol was added to the tube. The sample was incubated on ice for 20 minutes. After incubation, the tube was centrifuged at 10,000 g for 10 minutes to pellet DNA, and the supernatant was discarded. The remaining pellet was washed with 500 µL 70% ethanol (v/v) and allowed to dry. The pellet was resuspended in 200 µL TE buffer [10 mM Tris, 1 mM EDTA (pH 8)], and 20 µL of 3 M sodium acetate (pH 5.2) was added. The sample was re-precipitated twice more by the addition of 120 µL ice-cold 100% isopropanol, 20 minute incubation on ice, centrifugation at 10,000 g for 10 minutes, and washing with 70% ethanol (v/v). After the final precipitation, the pellet was dried and resuspended in 50 µL TE buffer. DNA concentration was measured on a NanoDrop 2000c (ThermoScientific; Waltham, MA).

Restriction digestion

All restriction digestions were performed in 20 to 50 µL reaction volumes at the manufacturer recommended temperature (Table 2.1). In most cases, single enzyme digestions were performed; however, when buffer compositions allowed, double digestions were also utilized. In all cases, the ratio of DNA to enzyme was 1 µg DNA/U of enzyme. The temperature was maintained by a T3 thermocycler (Biometra; Göttingen, Germany). Digestions were performed for 1 hour with a 65°C heat inactivation step immediately following. Vectors that were digested for ligation were also treated with 2 U

CIAP (calf intestinal alkaline phosphatase; Invitrogen; Carlsbad, CA) during the final restriction digestion.

Table 2.1 Restriction enzyme protocols

		Digestion		Inactivation	
Enzyme	**Manufacturer**	**Temp (ºC)**	**Time (min)**	**Temp (ºC)**	**Time (min)**
AscI	New England Biolabs	37	60	65	20
EcoRI	Invitrogen	37	60	65	20
PstI	Invitrogen	37	60	65	20
PvuII-FD	Fermentas	37	15	No Inactivation	
SmaI	Invitrogen	30	60	65	20
SwaI	New England Biolabs	25	60	65	20
XbaI	Invitrogen	37	60	No Inactivation	

FD – FastDigest enzyme; PvuII-FD and XbaI could not be heat inactivated

Ligation

Ligations were performed with 60 units of T4 ligase (Fermentas; Burlington, Canada) per 10 ng of vector DNA with temperatures controlled by a T3 thermocycler (Biometra; Göttingen, Germany). The insert:vector ratio was maintained at 5:1 for each reaction. The ligation of sticky-end vector and fragments was performed at 22°C for 2 hours, while blunt-ended vectors and fragments were ligated at 16°C for 8 hours. After ligation, the samples were incubated at 65°C for 20 minutes to inactivate the ligase prior to transformation.

RNAi element design

The ricin knockout segment was designed based on preproricin mRNA and castor genomic DNA sequences from the literature (Lamb et al. 1985; Chan et al. 2010). A 473 bp segment from 583 bp to 1055 bp of the preproricin mRNA (Genbank accession number X02388) was identified as a candidate for knockout because it could be isolated

from genomic DNA via polymerase chain reaction (PCR) rather than requiring cDNA synthesis from castor mRNA (Lamb et al. 1985). This sequence also aligned with the sequences of other putative copies of the ricin gene found within the castor genome (Figure 2.3) (Leshin et al. 2010). Some mismatches between the target mRNA and the RNAi element can be tolerated by the plant RNAi mechanism depending on where the mismatches occur in the 22 bp fragment generated by Dicer. As many as four mismatches has been shown to still generate siRNA capable of knocking out the target transcript (Tang et al. 2003). The following primers (5`-CTTCCAACTCTGGCTCGTTC-3` and 5`-CATGGCCACAACTGTATTGC-3`) were used to amplify the fragment twice with restriction endonuclease sites engineered on the 5`/3` ends corresponding to the insertion sites surrounding the intron region of the pFGC5941 plasmid (sense: AscI/SwaI; antisense: SmaI/XbaI). Pyrophage 3173 DNA WT (Lucigen; Middleton, WI) polymerase was used for amplification. The amplification protocol used 100 ng of castor DNA as template, 0.2 mM dNTPs, 2.5 U Pyrophage enzyme, and 3 µM of each primer. Thermocycler parameters were: 95°C for 2 minutes followed by 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute cycled 40 times followed by a 5-minute final elongation at 72°C. Pyrophage 3173 DNA polymerase WT was selected for its high-fidelity characteristics compared to standard Taq polymerases. The sample was treated with DNA polymerase I Klenow fragment (New England Biolabs; Ipswich, MA) according to the manufacturer's recommended protocol to create blunt ends.

After Klenow treatment, the fragment (RKO; Figure 2.3) was subjected to agarose gel electrophoresis on 1.2% low melting agarose (w/v) in 1x TAE buffer and stained with 0.5 µg/mL ethidium bromide. The resulting single band was cut from the gel with a clean

scalpel blade, and the product was isolated from the gel piece using the QUIquick gel extraction kit (Qiagen; Venlo, Netherlands) according to the manufacturer's instructions. After isolation, the DNA concentration of the sample was determined using a Nanodrop 2000c (ThermoScientific; Waltham, MA). The sample was ligated into a pUC19 vector which had been cleaved with SmaI. The pUC19+RKO plasmid was transformed into chemically competent *E. coli* DH5α. Vectors containing the RKO PCR product were identified via blue-white selection on LB medium containing 20 mg/L X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 0.1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside).

The vector was digested with AscI and SwaI according to the previously described protocol to extract the RKO fragment (Figure 2.3) and prepare it for insertion into the pFGC5941 vector (Figure 2.1a). The digestion mixture was electrophoresed on 1.2% low melting agarose (w/v) in 1x TAE buffer and stained with 0.5 µg/mL ethidium bromide. The band representing the fragment (483 bp) was removed and re-extracted as previously described. The pFGC5941 vector was likewise digested with AscI and SwaI to prepare it to accept the fragment. Calf intestinal alkaline phosphatase (2 U) was added to the vector digestion to prevent re-ligation of the vector without insertion of the fragment. The vector and fragment were ligated together via the previously described protocol. The ligation product was directly transformed into chemically competent *E. coli* DH5α. Transformed *E. coli* was then inoculated in 10 mL of liquid LB medium containing 50 µg/mL kanamycin, and incubated at 37ºC overnight with agitation at 150 rpm on an orbital shaker. Polymerase chain reaction using the primers designed for the RKO fragment was performed on the isolated plasmid from these colonies. Colonies that tested

positive for the insert were placed in 50 mL of liquid LB medium containing 50 µg/mL kanamycin and incubated at 37ºC overnight with agitation at 150 rpm on an orbital shaker. A portion was stored at -80ºC in 20% sterile glycerol (v/v), and the rest was used for plasmid isolation as described above.

Plasmid isolated from colonies that tested positive for RKO insertion were subjected to restriction digest by PvuII-FD to confirm the expected banding pattern produced. The procedure was repeated to insert the antisense fragment into the plasmid now containing the sense fragment using SmaI and XbaI restriction endonuclease sites. The end result was the pFGC5941 vector containing sense and antisense RKO fragments flanking the intron (Figure 2.1b) dubbed pFGC-RKO.

In order to isolate the RNAi element for insertion into pCambia1301, isolated pFGC-RKO was digested with PstI and EcoRI according to the procedure previously described. Similarly, isolated and digested pCambia1301 was prepared with the addition of 2 units CIAP to prevent re-ligation of the vector. The RKO element was ligated into the cleaved pCambia1301 vector via the procedure previously described. The ligation product was directly transformed into *E. coli* DH5α as previously described. Colonies were chosen and innoculated in 10 mL of liquid LB medium containing 50 µg/mL kanamycin and incubated at 37ºC overnight with agitation at 150 rpm on an orbital shaker. Plasmids were isolated from each of the desired colonies and analyzed via PCR to verify the presence of the insert. Colonies that tested positive for the insert were placed in 50mL of liquid LB medium antibiotic and incubated at 37ºC overnight with agitation at 150rpm on an orbital shaker. An aliquot was stored at -80ºC in 20% sterile glycerol (v/v), and the balance was used for plasmid isolation as described above. Isolated plasmids

were subjected to digestion with PvuII in order to verify the expected cleavage pattern. The end result was the pCambia1301 vector containing the RKO element from pFGC-RKO (Figure 2.4) dubbed pC1-RKO. The isolated vector was confirmed via sequencing by the School of Life Sciences DNA Laboratory at Arizona State University with the following primers (sense – 5`-GAGAGGACACGCTCGAGTATAA-3`; antisense – 5`-ACTTACACTTGCCTTGGAGTT-3`) in independent reactions. Figure 2.5 describes the process via flow diagram.

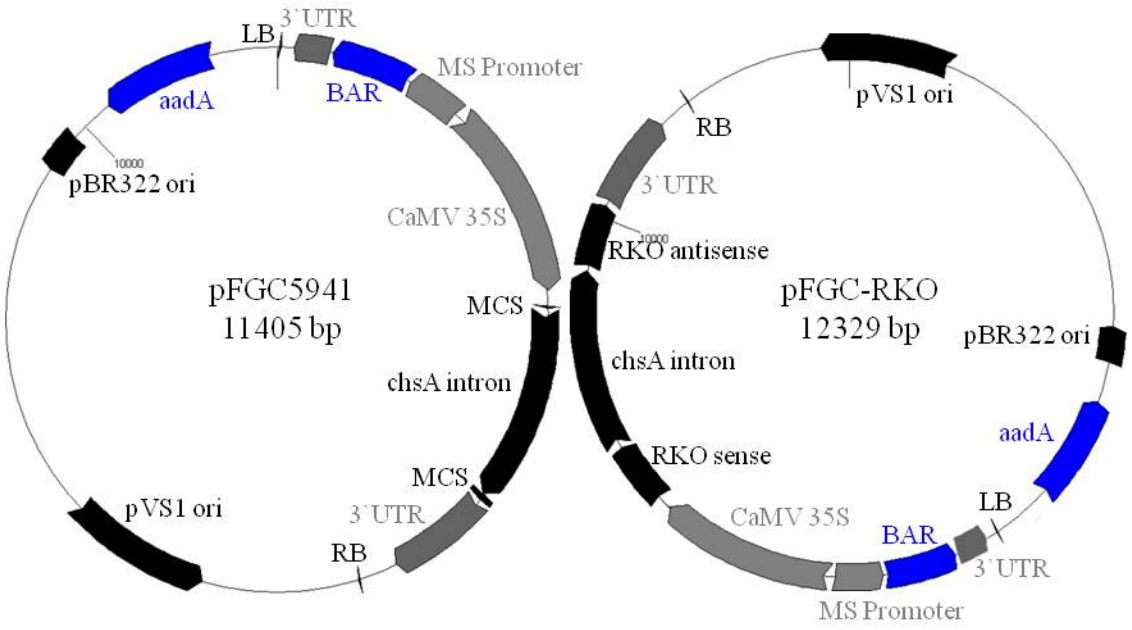

Figure 2.1 Maps of the pFGC5941 and pFGC-RKO vectors

A – pFGC5941 vector used for RNAi element construction; B – pFGC-RKO vectors as the intermediate for RNAi vector assembly; LB –T-DNA left border repeat sequence, RB – T-DNA right border repeat sequence, BAR – Basta herbicide resistance gene, MS Promoter – mannopine synthase promoter, CaMV 35S – 35S RNA promoter from cauliflower mosaic virus, MCS – synthetic multiple cloning site, chsA intron – intron from chalcone synthase, pVS1 ori – broad host range origin of replication from pVS1, pBR322 ori – high copy number origin of replication from ColE1, aadA - aminoglycoside phosphotransferase gene conferring kanamycin resistance, 3` UTR – 3` untranslated region of mannopine synthase (BAR) or octopine synthase (RNAi element)

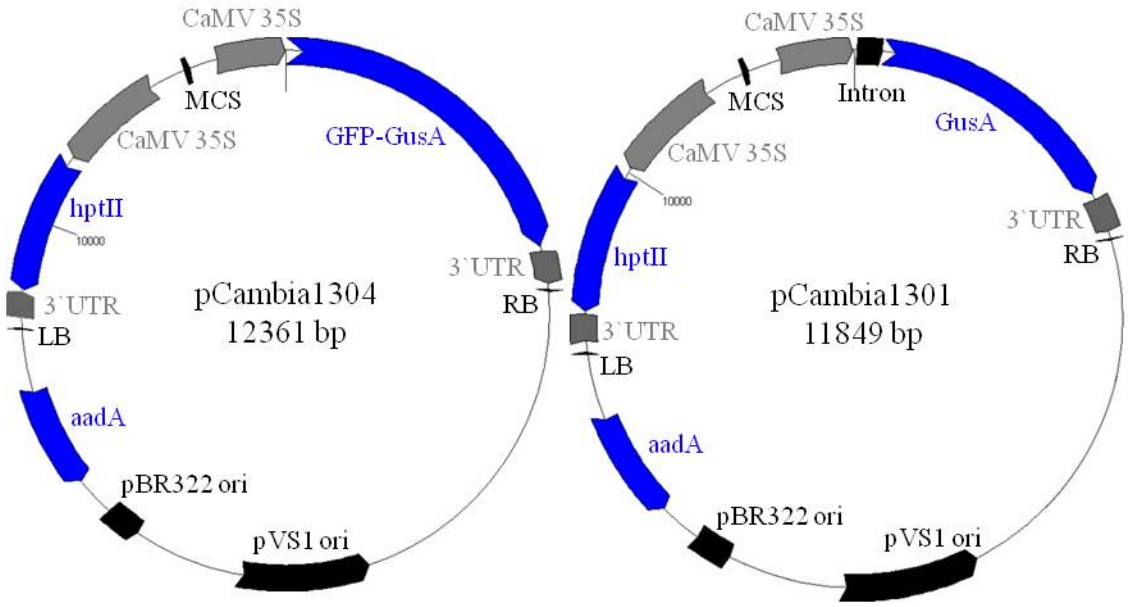

Figure 2.2 Maps of the pCambia1304 and pCambia 1301 vectors

A – pCambia1304 used as test vector; B – pCambia1301 used to construct RNAi vector; LB –T-DNA left border repeat sequence, RB – T-DNA right border repeat sequence, CaMV 35S – 35S RNA promoter from cauliflower mosaic virus, hptII – hygromycin phosophtransferase gene conferring hygromycin resistance, GusA - beta-D-glucuronidase marker gene, GFP-GusA – green fluorescent protein and GusA fusion gene, Intron – catalase intron from castor, MCS – synthetic multiple cloning site, pVS1 ori – broad host range origin of replication from pVS1, pBR322 ori – high copy number origin of replication from ColE1, aadA - aminoglycoside phosphotransferase gene conferring kanamycin resistance, 3` UTR – 3` untranslated region of CaMV 35S

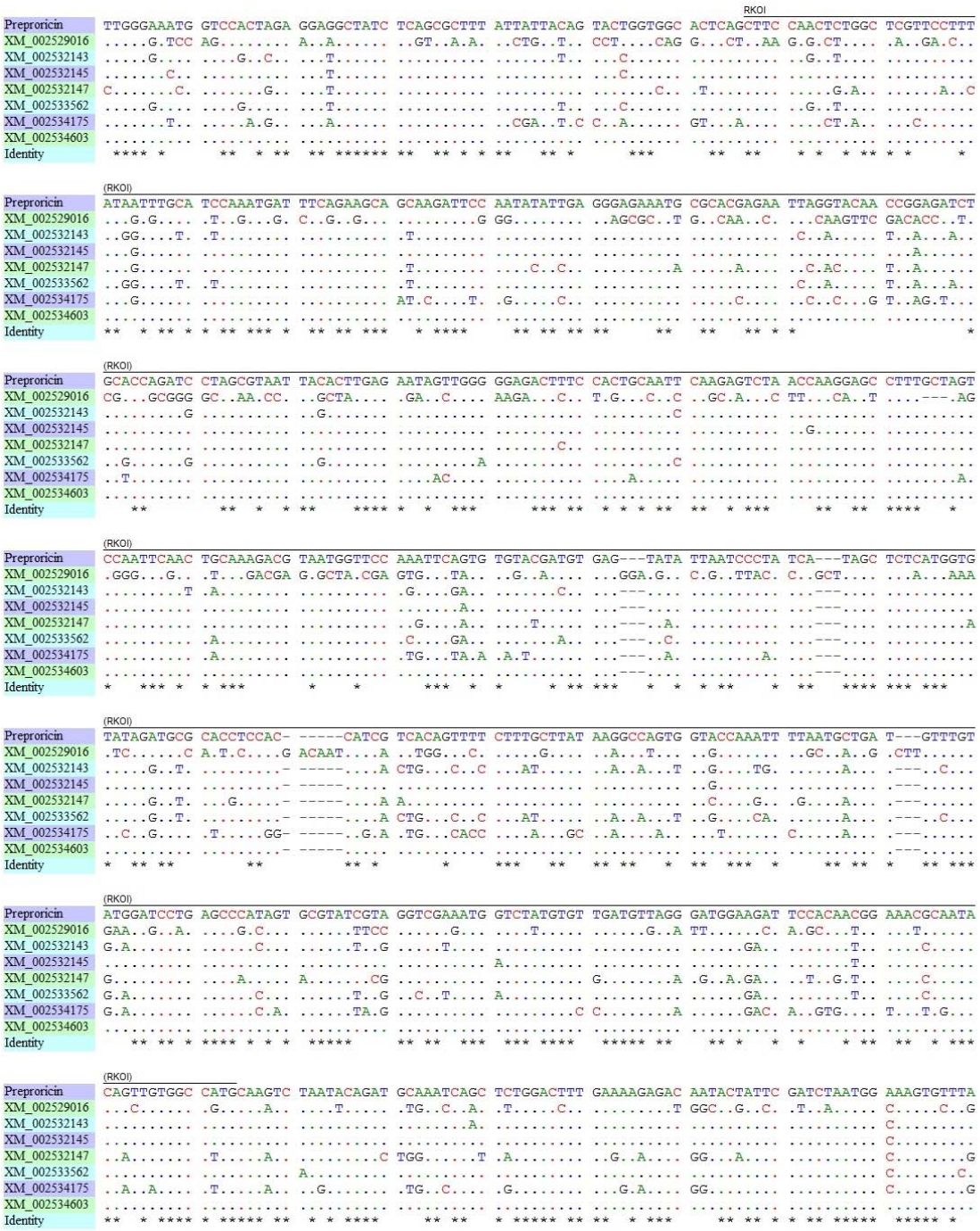

Figure 2.3 Multiple sequence alignment of ricin genes (truncated) showing the portion used for the design of the RKO sequence (480 bp)

Asterisks (*) in the 'Identity' row indicate matches between all sequences. This alignment shows a large degree of consensus within the selected area, indicating that this region would produce an RNAi element suitable for the confirmed copies of the ricin gene.

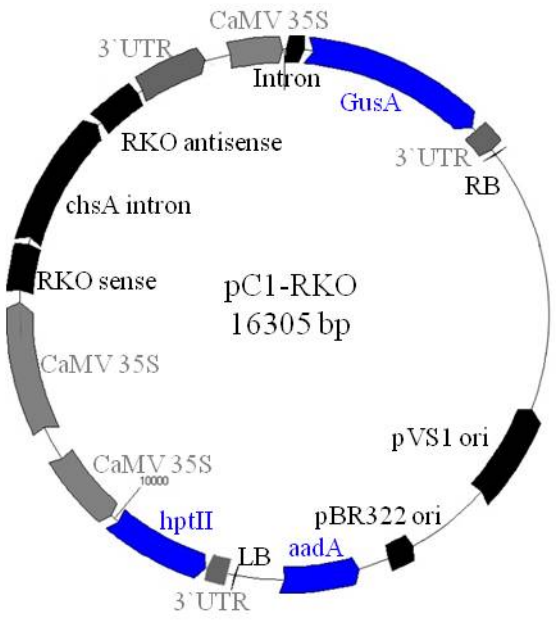

Figure 2.4 Map of the pC1-RKO vector

The final pC1-RKO vector carrying the RNAi element composed of the RKO sense and antisense sequences flanking the *chsA* intron, preceded by the CaMV 35S promoter, and followed by the 3`UTR from pFGC5941; LB –T-DNA left border repeat sequence, RB – T-DNA right border repeat sequence, CaMV 35S – 35S RNA promoter from cauliflower mosaic virus, hptII – hygromycin phosophtransferase gene conferring hygromycin resistance, GusA - beta-D-glucuronidase marker gene, Intron – catalase intron from castor, chsA intron – intron from chalcone synthase, pVS1 ori – broad host range origin of replication from pVS1, pBR322 ori – high copy number origin of replication from ColE1, aadA - aminoglycoside phosphotransferase gene conferring kanamycin resistance, 3` UTR – 3` untranslated region of mannopine synthase (RNAi element), CaMV35S (*hptII*), or nopaline synthase (GusA)

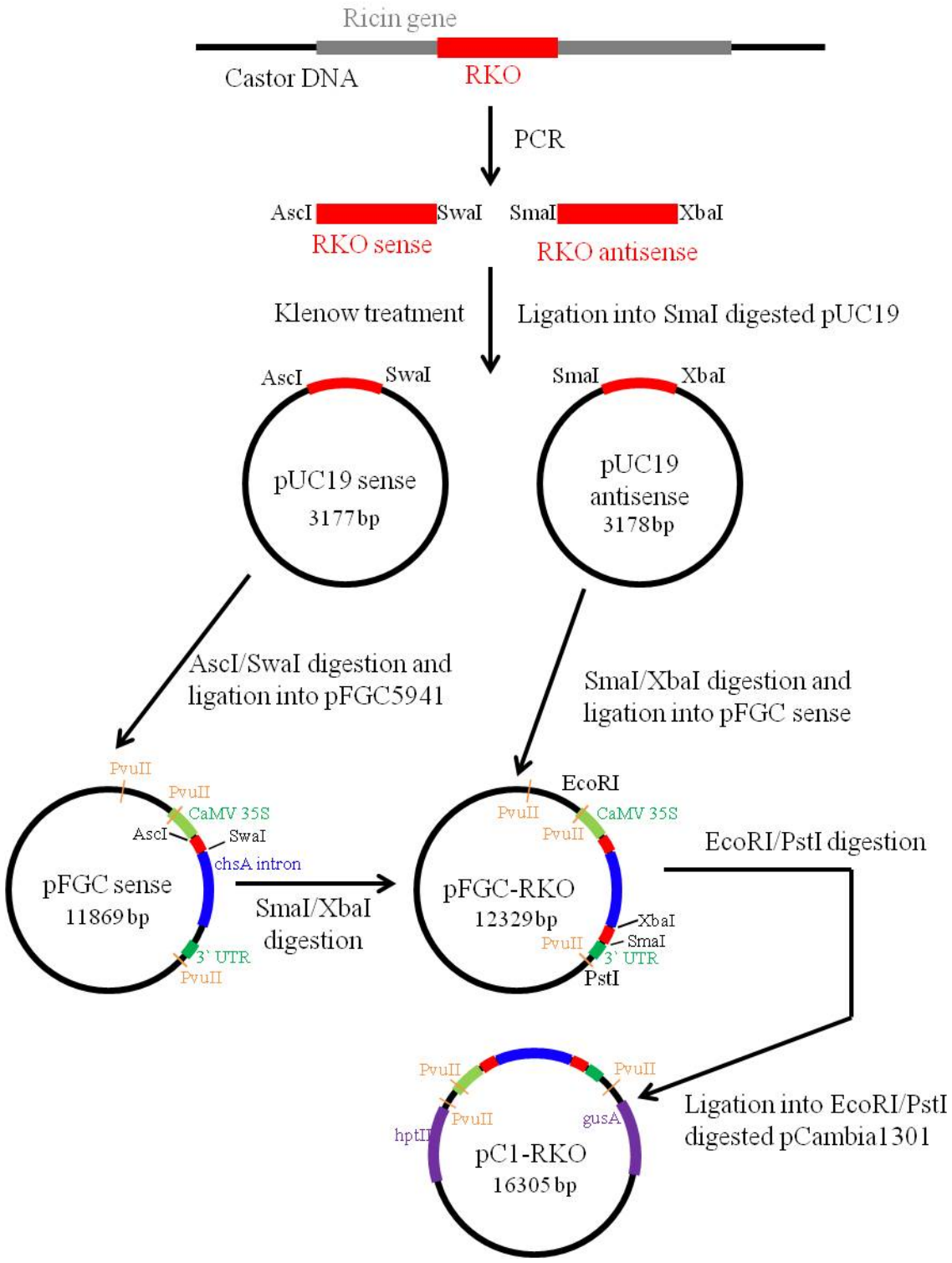

Figure 2.5 Flow diagram of pC1-RKO construction process

This is a pictorial representation of the cloning strategy used to create the pC1-RKO plasmid for this research.

Agrobacterium tumefaciens culture and transformation

A -80ºC freezer stock of *Agrobacterium tumefaciencs* EHA105, provided by Dr. Zhaohua Peng's lab (Mississippi State University; Starkville, MS), was streaked onto LB agar plates supplemented with 50 µg/mL rifampicin and cultured overnight at 28°C. Single colonies were inoculated into 50 mL liquid LB medium supplemented with 50 µg/mL rifampicin in 125 mL Erlenmeyer flasks and cultured overnight at 28°C with constant 150 rpm agitation. The cells were isolated by centrifugation at 3,000 g for 15 minutes. The pellet was resuspended in 50 mL 0.2 µm filter-sterilized 10% glycerol (v/v). Centrifugation followed by resuspension in 10% glycerol was repeated a total of 4 times, and the final pellet was resuspended in 1 mL sterile 10% glycerol (v/v). These cells were aliquoted into Eppendorf tubes for storage at -80°C or used immediately. Cells were transformed by electroporation as follows. In a 4 mm gap sterile electroporation cuvette (Fisher Biotech; Pittsburgh, PA), 48 µL of the prepared cells were added to 2 µL (approximately 50 ng) of plasmid DNA. The cuvette was placed in an ECM 630 electroporator (BTX; Holliston, MA) and pulsed with the following settings: 1.44 kV voltage, 50 µF capacitance, and 125 Ω resistance. After pulsing, 1 mL of liquid Circlegrow medium was added to the cells and transferred to a sterile 1.5 mL Eppendorf tube. The cells were incubated at 28°C for 2 hours with constant agitation and finally plated on LB agar plates supplemented with 50 µg/mL rifampicin and 50 µg/mL kanamycin. After overnight growth at 28°C, single colonies were chosen and inoculated into 10 mL liquid LB medium supplemented with 50 µg/mL rifampicin and 50 µg/mL kanamycin in 30 mL test tubes. These tubes were cultured overnight at 28°C with

constant 150 rpm agitation. The cells were aliquoted for -80° C freezer storage after the addition of 20% glycerol (v/v).

Vector verification

All verification PCR reactions were performed using 1 unit of EconoTaq (Lucigen; Middleton, WI) with 0.2 mM dNTP and 0.5 µmol of each primer in 0.2 mL PCR tubes using a T3 thermocycler (Biometra; Göttingen, Germany). The protocol for amplification was as follows: initial 95°C for 2 minutes followed by 95°C for 30 seconds, annealing temperature according to the primers being used for 30 seconds, and 72°C for 1 minute cycled 40 times followed by a 5-minute final elongation at 72°C. Primer sequences, annealing temperatures, and amplicon sizes are described in Table 2.2. Primers used for EHA105 verification (16S rDNA, pAT attS, pTi VirJ, pTi MoaA) were described in the literature (Broothaerts et al. 2005). Primers for *hptII* and *gfp* were designed using a combination of the online tool Primer3 and the computational genomics tool GENtle and the sequences for pCambia1301 and pCambia1304 (Manske 2006; Koressaar and Remm 2007; Untergasser et al. 2012). All primers were ordered from IDT-DNA (Coralville, IA), reconstituted in sterile distilled water, and diluted to create a 5 mM stock. The amplification products were electrophoresed on 1.2% 1x TAE agarose (w/v) and stained with 0.5 µg/mL ethidium bromide.

Table 2.2 PCR primer sequences, annealing temperatures, and amplicon sizes

Description	Sequence	Annealing Temp (ºC)	Amplicon size (bp)
16S rDNA forward	GAATAGCTCTGGGAAACTGGAAT	52	320
16S rDNA reverse	CGGGGCTTCTTCTCCGACT		
pAT attS forward	GTGCTTCGGATCGACGAAAC	54	642
pAT attS reverse	GGAGAATGGGAGTGACCTGA		
pTi VirJ forward	TCCTGTCATTGGCGTCAGTT	54	948
pTi VirJ reverse	TGACCTTGGCCAGGGAATTG		
pTi MoaA forward	CTCCCAAGAGGGTCGTTGAC	54	462
pTi MoaA reverse	ATGGATCCTGCCGTGGTCTCGTGTTCTGG		
hptII forward	AATTAATTCGGGGGATCTGG	52	535
hptII reverse	CTTGTATGGAGCAGCAGACG		
gfp forward	GTCAGTGGAGAGGGTGAAGG	50	558
gfp reverse	CTTTTCGTTGGGATCTTTCG		
RKO sense forward	**GGCGCGCC**CTTCCAACTCTGGCTCGTTC	56	491
RKO sense reverse	**CGATTTAAAT**CATGGCCACAACTGTATTGC		
RKO anti forward	**TCCCCCGGG**CTTCCAACTCTGGCTCGTTC	56	492
RKO anti reverse	**CTAGTCTAGA**CATGGCCACAACTGTATTGC		

16S rDNA, attS, VirJ, and MoaA primers were described by Broothaerts et al. (2005); Bolded sequence indicates engineered restriction enzyme cut-sites

Results and Discussion

The RNAi vector pC1-RKO was successfully generated using the process described. Sense and antisense fragments complementary to a 480 bp segment of ricin mRNA were inserted into the pFGC5941 vector at either side of the chalcone synthase intron, the construct was confirmed and named pFGC-RKO (Figures 2.4 and 2.6a). Digestion with PvuII-FD was used to verify the proper ligation of constructs throughout

the vector generation process (Figure 2.6). PvuII-FD digestion of pFGC5941, pFGC5941-sense, and pFGC-RKO indicated proper insertion of the sense and antisense RKO elements into the pFGC5941 backbone (Figure 2.6a). The banding patterns for pFGC5941 (6839 bp, 3463 bp, and 1103 bp), pFGC5941 with the sense RKO element (6839 bp, 3927 bp, and 1103 bp), and pFGC-RKO (6839 bp, 4387 bp, and 1103 bp) were generated as expected. Likewise, PvuII-FD digestion of pCambia1301, pFGC5941-RKO, and pC1-RKO confirmed the transfer of the RKO element from pFGC-RKO into the pCambia1301 backbone (Figure 2.6b). The banding patterns for pCambia1301 (11527 bp and 322 bp), pFGC-RKO (6839 bp, 4387 bp, and 1103 bp), and pC1-RKO (11527 bp, 4387 bp, and 391 bp) were generated as expected. The RKO sense and antisense elements of the pC1-RKO vector were sequenced by the School of Life Sciences DNA Laboratory at Arizona State University (https://sols.asu.edu/resources/core-laboratories) to verify the sequence and orientation of the elements (Figures 2.7 and 2.8). The asterisks (*) in the 'Identity' row of each alignment indicate matches between the sequence data and the expected data, indicating that the vector contains the designed sequences. Polymerase chain reaction was performed to verify the presence of the pC1-RKO plasmid via positive results for the RKO and *hptII* primer sets as well as the identity of the host via positive results for the EHA105-specific 16S rDNA, *attS*, *VirJ*, and *MoaA* primer sets (Figure 2.9). The presence of bands at 320 bp, 642 bp, 948 bp, 462 bp, 491 bp, and 535 bp for the 16S rDNA, *attS*, *VirJ*, *MoaA*, RKO, and *hptII* primers confirms that the pC1-RKO RNAi vector has been prepared as designed and is ready for transformation into castor.

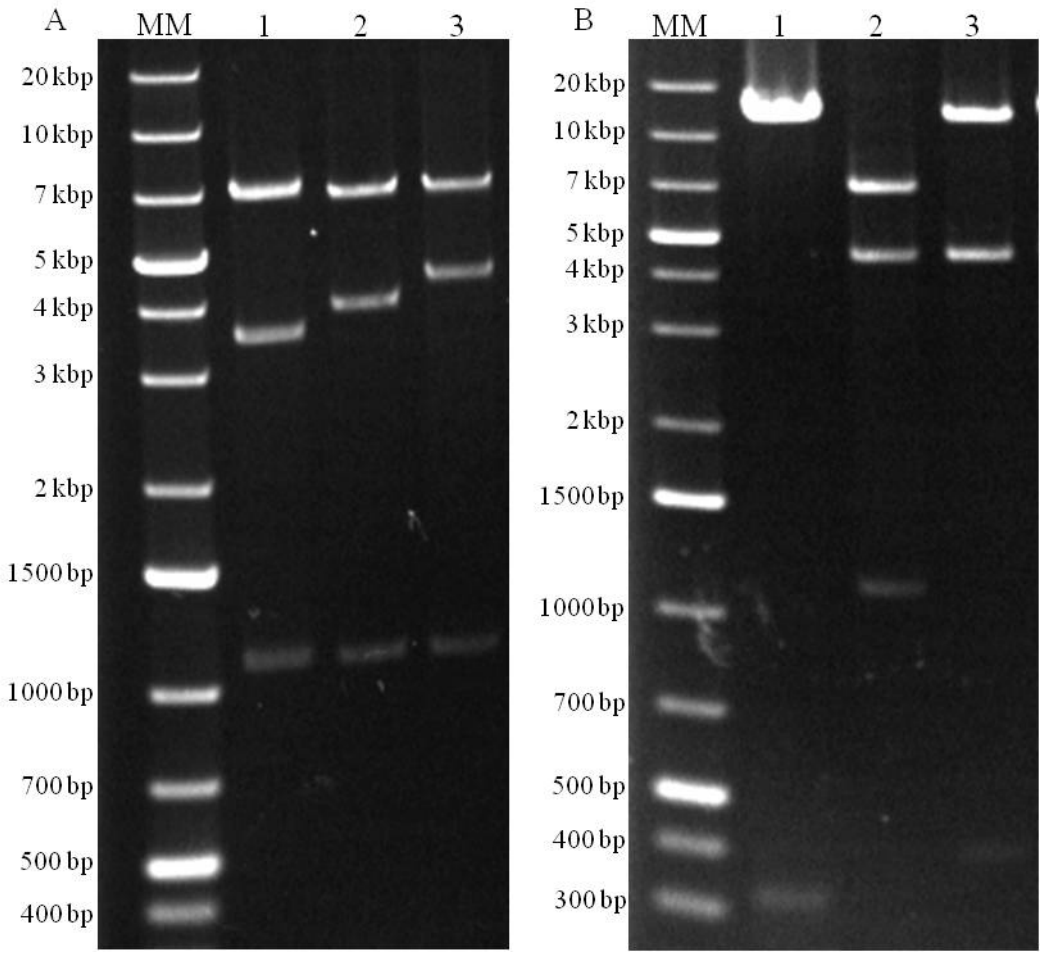

Figure 2.6 PvuII-FD restriction digestion profile of pFGC-RKO and pC1-RKO

MM – GeneRuler 1kb plus molecular marker (Fermentas; Burlington, Canada); **A** – Lane 1 contains PvuII-FD digested pFGC5941. Lane 2 is PvuII-FD digested pFGC5941 containing the sense RNAi element indicating the insertion of the 480 bp RKO sense element. Lane 3 is PvuII-FD digested pFGC-RKO indicating the insertion of the 480 bp RKO antisense element. The digestion patterns are as predicted using the GENtle software *in silica* digestion as follows: 6839 bp, 3463 bp, and 1103 bp in lane 1, 6839 bp, 3927 bp, and 1103 bp in lane 2, and 6839 bp, 4387 bp, and 1103 bp in lane 3; **B** – Lane 1 contains PvuII-FD digested pCambia1301. Lane 2 is PvuII-FD digested pFGC-RKO. Lane 3 is PvuII-FD digested pC1-RKO. The digestion patterns are as predicted using the GENtle software *in silica* digestion as follows: 11527 and 322 bp in lane 1, 6839 bp, 4387 bp, and 1103 bp in lane 2, and 11527 bp, 4387 bp, and 391 bp in lane 3; All lanes contain 1 µg of total DNA.

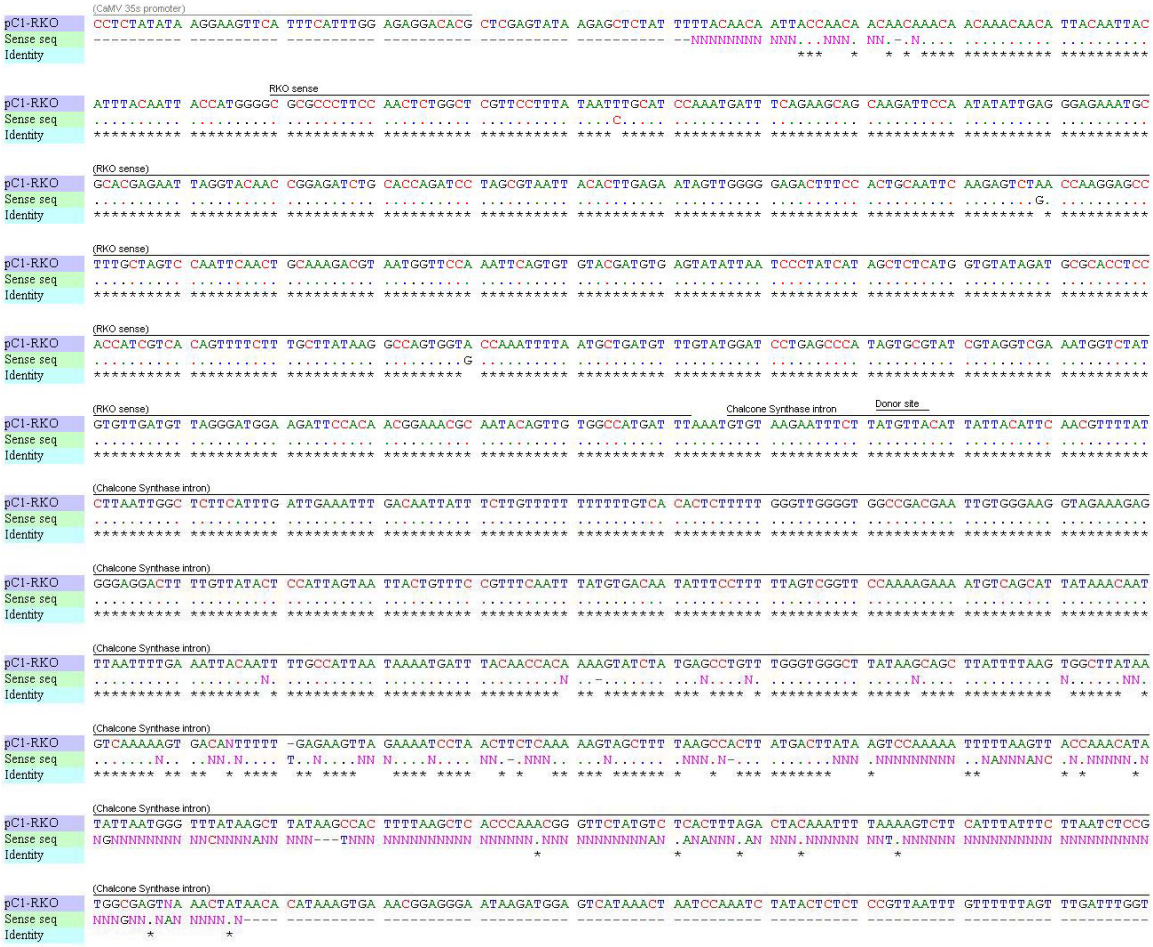

Figure 2.7 Alignment of RKO sense sequencing data with pC1-RKO

This alignment shows a 980 bp region of the pC1-RKO as sequenced matching the RKO sense element as well as 3' and 5' flanking regions to show orientation. Asterisks (*) in the 'Identity' row indicate homology between the sequenced vector and the expected pC1-RKO sequence. 'N' in the 'Identity' row indicates sequencing error.

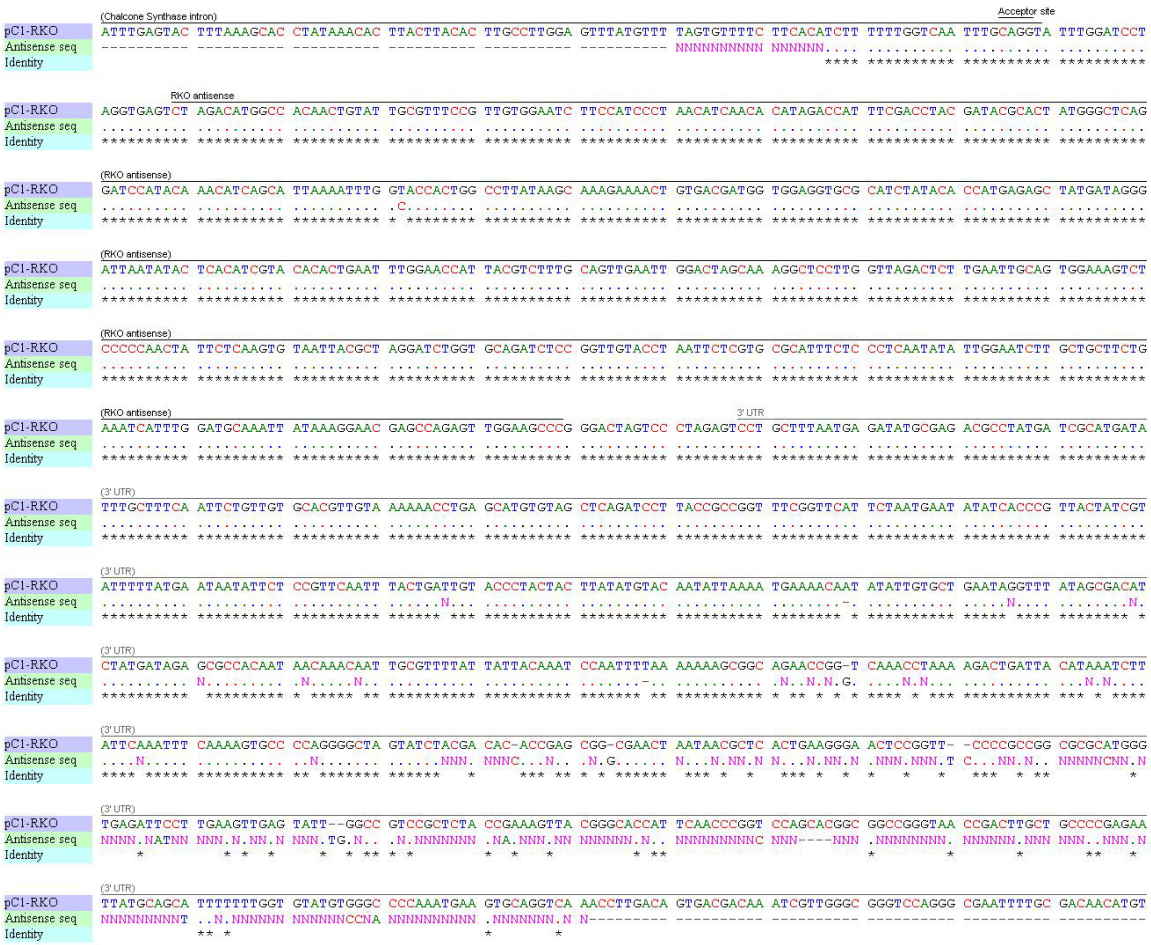

Figure 2.8 Alignment of RKO antisense sequencing data with pC1-RKO

This alignment shows a 949 bp region of the pC1-RKO as sequenced matching the RKO antisense element as well as 3' and 5' flanking regions to show orientation. Asterisks (*) in the 'Identity' row indicate homology between the sequenced vector and the expected pC1-RKO sequence. 'N' in the 'Identity' row indicates sequencing error.

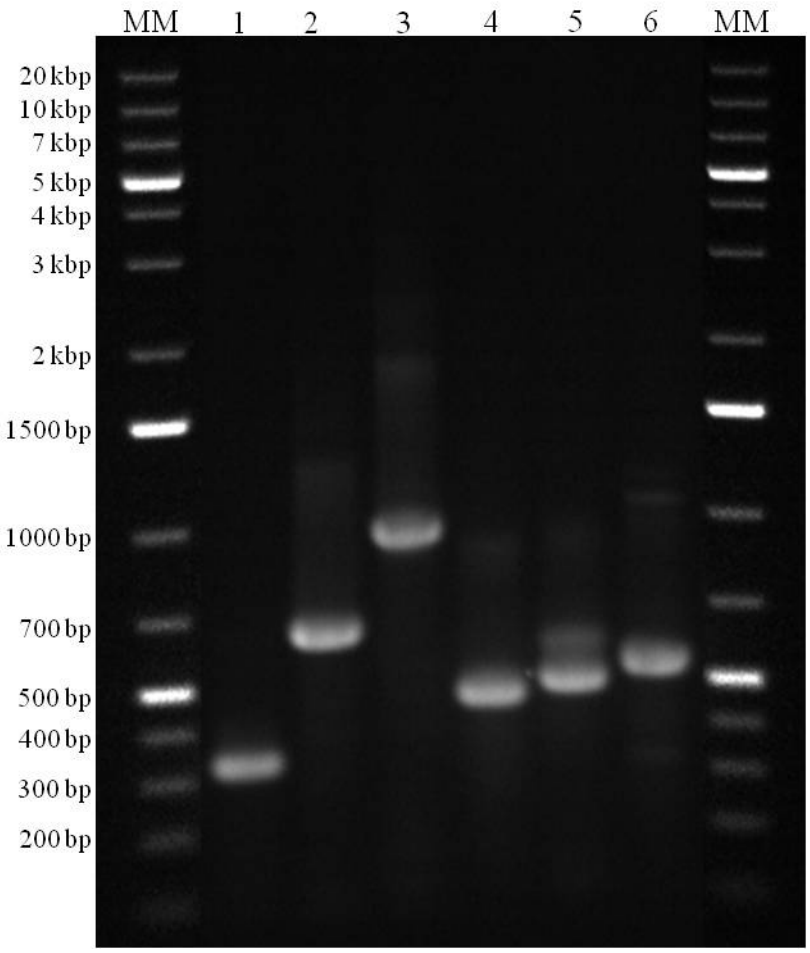

Figure 2.9 PCR verification of pC1-RKO and *A. tumefaciens* EHA105 host

MM – GeneRuler 1kb plus molecular marker (Fermentas; Burlington, Canada); Lanes 1-4 contain the amplification products of the EHA105-specific 16S rDNA, *attS*, *VirJ*, and *MoaA* primer sets described by Broothaerts et al. (2005). The bands appear at the expected amplicon sizes of 320 bp, 642 bp, 948 bp, and 462 bp respectively. These primers were used to verify the identity of the vector host; Lane 5 contains the amplification product of the RKO primer set designed for this experiment producing a 491 bp fragment. Lane 6 contains primers designed for the *hptII* gene of pCambia1301 producing a 535 bp fragment. The combination of these two primers is intended to verify the presence of the RKO element and the hygromycin resistance gene in the plasmid.

Summary

The procedure designed to insert the RNAi element within the pCambia1301 proved to be successful. The RNAi element was designed using multiple sequence alignment and amplified using specifically designed PCR primers. The element was inserted onto either side of an intron within the pFGC5941 oriented so as to create a hairpin structure when transcribed into RNA. It has been demonstrated that the RNAi element was inserted from pFGC5941, including the CaMV 35S promoter and 3` UTR, into the multiple cloning site of pCambia1301 via PvuII-FD digestion and sequencing. Polymerase chain reaction studies on *Agrobacterium tumefaciens* EHA105 colonies indicated that the complete pC1-RKO vector was present; allowing for attempts at *Agrobacterium*-mediated transformation of castor explants with this newly generated RNAi vector.

CHAPTER III

TISSUE CULTURE

Introduction

The establishment of a stable ricin knockout requires the development of a reproducible tissue culture protocol. Castor is known to be recalcitrant to re-differentiation from callus in tissue culture (Sujatha et al. 2008). Therefore, the most successful tissue culture strategies for castor have been direct organogenesis from tissue explants (La Rue 1944; Mohan-Ram and Satsangi 1963; Satsangi and Mohan Ram 1965; Brown et al. 1970; Johri and Srivastava 1972; Bahadur et al. 1991; Sarvesh et al. 1992; Rahman and Bari 2012). However, few micropropagation studies have shown promising and reproducible methods for generating castor shoots in tissue culture (Athma and Reddy 1983; Reddy et al. 1987; Sangduen et al. 1987; Reddy and Bhadur 1989; Molina and Schobert 1995; Sujatha and Reddy 1998; Ahn et al. 2007; Ahn and Chen 2008). Sujatha and Reddy (1998) tested embryo axes on media composed of Murashige and Skoog (MS) basal salts and vitamins, 30 g/L sucrose, and 7 g/L agar supplemented with a range of cytokinins including thidiazuron (TDZ), 6-benzylaminopurine (BA), kinetin, adenine, and zeatin, revealing that 45.4 μM TDZ produced the most shoots per plant (40.0) from embryo axis explants while 8.9 μM BA produced the most shoots per plant (46.7) when used with shoot apex (Murashige and Skoog 1962; Sujatha and Reddy 1998). This work also showed the formation of 6.3 shoots per explant on embryo axes

dissected from mature castor seed and cultured on 2.2 μM BA-supplemented medium. The comparison of TDZ versus BA was taken further by Ahn et al. (2007) who showed that mesocotyl explants, composed of the embryo axes with the radical tip removed which the author refers to as hypocotyl explants, dissected from mature castor seed on media composed of MS basal salts, MS vitamins, 30 g/L sucrose, and 0.5 g/L MES at pH 5.7 supplemented with 1 μM TDZ produced three times as many shoots per plant (24.2) compared to the same explant on medium supplemented on 20 μM BA (6.8). Their study also showed that pretreatment of the explants in dark conditions for the first 7 days of culture enhanced the number of shoots generated by as much as 82% (Ahn et al. 2007). Ahn and Chen (2008) later showed that cotyledon explants dissected from mature seed and placed distal end down on media containing MS basal salts, MS vitamins, 30 g/L sucrose, and 0.5 g/L MES at pH 5.7 supplemented with 5 μM TDZ developed adventitious shoots at a rate of 25 shoots per explant when cultured in dark conditions for the first 7 days (Ahn and Chen 2008). Furthermore, Sujatha and Sailaja (2005) reported a protocol utilizing embryo axes dissected from mature seed placed on a medium composed of MS basal salts, MS vitamins, 30 g/L sucrose, and 8 g/L agar at pH 5.8 supplemented with 0.44 μM BA for 2 weeks followed by 7 days on medium supplemented with 2 μM TDZ and elongation on medium supplemented with 2.2 μM BA produced several shoots.

In addition to shoot formation, rooting and acclimatization protocols were also studied. Sujatha and Reddy (1998) transferred newly generated shoots onto half-strength MS media composed of 1/2x MS basal salts, 1/2x MS vitamins, and 30 g/L sucrose at pH 5.8 supplemented with either 5.4 μM naphthaleneacetic acid (NAA) or 4.9 μM indole-3-

butyric acid (IBA). Rooting occurred after 10 days of culture at rates of 53.2% and 66.7% for NAA- and IBA-supplemented media respectively. The rooted shoots were then acclimatized in sterile vermiculite after a 10 minute soak in Bavistin fungicide (Sujatha and Reddy 1998). Similarly, Ahn et al. (2007) found that shoots generated from mesocotyls and cotyledons placed on full-strength MS medium containing MS basal salts, MS vitamins, 30 g/L sucrose, and 0.5 g/L MES at pH 5.7and supplemented with 5 μM NAA or 5 μM IBA showed a rooting frequency of 84.3% and 87.4% respectively after 4 weeks of culture. These rooted shoots were then transferred to a peat-vermiculite growth mixture and acclimatized in the greenhouse (Ahn et al. 2007; Ahn and Chen 2008).

These protocols were selected primarily for the higher relative level of detail provided when compared to the majority of castor tissue culture literature. Many of the protocols listed in the literature did not go into sufficient depth to be repeated. These protocols also utilized equipment and explants that were readily available in the facilities used for this research.

Materials and Methods

Seed disinfestation

Castor seed (cv. Hale and Ultradwarf) were obtained from fields maintained by Dr. Brian Baldwin (Mississippi State University; Starkville, MS). The spiky husks were removed manually, and seed coats were carefully cracked by manual pressure via a screw-clamp taken from a ring-stand. Cracked seed coats were manually removed revealing the whole, undamaged seed. Any visually cracked or otherwise damaged seed were discarded. The decoated seed were then treated via one of the following protocols:

- One hundred seed were placed in a 250 mL glass jar with 50 mL of 10% commercial bleach (v/v; 0.6% sodium hypochlorite final concentration) and agitated on a rotary shaker at 150 rpm for 30 minutes. Seed were rinsed 5 times for 5 minutes with 50 mL of autoclaved distilled water.
- One hundred seed were placed in a 250 mL glass jar with 50 mL of 70% ethanol (v/v) and agitated on a rotary shaker at 150 rpm for 2 minutes followed by a 30-minute wash in 50 mL of 10% commercial bleach (v/v; 0.6% sodium hypochlorite final concentration) and a 30-minute wash in 0.1% SDS (w/v) in autoclaved distilled water. Seed were rinsed 5 times for 5 minutes with 50 mL of autoclaved distilled water.
- One hundred seed were placed in a 250 mL glass jar with 50 mL of 0.1% mercuric chloride (w/v) and agitated on a rotary shaker at 150 rpm for 10 minutes. Seed were rinsed 3 times for 5 minutes with 50 mL of autoclaved distilled water.

Seed dissection

The freshly disinfested, naked seed were either dissected immediately or allowed to imbibe in sterile distilled water overnight. Seed that had been allowed to imbibe overnight were softer and easier to dissect; however, imbibition was not necessary for dissection. Seed were carefully dissected on an autoclaved 10 cm glass Petri dish using sterile forceps and No. 11 surgical scalpel blades (Feather Safety Razor; Osaka, Japan) under a dissecting microscope at 10x magnification. The first incision was made along the perimeter of the endosperm following the line created by the junction of the two endosperm halves. Once the first incision was complete, the endosperm was separated

along the intersection by carefully prying with forceps. Care was taken to preserve the cotyledons and embryo at this point as the separation of the lobes exposes these tissues. The cotyledons were removed from the embryo at the cotyledonary node using the surgical scalpel blade. This resulted in two cotyledons, one embryo axis, and two endosperm halves (Figure 3.1). For some experiments, the embryo axis was further dissected to produce a mesocotyl by removing 1 mm of the radicle tip (Figure 3.1c). Cotyledon explants were cultured on MS medium supplemented with 5 µM TDZ (Ahn and Chen 2008). The cotyledons were excised from the mature seed and placed vertically with the distal end in contact with the medium (3 explants per 3 cm Petri plate). Embryo axis explants were cultured on MS medium supplemented with 2.2 µM BA (Sujatha and Reddy 1998). Embryo axes were dissected from mature seed and placed vertically with the radicle in contact with the medium (3 explants per 3 cm Petri plate). Mesocotyl explants were cultured on MS medium supplemented with 1 µM TDZ (Ahn et al. 2007). Mesocotyl explants were prepared the same as embryo axes with 1 cm of the radical end excised. The explant was then placed vertically on medium with the cut end in contact with the medium (3 explants per 3 cm Petri plate).

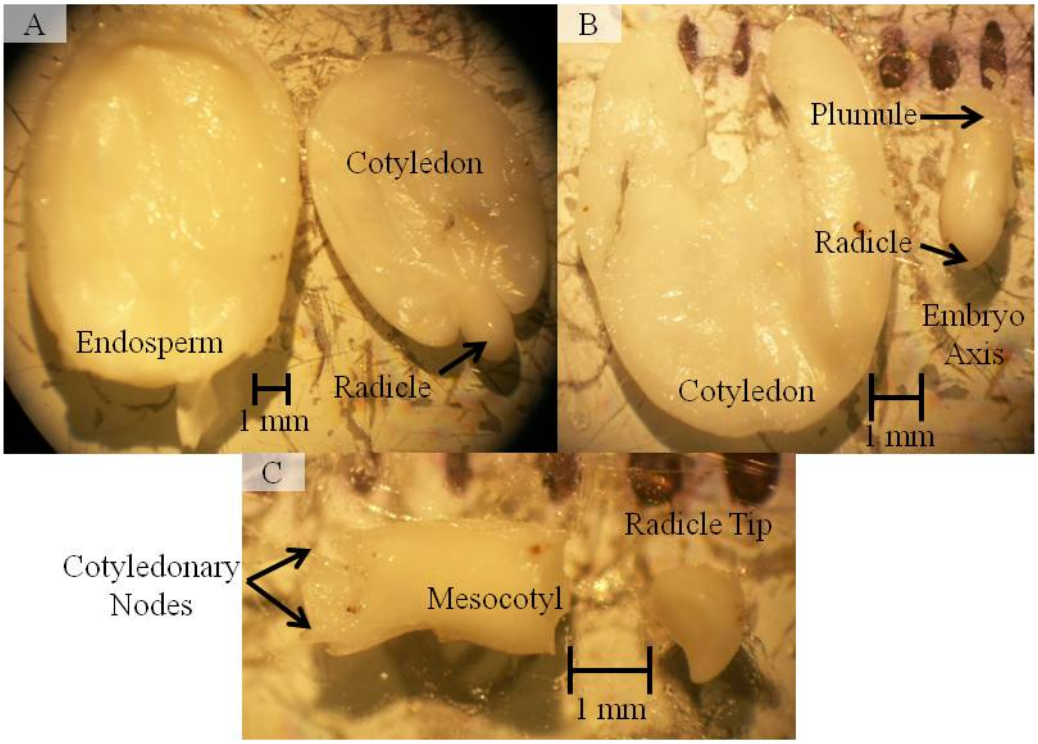

Figure 3.1 Dissected castor seed tissues

A – Dissected castor seed showing the endosperm on the left and the embryo and cotyledon on the right; **B** – The embryo dissected away from the cotyledon at the cotyledonary node; **C** – The removal of approximately 1mm of the radical tip generates the mesocotyl explant.

Nodal segment collection

Nodal segments from castor cultivars Hale and Energia and germplasms Ultradwarf, Memphis, and Brigham were collected from fields maintained by Dr. Brian Baldwin. Segments (Figure 3.2b) were collected by harvesting the most distal shoots on mature castor plants (5 months after planting). Leaves and internodal regions were removed with scissors. Nodal explants were disinfested by 30-minute incubation in 0.1% mercuric chloride (w/v) on a rotary shaker at 150 rpm and subsequent 5-minute sterile water wash repeated 3 times. The explants were placed distal end up on culture media.

Figure 3.2 Castor nodal segment dissection

A – Intact distal shoot from a mature castor plant; **B** – Node segments dissected from the shoot shown in close-up.

Media preparation

Explants were maintained in culture on a variety of media. The standard culture medium utilized was 4.33 g/L Murashige & Skoog (MS) basal salts (Catalog # M524; Phytotechnology Laboratories; Shawnee Mission, KS), 103.1 µg/L MS vitamins (Catalog # M533; Phytotechnology Laboratories; Shawnee Mission, KS), 30 g/L sucrose, 0.5 g/L MES, and 8 g/L agar (Catalog # A296; Phytotechnology Laboratories; Shawnee Mission, KS). This is the medium composition referred to as 'MS medium' in this paper unless

otherwise stated. All media were prepared in Erlenmeyer flasks, adjusted to pH 5.7 with 0.1N NaOH, sealed with aluminum foil, and autoclaved at 121°C at 15 psig for 30 minutes (volumes less than 1 L) or 35 minutes (volumes 1 L and greater). Following autoclaving, the medium was moved to a 55°C water bath to cool before pouring. Media were poured into sterile 3 cm Petri plate, 10 cm Petri plates, baby food jars, or Magenta boxes, depending on the requirements of the explant. Petri plates arrived sterile from the manufacturer. Baby food jars and Magenta boxes required sterilization by autoclaving the vessel at 121°C at 15 psig for 1 hour and allowing them to cool in the laminar flow hood prior to addition of sterile media.

These media were typically supplemented with plant growth regulators (PGRs). The PGRs used in this study were 6-benzylaminopurine (BA), thidiazuron (TDZ), 1-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), and gibberellic acid (GA_3). The BA and NAA stock solutions were prepared by dissolving 10 mg of PGR in 1 mL of 1 N sodium hydroxide and adding 9 mL of distilled water, producing 10 mL of a 1 mg/mL stock PGR solution (44.4 mM BA, 53.7 mM NAA). The TDZ stock solution was prepared by dissolving 10 mg of TDZ in 10 mL of 100% dimethyl sulfoxide, producing 10 mL of a 1 mg/mL (45.4 mM) stock solution. These solutions were 0.2 µm filter sterilized, aliquoted into 1.5 mL Eppendorf tubes, and stored at 4°C for up to 1 month. When media were supplemented, PGR was added prior to final pH adjustment (pH 5.7). and autoclaving. Gibberellic acid (GA_3) and indole-3-butyric acid (IBA) stock solutions were prepared by dissolving 10 mg of PGR in 10 mL of absolute ethanol, producing a 1 mg/mL stock solution (28.9 mM GA_3, 49.2 mM IBA). This solution was 0.2 µm filter sterilized, aliquoted into 1.5 mL Eppendorf tubes and stored at -20°C for up to 1 month.

In the case of GA_3 and IBA, each filter-sterilized PGR was added after the medium had cooled to 55°C.

Some media were supplemented with hygromycin or cefotaxime. For hygromycin, 100 mg of hygromycin B (Sigma-Aldrich; St. Louis, MO) was dissolved in 5 mL of distilled water, producing a 20 mg/mL stock solution. The solution was 0.2 µm filter sterilized and stored at 4°C for up to 1 week. Cefotaxime was received in vials (Claforan®; Sanofi Aventis; Paris, France) containing 1 g of cefotaxime per vial. To prepare the stock solution, 10 mL of distilled water was added to a vial to produce a 100 mg/mL cefotaxime stock solution, which was 0.2 µm filter sterilized and stored at 4°C for up to 1 month. Both hygromycin and cefotaxime were added to media after the medium had cooled in a 55°C water bath.

After explants had been placed on the solidified media, the perimeter of the vessel was sealed with parafilm and transferred to the growth chamber. The culture conditions were dependant on the explant type. Mesocotyl and embryo axis explants were maintained at 26°C in the dark for 1 week prior to transfer to 26°C with a 16h/8h light/dark cycle under cool white fluorescent lighting at an intensity of 30 mmol/m^2s. Cotyledon explants and nodal explants were maintained at 26°C with a 16h/8h light/dark cycle under cool white fluorescent lighting at an intensity of 30 mmol/m^2s.

Rooting and acclimatization

When shoots had reached approximately 1 cm in length, they were transferred to rooting medium [2.16 g/L MS basal salts, 51.5 µg/L MS vitamins, 30 g/L sucrose, 0.5 g/L MES (pH 5.7), 8 g/L agar, and 2 mg/L NAA]. This medium is referred to as 'half-strength MS medium' and 'rooting medium' within this paper. The shoots were

maintained on this medium with changes to fresh medium every 7 days until root growth was observed. When roots were observed, the shoots were transferred to PGR-free rooting medium in Magenta boxes to allow the roots to elongate. After 1 week, the rooted shoots were transferred to Magenta boxes containing autoclaved, saturated vermiculite for 10 days. Once established, the rooted shoots were soaked overnight in an 1.78 a.i./L thiophanate methyl systemic fungicide (Halt™; Ferti-lome; Bonham, TX) solution and transferred to autoclaved soil mix [2 parts Sunshine Mix No. 1 (SunGro Horticulture; Vancouver, Canada), 1 part sand, and 1 part mulch] in 4-inch square pots, covered with a clear plastic cloche to maintain humidity, and sustained in the growth chamber. After 1 week, the cloche was removed daily for increasing lengths starting at 1 hour until the plant was sufficiently acclimatized to survive without it entirely. Once acclimatized, the plants were transferred to larger pots and placed in the greenhouse.

Results

Seed disinfestation

Disinfestation of the explant tissue was an ongoing concern for the project described here. Seed samples from the field often carried fungi that were not eradicated during standard disinfestation procedures. Fungi were often quiescent for up to a week before proliferating and spreading through the fresh culture plates infecting many samples. In order to establish a protocol that mostly or completely removed contamination from field-derived samples, protocols involving combinations of treatments with several disinfection agents were tested. Among these agents were 10% commercial bleach (v/v), 70% ethanol (v/v), 0.1% SDS (w/v), and 0.1% mercuric chloride (w/v). Since the primary contamination was fungal (Figure 3.3), the addition of

50 mg/L benomyl (Benlate; DuPont) added as an 0.2 μm filter sterilized concentrate to the cooled, autoclaved medium were also tested (Shields et al. 1984). Treatment with commercial bleach, ethanol, SDS, and combinations thereof showed little impact on the level of fungal contamination compared to commercial bleach alone. The addition of benomyl to the culture medium only slowed fungal growth at the start of culture. After the first week, the contamination levels of benomyl-treated media were equivalent to non-treated media. This could possibly be due to the hydrolytic breakdown of benomyl in the media, resulting in the generation of butyl isocyanate and methyl-2-benzimidazole (MBC). Butyl isocyanate is an active fungitoxin, whereas MBC inhibits fungal growth by disrupting the formation of microtubules, preventing cell division (Shields et al. 1984; Hauptmann et al. 1985). The only treatment that showed a marked reduction in contamination was the 10-minute wash in 0.1% mercuric chloride. This treatment nearly completely removed contamination with only the occasional contaminated explant noted.

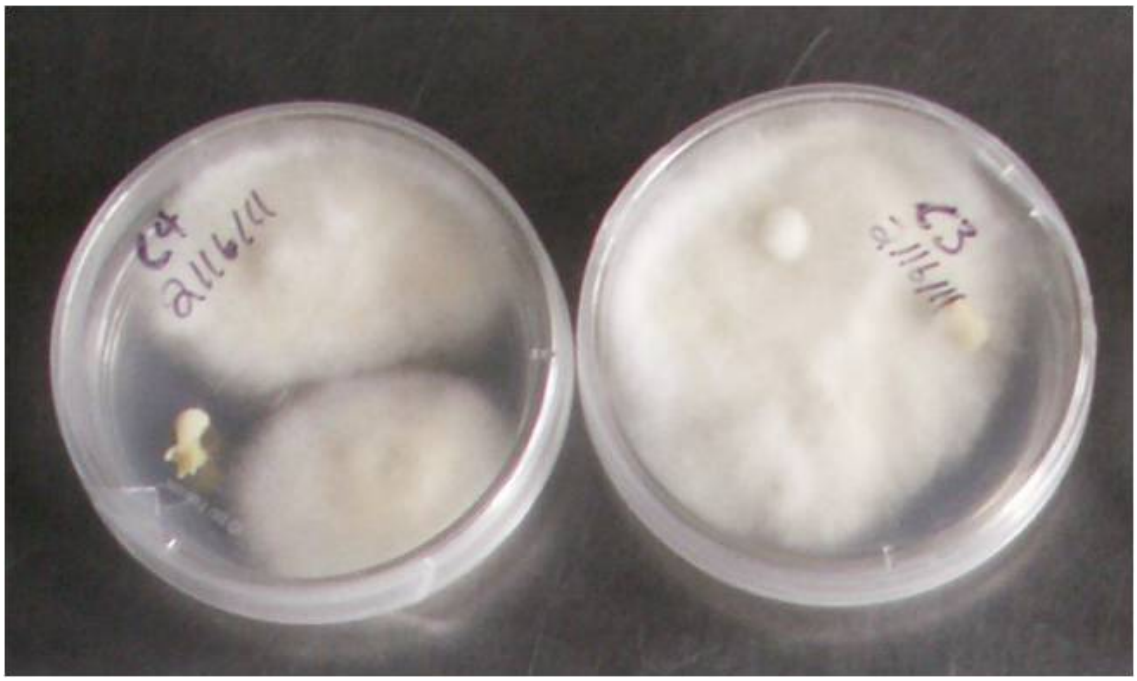

Figure 3.3 Fungal contamination typically seen on MS media 7 days after explant introduction

Mesocotyl explants on MS medium supplemented with 30 g/L sucrose, 0.5 g/L MES (pH5.7) exhibiting advanced fungal growth 7 days after culture initiation.

Protocol screening

Each dissected castor seed contains a variety of explant tissues. The literature has described micropropagation protocols from cotyledon, embryo axis, and mesocotyl explants dissected from mature castor seed (Sujatha and Reddy 1998; Ahn et al. 2007; Ahn and Chen 2008). Protocols using these three tissues were examined individually in order to identify the tissue that would generate the largest number of healthy shoots in culture. These protocols were chosen specifically because of their reported success in generating healthy castor plants via tissue culture as well as the depth into which the protocol was described compared to most castor tissue culture literature. Of the three protocols screened, only the embryo axes and mesocotyls showed shoot formation when

cultured as described by the literature. Of these two tissues, mesocotyls on 1 μM TDZ supplemented MS medium generated far more shoot buds compared to the embryo axes on 2.2 μM BA supplemented MS media. Embryo axes generated long radicles that had to be cut away after the first week (Figure 3.4a). Cotyledon explants on 5 μM TDZ supplemented MS medium expanded and generated green, friable tissue, but shoot formation was never observed. For these reasons, embryo axes and mesocotyl explants were used for the remainder of the project.

The literature suggests that the use of TDZ induces a more aggressive cytokinin response in explants compared to BA (Huetteman and Preece 1993). This can be seen in the sheer number of shoot buds present on mesocotyl explants placed on 1 μM TDZ supplemented MS compared to explants placed on 2.2 μM BA supplemented MS (Figures 3.4b and 3.4c). At 4 weeks, TDZ treated explants showed 9.8 ± 1.8 shoot buds per explant whereas BA treated explants showed only a single shoot. However, shoots generated by TDZ treated explants were shortened and were so densely arranged that separating the shoots in order to sub-culture them became difficult (Figure 3.4d). Therefore, the shoots would elongate slowly and eventually senesce as the clusters of shoots were presumably too dense to allow sufficient nutrient flow. Sub-culturing of TDZ-treated shoots often resulted in the death of the explant as each shoot was too small to be precisely cut from the rest and damage to the shoot was likely. Clusters of shoots could be sub-cultured; however, this still seemed to damage the explant resulting in the death of many shoots. Sterile GA_3 solution was added to the medium to a final concentration of 0.7 μM in order to increase shoot elongation; however, this resulted in long, fragile shoots that senesced rapidly after sub-culture.

While BA-treated embryo axes generated fewer shoots, the shoots were generally much healthier in culture. Therefore, an alternate protocol described by Sujatha and Sailaja (2005) was explored. In this protocol, embryo axes were cultured for the first 3 weeks in 0.44 µM BA-supplemented MS, transitioned to 2 µM TDZ-supplemented MS for 14 days to increase shoot proliferation, and transitioned to 2 µM BA supplemented MS for shoot elongation. Using this method, the number of shoots could be increased from a single shoot to 2-3 shoots generated per explant. These shoots elongated well in culture and could be sub-cultured by cutting at the base and placing the shoot upright on fresh medium (Figure 3.4e). A summary of the protocol screening is presented in Table 3.1.

Table 3.1 Summary of the castor tissue culture protocols screened in this research

Explant tissue	PGR	Shoots/explant	Observations	Reference
Cotyledon	5 µM TDZ	0 (n=100)	Developed green friable tissue	Ahn and Chen 2008
Mesocotyl	1 µM TDZ	9.8 ± 1.8 (n=100)	Densely packed shoot clusters did not survive sub-culturing	Ahn et al. 2007
Embryo axis	2.2 µM BA	1 (n=100)	Single healthy shoot	Sujatha et al. 1998
Embryo axis	0.44 µM BA 3w 2 µM TDZ 2w 2 µM BA elongation	2-3 (n=120)	Multiple shoots that withstood sub-culturing	Sujatha and Sailaja 2005

PGR - plant growth regulator; 3w – 3 weeks; 2w – 2 weeks

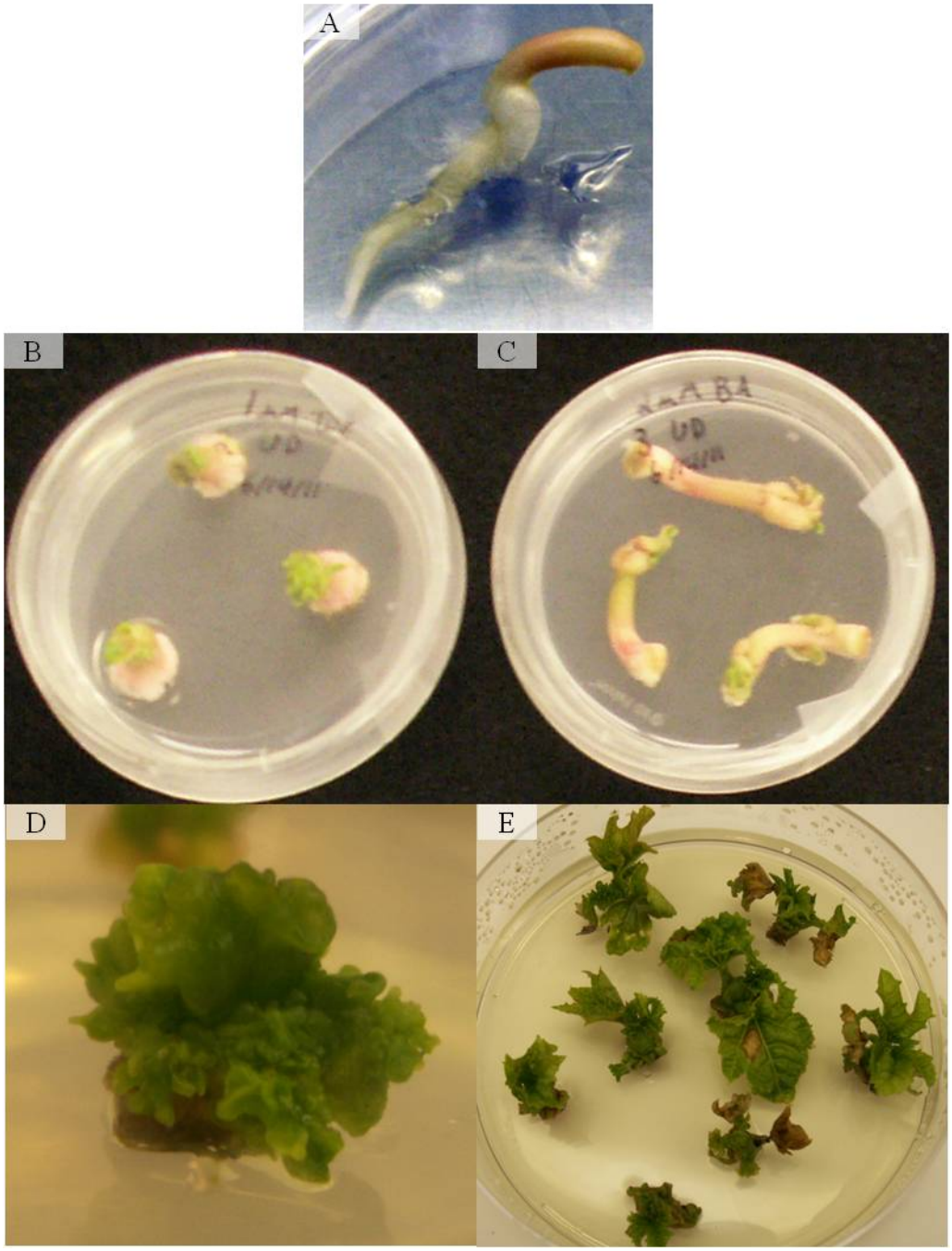

Figure 3.4 Shoot formation in Ultradwarf castor cultures produced via the protocols screened in this research

A – One-week-old embryo axis on 2.2 µM BA exhibiting long radical; **B** – Four-week-old mesocotyl explants on 1 µM TDZ; **C** – Four-week-old embryo axes explants on 2.2 µM BA; **D** – Eight-week-old mesocotyl explants initiated with 1 µM TDZ exhibiting densely clustered shoots; **E** – Eight-week-old mesocotyl explants initiated on 0.44 µM BA transitioned to 2 µM TDZ for 14 days then transferred to 2 µM BA.

Variety selection

At the onset of this project, there were multiple issues with the reproducibility of the experiments. The loss of many samples to contamination as previously discussed accounts for many of these issues. However, the homogeneity of the seed lot being used for dissection was also questionable. Therefore, a series of experiments with the two cultivars Hale and Ultradwarf were conducted to identify any varietal differences in culture. Seed from both cultivars were dissected to produce 100 mesocotyl explants that were then cultured on 2 μM BA supplemented MS for 1 week in the dark. The explants were transferred to a 16h/8h light/dark cycled growth chamber and sub-cultured every 14 days on fresh MS media.

The results of this experiment showed marginal differences between the two cultivars. The Hale explants appeared to produce qualitatively longer and more robust shoots when compared to the Ultradwarf explants (Figure 3.5). This resulted in a slightly higher rate of survival for Hale explants (45 at 8 weeks) compared to Ultradwarf explants (42 at 8 weeks) during sub-culture. This was somewhat explained by the fact that Hale is a semi-dwarf variety whereas Ultradwarf is a dwarf variety. However, as the project progressed, larger quantities of more homogenous Ultradwarf seed became available, making Ultradwarf the cultivar of choice for downstream experiments.

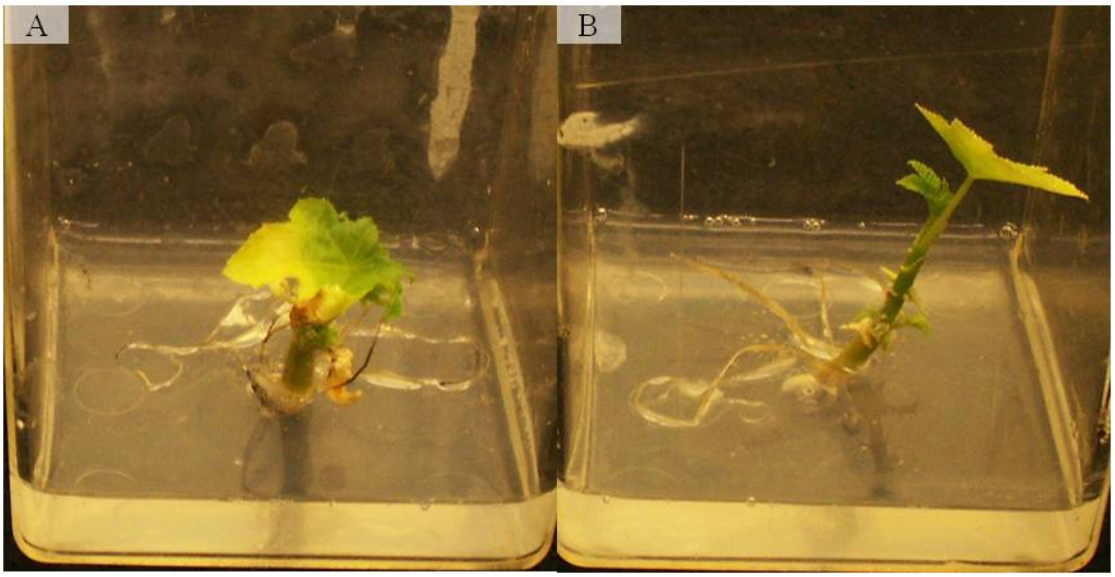

Figure 3.5 Visual comparison of Ultradwarf and Hale castor shoots

A – Eight week old Ultradwarf mesocotyl explant cultured on MS medium supplemented with 2 µM BA; **B** – Eight week old Hale mesocotyl explant cultured on MS medium supplemented with 2 µM BA

Nodal segments

As previously mentioned, the homogeneity of the seed lot being used for generation of explants for culture was of concern, so nodal segments taken from mature plants were experimented with since many segments could be collected from a single mature plant. This would ensure genetic homogeneity of the explants, allowing for more consistent results during culture. Nodal segments (50 for each variety) were dissected from the most distal shoots of four castor varieties (Hale, Ultradwarf, Energia, and Memphis). After disinfestation, the node segments were placed on 5 µM TDZ-supplemented MS medium and cultured under a 16h/8h light/dark cycle at 26ºC. New shoots began to form at the axillary buds within 7 days. These shoots could then be sub-cultured onto fresh 2 µM BA-supplemented MS medium and maintained in culture

during elongation. Unfortunately since the nodes were collected from the field, many of the samples were contaminated even after disinfestation with 0.1% mercuric chloride (w/v). In addition only 1-2 shoots per explant were collected, making this technique a poor choice for micro-propagation protocols requiring large numbers of samples. However, the surviving shoots generally grew vigorously and rooted quickly. No apparent differences in tissue culture protocols were seen between the cultivars selected for this experiment; however, the Energia nodal segments possessed a much more pronounced waxy coating making disinfestation less effective. The addition of 10 mg/L of 0.2 μm filter-sterilized silver nitrate (Sigma-Aldrich; St. Louis, MO) to the autoclaved media reduced contamination and media browning. It is not clear if the addition of silver nitrate to the media increased the efficiency of shoot regeneration since no study was designed to specifically test this. However, the literature suggests that silver nitrate increases shoot formation due to its action as an ethylene inhibitor (Biddington 1992; Pua et al. 1996; Mohiuddin et al. 1997).

Rooting and acclimatization

In order to optimize the protocol for rooting castor shoots, two methods from the literature were compared (Sujatha and Sailaja 2005; Ahn and Chen 2008). Both methods utilized half-strength MS medium supplemented with either 2 μM NAA or 5 μM IBA. Shoots originating from both 1 μm TDZ- and 2.2 μm BA-initiated cultures were placed in Magenta boxes containing 40 mL of solidified rooting medium (1 explant per box; 30 each of TDZ- and BA-initiated shoots per rooting medium repeated 3 times). Both rooting media showed rooting in approximately 50% of shoots within (NAA: 48% TDZ, 55% BA; IBA: 46% TDZ, 51% BA) the first 2 weeks. Shoots cultured on NAA-

supplemented medium produced larger-diameter, shorter roots that were often more sturdy compared to the thinner, longer roots of shoots cultured on IBA-supplemented medium (Figure 3.6). The thicker roots of the NAA samples were more resilient during transfer to new medium or soil when compared to IBA samples. The roots generated by IBA samples would often break during transfer sometimes causing the death of the shoot.

Shoots that had been cultured on BA supplemented media for longer periods of time rooted much more slowly. This could be due to the accumulation of BA inside the explant inhibiting root formation (Stenlid 1982; Cary et al. 1995). To combat this, 0.02% activated charcoal (Sigma-Aldrich; St. Louis, MO) was added to pre-autoclaved half-strength MS media. Activated charcoal adsorbs a variety of compounds from the media, and the removal of accumulating ethylene aids in the root formation process (Thomas 2008). Shoots were cultured on this medium for 2 weeks prior to transfer to rooting media. This treatment increased the rate of shooting from 47% (n=200) to 78% (n=200) in both IBA and NAA treated samples.

Regardless of treatment, shoots showing root growth were transferred to autoclaved vermiculite in Magenta boxes and grown for 10 days. Initially, the rooted shoots were directly transferred to autoclaved soil mix in 4-inch pots and acclimatized under a cloche at 26ºC (Figure 3.7). However, many of the rooted plants quickly succumbed to fungal infection due to the high-humidity environment. Unfortunately, the cloche was necessary for proper acclimatization since plants migrated directly to soil with no cloche quickly dried out and died. Therefore, the rooted shoots were soaked overnight in thiophanate methyl systemic fungicide solution prior to planting in soil. This resulted in a reduced number of plants killed by fungal infection.

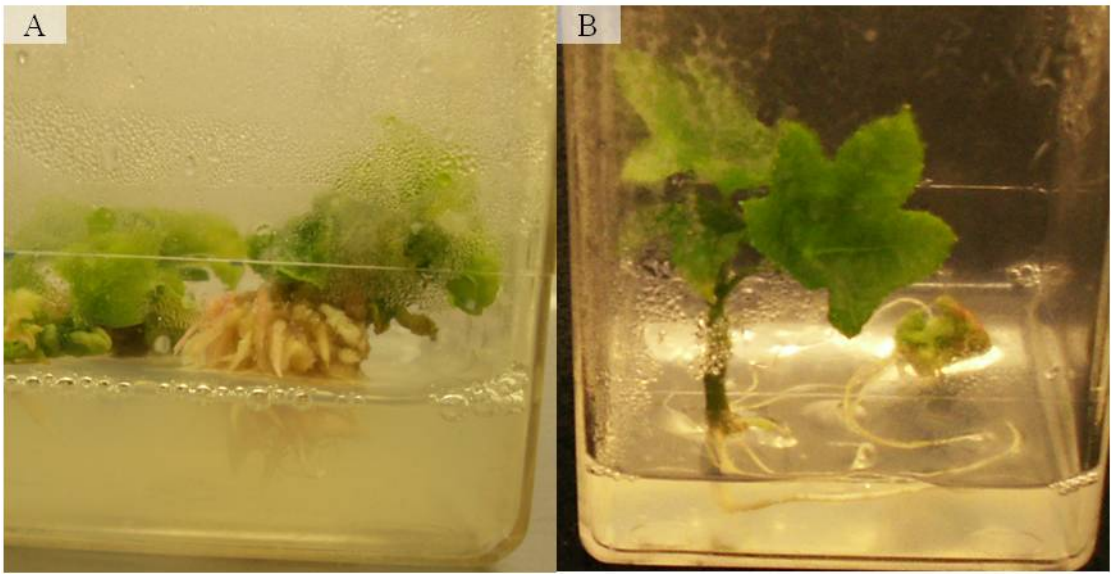

Figure 3.6 Rooting of Ultradwarf castor shoots in MS medium supplemented with NAA or IBA

Shoots are from 2 µm BA-initiated explants. **A** – Clustered, thin root formation initiated by 2 µM NAA; **B** – Thin, long root formation initiated by 5 µM IBA

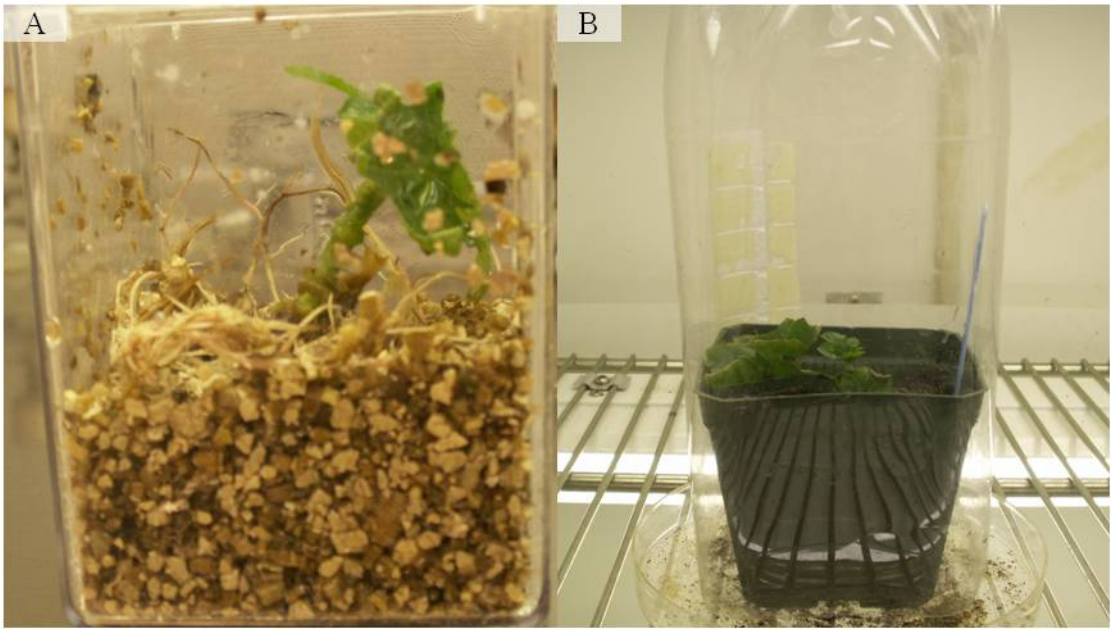

Figure 3.7 Ultradwarf castor shoots rooted in NAA-supplemented medium undergoing acclimatization

Shoots are from 2 µm BA-initiated explants. **A** – Rooted castor shoot on sterile, saturated vermiculite; **B** – Rooted castor shoot that has undergone vermiculite treatment is planted in soil with humidity controlled by cloche (plastic bottle)

Discussion

During the course of the tissue culture protocol development portion of this project, several important decisions were made. First, field-derived samples should be disinfested with 0.1% mercuric chloride (w/v) prior to use in culture. This treatment, while requiring the use of a hazardous heavy metal salt, is the only method discovered that could remove nearly all fungal contamination. Many of the protocols listed in the literature utilize mercuric chloride for seed disinfestation (Sujatha and Reddy 1998; Sujatha and Sailaja 2005; Malathi et al. 2006). However, the use of heavy metals was avoided in the beginning of this research due to its toxicity. Some protocols have been published that utilize commercial bleach to surface-sterilize castor seed (Ahn et al. 2007; Ahn and Chen 2008); unfortunately, this procedure was not sufficient for the seed used in this research. This may be due to the source of the seed in question. It stands to reason that seed that are derived from fungicide-treated crops would not present the same fungal contamination issues. While the literature does not suggest this, it is possible that seed successfully decontaminated with commercial bleach may have come from treated fields.

Mesocotyl and embryo axis explants both generated new shoots when cultured on TDZ and BA supplemented medium respectively. Thidiazuron-treated mesocotyls generated far more shoots than BA treated axes; however, the TDZ derived shoots elongated very slowly and senesced more rapidly compared to BA derived shoots. Therefore, a protocol from the literature, involving the transfer of BA derived shoots onto TDZ for a short culture prior to elongation on BA media, was evaluated and shown to be effective (Sujatha and Sailaja 2005). The relative potency of TDZ as a cytokinin compared to BA has been reported (Huetteman and Preece 1993). Indeed the castor tissue

culture literature contains examples of TDZ providing more shoots compared to BA (Sujatha and Reddy 1998; Ahn et al. 2007). However, none have reported on the fragile nature of shoots produced by TDZ treatment. This is likely a technique issue and could be solved by more delicate treatment of the shoots and shoot clusters during sub-culturing. It is apparent that TDZ is capable of generating many shoots on a variety of castor explants, but the rate of survival of these shoots is of optimal concern when proceeding with propagation.

Shoots were successfully generated from nodal segments cultured on 5 μM TDZ supplemented MS media. This technique may be useful for generating a tissue culture line as it produces new tissue in an axenic environment; however, this technique would not be of much use when large numbers of sample were needed as only one or two shoots were generated per explant. Further modification of this procedure may produce more shoots from each explant or present a source for new explant types for further tissue culture.

The rooting and acclimatization protocol required pretreatment of shoots on half-strength MS medium containing 0.02% activated charcoal prior to transfer to half-strength MS medium supplemented with 5 μM NAA was shown to produce robust rooted shoots. The use of NAA for root induction is consistent with some of the published literature (Reddy and Bhadur 1989; Ahn et al. 2007). However, many published protocols indicated that IBA was more effective for rooting castor shoots (Sujatha and Reddy 1998; Ahn et al. 2007; Ahn and Chen 2008). Many of the issues with rooting faced in this research did not come from the induction of rooting itself but, rather, the root morphology. The roots induced by IBA were thin and fragile compared to the robust

roots induced by NAA. Much of the issue with this may be solved through more careful handling or adjustments to the solidification medium used. Reduced agar concentration or the use of gelling media with lower tensile strength may aid in the recovery of rooting shoots. Activated charcoal was used in the medium to adsorb accumulating toxic compounds, allowing the explants to root more reliably. This treatment has not been reported in castor tissue culture literature but is a common practice in general plant tissue culture (Stenlid 1982; Cary et al. 1995).

More up-to-date and in-depth review of the literature on the tissue culture of castor and related species has provided new potential avenues for enhanced micropropagation procedures. This project focused on screening of only a few protocols. The research plan was to replicate the success of these protocols for use in the transformation procedure. However, the efficiency of the tissue culture procedure is paramount to any transformation research. Sarvesh et al. (1992) reported success in inducing callus in castor epicotyls cultured on MS salts with Gamborg's B5 vitamins (Gamborg et al. 1968) supplemented with 8.8 µM BA and 5.4 µM NAA. This callus then showed a 20% efficiency of shoot formation when placed on media supplemented with 11 µM BA, 0.5 µM NAA, and 1.1 µM GA_3 (Sarvesh et al. 1992). More recently Kumari et al. showed callus induction of cotyledon cultures placed on MS salts with Gamborg's B5 vitamins supplemented with 8.8 µM BA and 4.3 µM NAA. Somatic organogenesis (85% efficiency) was achieved on MS medium containing 11.3 µM TDZ, 2.1 µM NAA, and 15 mg/L glutamine (Kumari et al. 2008). These protocols, in addition to the protocols described in for *Jatropha*, cassava, and rubber tree culture in Chapter I may present an opportunity for more efficient castor culture (Cailloux et al. 1996; Li et al. 1996; Etienne

et al. 1997; Blanc et al. 1999; Kumari Jayasree et al. 1999; Jha et al. 2007; Deore and Johnson 2008; Bull et al. 2009; Kumar et al. 2010; Misra et al. 2010; Singh et al. 2010; Cai et al. 2011; Sharma et al. 2011).

Summary

Seed disinfestation required the use of 0.1% mercuric chloride for the removal of fungal contamination. Four protocols for the generation of castor shoots in tissue culture were examined. It was found that, while TDZ-treated mesocotyl explants produced the most shoots, these shoots did not survive in tissue culture due to damage during sub-culture. Cotyledon explants treated with TDZ failed to produce shoots. Embryo axes treated with BA produced only single shoots, but these shoots were healthy and elongated readily. A protocol using mesocotyl explants pre-cultured on BA media, treated on TDZ medium for 7 days, then elongated on BA medium showed the most promise for shoot generation. Nodal segments generated healthy shoots when treated on TDZ-supplemented MS media. Shoots were then rooted with half-strength MS medium supplemented with NAA and activated charcoal. Rooted shoots were soaked in thiophanate methyl fungicide overnight and transferred to autoclaved vermiculite in Magenta box for 10 days before transfer to sterile soil mix kept beneath a cloche. These methods formed the framework upon which the transformation experiments were developed.

CHAPTER IV

TRANFORMATION

Introduction

The final stage of this project required the introduction of the constructed pC1-RKO RNAi vector into the castor genome. Few works have shown successful transformation of castor. A United States patent was issued in 2003 for the transformation of castor via *Agrobacterium* infiltration of mature inflorescence (McKeon and Chen 2003). Sujatha and Sailaja (2005) developed a castor transformation protocol utilizing the DCS-9 cultivar and the pCambia1304 vector. Embryo axes from mature seed were dissected and placed on MS medium supplemented with 0.44 µM BA 7 days prior to wounding with a surgical blade, incubation with *Agrobacterium* EHA105, and co-cultivation for 2-10 days followed by 250 mg/L cefotaxime treatment. The explants were then placed on selection medium for 10 days at concentrations of 20, 40, and 60 mg/L hygromycin successively. This protocol reported a transformation efficiency of 0.08% (Sujatha and Sailaja 2005). Malathi et al. (2006) produced a protocol for *Agrobacterium*-mediated castor transformation using the pTOK233 and pSB111 vectors. They were able to generate a semilooper resistant castor line expressing the transformed cryIAb gene with 0.4% transformation efficiency. Their protocol utilized embryo axes dissected from seed of the Jyothi and VP1 castor cultivars placed on MS medium supplemented with 2.2 µM BA. The explants were then co-cultured with *Agrobacterium* for 3 days followed by

cefotaxime treatment and selection on 30 mg/L hygromycin (Malathi et al. 2006). Sailaja et al. (2008) reported stable transformation of embryo axes (1.4% transformation efficiency) with the pCambia1305 vector via particle bombardment (Sailaja et al. 2008). Sujatha et al. (2009) later showed the stable transformation of castor with the *cry1EC* gene with a transformation efficiency of 0.82% using the method published by Sujatha and Sailaja (2005) (Sujatha et al. 2009).

This research was based on the protocol described by Sujatha and Sailaja (2005) due to its reported success and the amount of information presented in the work. Ultradwarf castor was chosen for its availability and desirability as a production variety (i.e. short plant stature, shatter resistance, disease resistance). However, the protocol described by Sujatha and Sailaja (2005) utilized the DCS-9 castor cultivar, which proved difficult to reproduce with Ultradwarf castor. As previously described in Chapter 3, many of the culture methods had to be adjusted for Ultradwarf castor. Likewise, the transformation protocol was refined to suit the requirements of Ultradwarf. Therefore, the final protocol reported herein closely resembles that of Sujatha and Sailaja (2005), with some modifications.

The pCambia1304 plasmid served as a test plasmid for generation of the transformation protocol. Prior to the completion of the final pC1-RKO vector, the pCambia1304 vector was used to troubleshoot the transformation protocol. This is the same vector used in the referred work and was readily available for research use. Additionally, the pCambia1304 vector contains the selectable marker, *hptII*, for hygromycin resistance selection as well as the reporter, *gusA*, for GUS assaying. These are the same markers found in the pC1-RKO RNAi vector. Therefore, techniques

developed for identification of pCambia1304 transformants were adaptable for use with pC1-RKO transformants.

Materials and Methods

Agrobacterium tumefaciens culture

Agrobacterium tumefaciens strain EHA105 containing either pCambia1304 or pC1-RKO was removed from the -80ºC freezer and streaked on fresh LB (Qbiogene; Quebec, Canada) agar plates supplemented with 50 mg/L kanamycin (Sigma-Aldrich; St. Louis, MO) and 50 mg/L rifampicin (Phytotechnology Laboratories; Shawnee Mission, KS). Plates were incubated in the dark at 28ºC overnight. Single colonies were isolated and inoculated into 50 mL of liquid LB medium supplemented with 50 mg/L kanamycin and 50 mg/L rifampicin in a 125 mL Erlenmeyer flask and incubated overnight with 150 rpm agitation at 28ºC. Next, 5 mL of this overnight culture was inoculated into 50 mL of liquid LB medium supplemented with 50 mg/L kanamycin and 10 mg/L rifampicin in a 125 mL Erlenmeyer flask and incubated with 150 rpm agitation at 28ºC until the $O.D._{600}$ reached 0.6-0.8. The culture was transferred to a sterile 50 mL centrifuge tube and pelleted by centrifugation at 3,000 g for 30 minutes. The cell pellet was resuspended in 25 mL autoclaved liquid MS medium supplemented with 30 g/L sucrose. The $O.D._{600}$ was reanalyzed, and the culture was diluted to 0.3-0.5 $O.D._{600}$ with MS media. Filter-sterilized (0.2 μm) 200 mM acetosyringone stock (Sigma-Aldrich; St. Louis, MO) was prepared in 100% ethanol and added to the medium at a final concentration of 200 μM.

Inoculation and co-culture

Embryo axis explants dissected from mature castor seed were pre-cultured for 2-7 days on 0.44 µM BA supplemented MS medium [4.33 g/L MS basal salts (Catalog # M524; Phytotechnology Laboratories; Shawnee Mission, KS), 103.1 µg/L MS vitamins (Catalog # M533; Phytotechnology Laboratories; Shawnee Mission, KS), 30 g/L sucrose, 0.5 g/L MES, and 8 g/L agar (Catalog # A296; Phytotechnology Laboratories; Shawnee Mission, KS) at pH 5.7] were injured at the plumule three times at a depth of approximately 0.5 mm (as judged via 1 mm reference markings under a dissecting microscope at 10-20x magnification) with a sterile 25g syringe needle (Becton Dickinson) and incubated with the *A. tumefaciens* solution in a sterile baby food jar for 30 minutes with 175 rpm agitation. After incubation, the explants were blotted on autoclaved filter paper (Whatman; Little Chalfont, UK) and placed upright on fresh MS medium supplemented with 0.44 µM BA. After 5 days of co-cultivation at 26ºC in a dark growth chamber, the explants were removed from the MS medium and rinsed in a sterile glass jar for 5 minutes with 500 mg/L cefotaxime in sterile water followed by 3 rinses in sterile water. The explants were blotted dry on autoclaved filter paper and placed back on fresh MS culture medium supplemented with 500 mg/L cefotaxime and 0.44 µM BA. After 2 weeks, the explants were moved to MS culture medium supplemented with 500 mg/L cefotaxime and 2 µM TDZ. Two week later, the explants were transferred to hygromycin selection medium; MS medium containing 0.44 µM BA, 500 mg/L cefotaxime, and 20, 40, or 60 mg/L hygromycin.

Hygromycin selection

Putatively transformed explants were transferred to hygromycin selection medium 4 weeks after removal from co-cultivation media. Explants which had been exposed only to *A. tumefaciens* not containing a plasmid were used as controls. The selection scheme utilized was adapted from Sujatha and Sailaja (2005). First the explants were placed on MS culture medium containing 20 mg/L hygromycin (Sigma-Aldrich; St. Louis, MO), 0.44 μM BA, and 500 mg/L cefotaxime. After 2 weeks, shoots that remained green and non-necrotic were transferred to MS culture medium containing 40 mg/L hygromycin, 0.44 μM BA, and 500 mg/L cefotaxime. After an additional 2 weeks, shoots that were still green color and did not appear necrotic were transferred to MS culture medium containing 60 mg/L hygromycin, 0.44 μM BA, and 500 mg/L cefotaxime. This was the final selection, after 2 weeks surviving shoots were transferred to PGR-free recovery medium [4.33 g/L MS salts, 103.1 μg/L MS vitamins, 30 g/L sucrose, 0.5 g/L MES (pH 5.7), 8 g/L agar, with or without 0.02% activated charcoal (w/v) and 10 mg/L 0.2 μm filter-sterilized silver nitrate]. The shoots were maintained on recovery medium for 2 weeks transferred to standard MS culture medium supplemented with 2 μM BA. Surviving shoots were rooted and acclimatized as described in the rooting and acclimatization section of Chapter 3.

GUS assay

Tissue samples from putatively transformed shoots were carefully removed and transferred to 1.5 mL Eppendorf tubes. The tissue was covered with GUS staining solution [1 mM X-gluc (Phytotechnology Laboratories; Shawnee Mission, KS), 50 mM phosphate buffer (pH 7.0), 0.02% Triton X-100 (v/v)], and the open tubes were subjected

to 25 mmHg vacuum pressure for 30 seconds. The tubes were capped and transferred to a 37°C incubator overnight. After incubation, the GUS staining solution was aspirated off, and the samples were bleached with 70% ethanol (v/v) for approximately 2 hours or until the chlorophyll had been mostly removed. Blue coloration indicated that the *gusA* gene was active in the sample.

PCR verification

Verification PCR procedure closely mirrored those described in Chapter 2. Each reaction was comprised of the following: 1 unit of EconoTaq (Lucigen; Middleton, WI), 100 ng template DNA, 0.2 mM dNTP, and 0.5 μmol of each primer in 0.2 mL PCR tubes using a T3 thermocycler (Biometra; Göttingen, Germany). Template DNA was prepared as described in Chapter 2 via the CTAB extraction method but needed further purification with the DNeasy Plant Mini kit (Qiagen; Venlo, Netherlands) following manufacturer's recommendations. A touchdown PCR protocol as follows was used for all verification reactions: 94ºC for 2 min; 5 cycles of 94ºC 15 sec, 62ºC 30 sec, 68ºC 30 sec; 5 cycles of 94ºC 15 sec, 61ºC 30 sec, 68ºC 30 sec; 5 cycles of 94ºC 15 sec, 60ºC 30 sec, 68ºC 30 sec; 5 cycles of 94ºC 15 sec, 59ºC 30 sec, 68ºC 30 sec; 15 cycles of 94ºC 15 sec, 68ºC 30 sec, 68ºC 30 sec; 68ºC for 5 min. Table 4.1 displays the sequences for the primers used for verification. Ten nanograms of purified pC1-RKO plasmid DNA was used as a positive control for all reactions.

Table 4.1 Primers used for PCR and RT-PCR verification of pC1-RKO integration in mature leaf DNA and cDNA

Description	Sequence	Amplicon size (bp)
hptII forward	CACAATCCCACTATCCTTCGC	465
hptII reverse	GCAGTTCGGTTTCAGGCAGGT	
actin forward	CCTTGTATGCCAGTGGTCGT	360
actin reverse	GGCAGTCTCAAGTTCCTGCT	
RKO forward	CTTCCAACTCTGGCTCGTTC	473
RKO reverse	CATGGCCACAACTGTATTGC	
hptII mRNA forward	GTGCTTGACATTGGGGAGTT	465
hptII mRNA reverse	GATGTTGGCGACCTCGTATT	
RB span 2 forward	TGGACTGGCATGAACTTCGG	572
RB span 2 reverse	TGAACGTCAGAAGCCGACTG	
RB prox 1 forward	TCGGCTTCTGACGTTCAGTG	558
RB prox 1 reverse	GGGTGCGGTCGATGATTAGG	
RB mid 1 forward	AAGCTGAAGATGTACGCGGT	588
RB mid 2 reverse	GCTTCGACAGACGGAAAACG	

RT-PCR

Sections of mature leaf tissue (0.1 g) were placed in 1.5 mL Eppendorf tubes and ground with an RNAse Away (Molecular BioProducts; Waltham, MA) treated micro-pestle. The tissue was then prepared with 1mL TRIzol® reagent (Life Technologies; Carlsbad, CA) and purified with the PureLink™ RNA mini kit (Ambion; Carlsbad, CA) as per manufacturer's recommendations. The purified total RNA was then quantified using a Nanodrop 2000c (ThermoScientific; Waltham, MA) prior to cDNA synthesis with the SuperScript First Strand synthesis kit (Invitrogen; Carlsbad, CA) as per

manufacturer's recommendations using the supplied oligo-dT primers and 40 ng of total RNA. DNase treatment was performed during purification as per manufacturer's instructions using amplification grade DNase I (Life Technologies; Carlsbad, CA). Ten microliters of the cDNA product were then used for touchdown PCR as described above.

Western blotting

Testa were removed from three randomly chosen seed from each mature castor plant, submerged in liquid nitrogen, and ground with a mortar and pestle until homogenous. The ground samples were then weighed and separated into 0.2 g aliquots and placed in 1.5 mL Eppendorf tubes. Next 1 mL of complete protein extraction buffer [50mM Tris Base, 120mM sodium chloride, 0.5% Triton X-100, 1 mM Na-EDTA, 1 mM DTT, 1 tablet/25mL Complete™ Mini protease inhibitor cocktail tablets (Roche; Basel, Switzerland) pH 7.4] was added to each sample tube (Barnes et al. 2009a). The tubes were then vortexed for 1 hour at 20ºC. After vortexing, the tubes were centrifuged at 10,000 g for 5 minutes at 20ºC in order to pellet solids. The resulting supernatant was then carefully removed via micropipet and placed in a Nanosep® MF 0.45µm pore-size centrifugal-filter (Pall Life Sciences; East Hills, NY) spin-filter tube and centrifuged at 14,000 g for 1 minute at 20ºC to remove any remaining solid material. The protein content of each sample was then calculated using a Micro BCA™ Protein Assay kit (Pierce; Rockford, IL) as per the manufacturer's instructions.

Protein samples were reduced by heating aliquots to 90ºC for 10 minutes in the presence of loading dye (2% SDS, 12.5% glycerol, 0.002% bromophenol blue, 0.002% acid orange G, 50mM dithiothreitol, 62.5mM Tris-HCl, pH 6.8). The reduced samples were then loaded (50 ng per well) onto a 1 mm thick 4-20% TruPAGE precast 12-well

gel (Sigma-Aldrich; St. Louis, MO) for separation. An RCA60 positive control (Vector Labs; Burlingame, CA) was loaded to serve as a positive identifier of ricin content. Gels were run in 1x TruPAGE TEA-Tricine SDS running buffer (Sigma-Aldrich; St. Louis, MO) at constant 180V for 50 minutes or until the bromophenol blue loading dye was within 1 cm of the bottom of the gel. Separated proteins within the gel were transferred to an Immobilon™-FL PVDF membrane (Millipore; Billerica, MA) using a Panther™ semidry transfer apparatus (Owl; Rochester, NY) and 1x TruPAGE transfer buffer (Sigma-Aldrich; St. Louis, MO) for 1h at 0.8 mA/cm^2. Transfer membranes were blocked overnight (12-16 h) in NZAB-Teleostean gelatin blocking buffer [2% casein enzymatic hydrolysate, 2% Teleostean gelatin, 50 mM phosphate-buffered saline pH 7.4] on a rocker (25rpm) at 20ºC. Blocking buffer was removed, and a 1:1,000 solution of RCA-52B anti-RCA60 mouse IgG primary antibodies (US Biological; Swampscott, MA) diluted in Tween Tris-buffered saline (TTBS; 50 mM Tris pH 7.4, 150 mM NaCl, 0.05% Tween-20) was incubated with the membrane for 1 hour at 20ºC. These antibodies bind specifically to the A-chain of ricin (RCA60) although they also exhibit some cross-reactivity to ricin agglutinin (RCA120). After incubation the primary antibody solution was removed, and the membrane was washed in TTBS for 5 minutes 4 times at 20ºC. A 1:100,000 solution of horseradish peroxidase conjugated Fc-specific anti-mouse goat IgG (Sigma-Aldrich; St. Louis, MO) diluted in TTBS was placed on the membrane and allowed to incubate for 1 hour at 20ºC. After incubation, the membrane was washed again in TTBS for 5 minutes 4 times at 20ºC. Residual TTBS was removed from the membrane, and a 1:1 solution of luminol and enhancer solution from the Supersignal© West Femto kit (Pierce; Rockford, IL) was overlaid onto the membrane. The membrane

was then transferred to a film cassette and exposed (5-10 seconds) onto CL-XPosure™ film (Pierce; Rockford, IL) in a dark room to imprint the signal onto the film. The exposed film was then developed using an M35A X-Omat™ processor (Kodak; Rochester, NY).

Dot-blotting

Dot-blotting was performed on a Bio-Dot® unit (Bio-Rad; Hercules, CA) by loading 150 µL of 1.5x serially-diluted protein sample (5.85-100 ng/well) in 1x TruPAGE transfer buffer (Sigma-Aldrich; St. Louis, MO) into each well of the apparatus housing the Immobilon™-FL PVDF membrane (Millipore; Billerica, MA). Vacuum pressure was applied to the apparatus causing the sample to be pulled across the membrane. Each well of the apparatus was then washed 3 times with 150 µL of 1x TruPAGE transfer buffer (Sigma-Aldrich; St. Louis, MO). The membrane was then removed from the apparatus and treated with blocking buffer and antibodies the same as a Western blot.

Results

The optimization of several parameters was necessary when refining the *Agrobacterium tumefaciens*-mediated transformation protocol for use with Ultradwarf castor. For these tests, *A. tumefaciens* EHA105 bearing the pCambia1304 vector was utilized. It was noted early in the transformation trials that the older the explant was when the transformation procedure was performed, the slower the explant grew after transformation. This could be due to the damage caused by the transformation process by either or both the needle causing mechanical damage or *A. tumefaciens* itself causing

damage. The older, more developed tissue may not be able to overcome the damage as readily as younger tissues; although, the reverse was expected. Therefore, the transformation procedure was performed on embryo axis explants pre-cultured on 0.44 μM BA-supplemented MS medium for 2, 5, and 7 days to determine at what stage the inoculation should be performed. This test counted the number of explants that had grown in the first week after culture for each age, and it was discovered that of the 2, 5, and 7 day pre-cultured explants 87.4%, 75.8%, and 64.7%, respectively, showed growth within the first week after inoculation (Table 4.2).

Table 4.2 Survival frequency of pre-cultured Ultradwarf embryo axis explants 1 week after inoculation

Pre-culture period	Explants inoculated	Explants showing growth 1 week after inoculation
2 days	183	160 (87.4%)
5 days	178	135 (75.8%)
7 days	190	123 (64.7%)

The next test examined the co-cultivation period. It was observed in the transformation trials that explants co-cultivated with *A. tumefaciens* for 7 days exhibited heavy *A. tumefaciens* growth around the explant on the media. This heavy growth was difficult to remove and often could not be controlled with cefotaxime rinses and cefotaxime supplemented media. It was observed that 5 days of co-cultivation produced far less contamination and subsequent cefotaxime rinses were sufficient to remove the *A. tumefaciens* contamination. Similarly, inoculation with *A. tumefaciens* cultures with an $O.D._{600}$ above 0.5 increased downstream contamination. Likewise, it was noted in this same procedure that 250 mg/L cefotaxime was not sufficient to control *A. tumefaciens*

growth. Therefore, the concentration of cefotaxime in the rinses and medium supplement was increased to 500 mg/L which was sufficient to control *A. tumefaciens* growth.

Finally, the selection procedure for putative transformants was resolved. Fortunately, the protocol described by Sujatha and Sailaja (2005) was directly transferable to use with Ultradwarf castor. This protocol utilizes a tiered selection process in which the putative transformants are subjected to MS medium containing 20, 40, and 60 mg/L hygromycin B in 2 week intervals. In the trials performed during this project, all control individuals were killed by the end of the third tier (60 mg/L) treatment. Of the 870 total explants tested using this protocol with the pCambia1304 vector, only 2 survived the selection process. These explants were rooted and acclimatized, and leaf cuttings were collected and tested for GUS activity (Figure 4.1). The tissue from both individuals showed blue coloration when subjected to the GUS assay. Unfortunately, both died in a growth chamber malfunction before DNA was collected for PCR analysis to ensure the inclusion of the test plasmid. Since the pCambia1304 vector does not possess an intron in the gusA gene, it is possible that the blue coloration is due to expression by *A. tumefaciens* still present in the plant. Without PCR results, these individuals could not be confirmed to be transformed.

Table 4.3 Survival of Ultradwarf embryo axis explants throughout transformation and selection

		Number of explants at each stage				
Plasmid	**Initial explants**	**Inoculated**	**20 mg/L Hygromycin**	**40 mg/L Hygromycin**	**60 mg/L Hygromycin**	**Rooting**
pCambia1304	870	754	471	223	29	2
pC1-RKO	2,500	2,153	1,355	606	156	6

Figure 4.1 pCambia1304 putative transformants of Ultradwarf castor

A – GUS stained control sample showing no *gusA* expression; B,C – GUS stained tissue from pCambia1304 putative transformants, the blue coloration indicates *gusA* activity; D,E – Rooted and acclimatizing pCambia1304 putative transformants

Time constraints shifted the focus of the project to insertion of the pC1-RKO vector into Ultradwarf castor. This phase of the project included the transformation of 2,500 Ultradwarf castor embryo axis explants dissected from genetically homogenous breeder's seed. A flow chart comparing the protocol described in this paper and the

protocol as reported by Sujatha and Sailaja (2005) is presented in Figure 4.2. One alteration to the protocol generated with the test plasmid was the addition of 0.02% activated charcoal (w/v) and 10 mg/L silver nitrate to the recovery medium after the final stage of hygromycin resistance screening. This addition was designed to assist in its recovery from the hygromycin selection process by adsorbing ethylene and other inhibiting compounds from the medium and preventing further damage to the explant. Six of the 2,500 original explants (Table 4.3) survived the transformation and screening process (3/25 1a, 3/25 2b, 4/23 1a, 4/23 2b, 4/24 1, and 4/24 4a). All have successfully rooted and been transferred to the greenhouse; however, only 3 (4/23 1a, 4/24 1, and 4/24 4a) of the original 6 are healthy due to a mealybug infestation (Figure 4.3). Analysis of GUS activity of these three individuals initially indicated positive expression of the *gusA* gene (Figure 4.4). However, subsequent examination of mature leaf tissue showed no positive result for *gusA* expression.

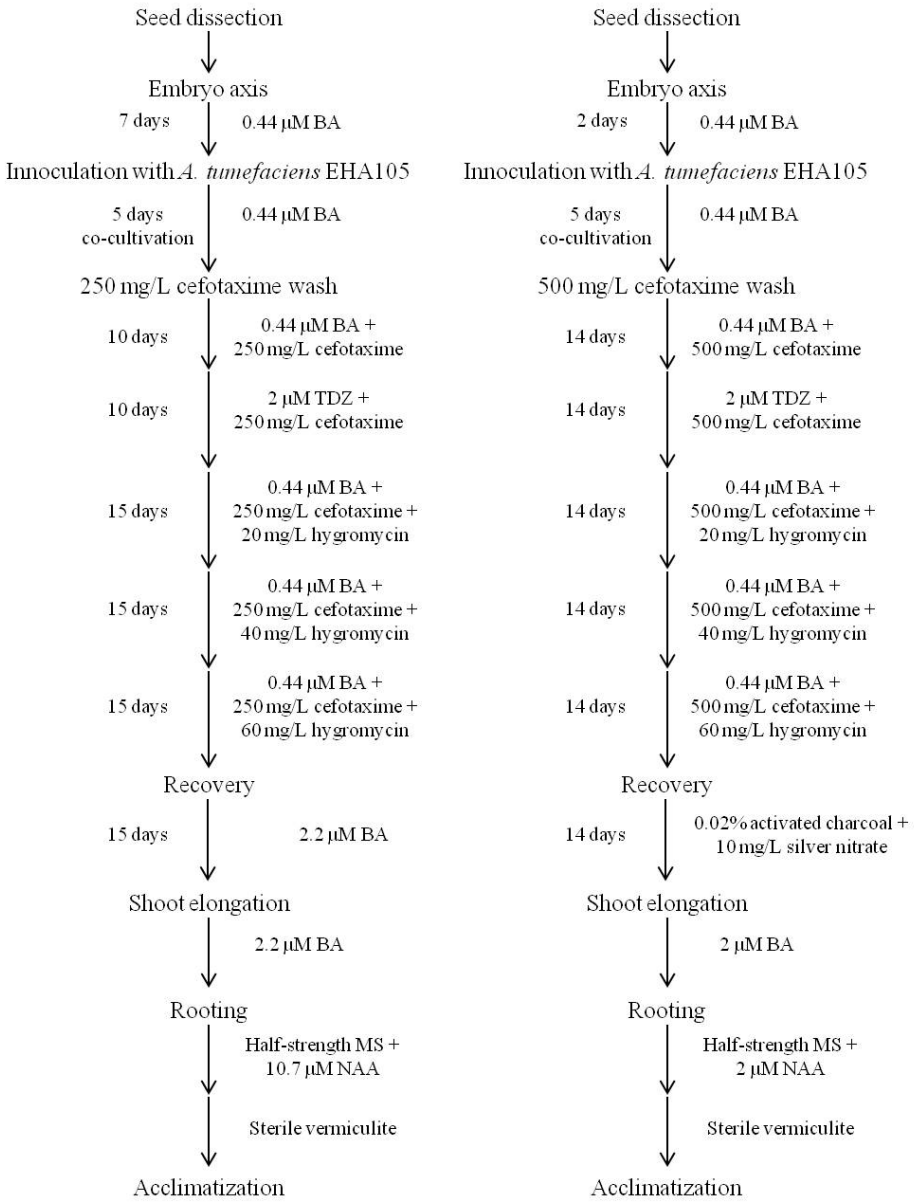

Figure 4.2 Side-by-side comparison of Sujatha and Sailaja (2005) protocol and Barnes protocol

A side-by-side comparison of the protocol reported by Sujatha and Sailaja (2005) [left] and the modified protocol described in this work [right].

Figure 4.3 pC1-RKO-treated Ultradwarf castor individuals maintained in the greenhouse

The 3 healthy, rooted individuals being maintained in the greenhouse

Figure 4.4 Examples of GUS stained tissue from pC1-RKO-treated individuals

Left-most image is of a GUS-stained leaf from a non-transformed control plant. The remaining three are GUS-stained leaves from putative transformants.

As the 6 surviving individuals matured in the greenhouse, DNA samples were extracted from mature leaf tissue for PCR screening. Initial results showed that individual '4/24 1' tested positive for the *hptII* gene via the production of a 465 bp amplicon (Figure 4.5). However, additional screening with multiple DNA samples showed inconsistent results as 2 DNA samples from the same individual showed differing amplification of the 465 bp amplicon while both tested positive for the 320 bp actin amplicon (Figure 4.6). Further analysis of positive '4/24 1' DNA samples with primers designed for pC1-RKO vector backbone (RB span 2, RB prox 1, and RB mid 2) showed the presence of intact non-T-DNA vector sequence via the production of 572 bp, 558 bp, and 588 bp amplicons (Figures 4.7 & 4.8). The primers define amplicons in the following regions: RB span 2 defines a 572 bp region which includes the intact right border sequence, RB prox 1 defines a 558 bp region directly adjacent to the right border sequence on the non-T-DNA side, RB mid 2 defines a 588 bp region 2 kbp from the right border sequence within the non-T-DNA region. While some incorporation of backbone sequence is common due to incomplete cleavage at the left border region during single-stranded DNA generation, the inclusion of all three of the regions probed indicates that the vector is intact (Gelvin 2003). The amplification of the RB span 2 amplicon is especially conclusive as it spans the right border sequence, so amplification of that region indicates intact right border sequences. This indicates that the right border was never cleaved to form single stranded DNA for insertion into the host genome. The false positive was then confirmed via end-point reverse transcriptase PCR (RT-PCR) on RNA extracted from leaf tissue of the 6 individuals (Figures 4.9 & 4.10). The appearance of bands corresponding to RKO and actin mRNA in Figure 4.10 and the absence of bands corresponding to *hptII* mRNA in

Figure 4.9 clearly indicates that the *hptII* gene is not expressed in any of the 6 individuals.

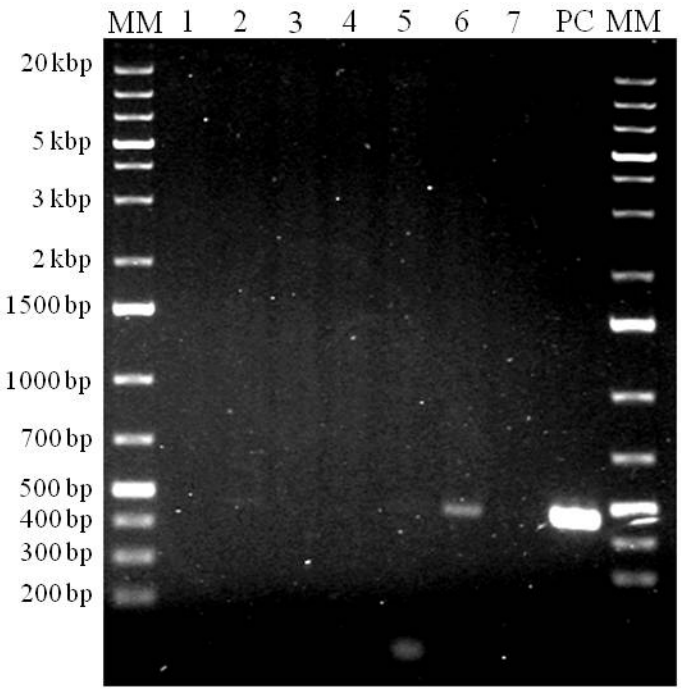

Figure 4.5 PCR amplification of mature leaf DNA from pC1-RKO-treated individuals using *hptII* primers

Each PCR amplification included 100 ng of genomic DNA for the samples or 10 ng of pC1-RKO plasmid DNA for the positive control (PC). Amplifications performed with the *hptII* primer pair, with an expected 465 bp amplicon. Lane 6 contains DNA from '4/24 1' showing a positive band at 465 bp corresponding to the positive control. **MM** - GeneRuler 1kb plus molecular marker (Fermentas; Burlington, Canada); **PC** – pC1-RKO positive control; **1** – non-transformed control castor; **2** – 3/25 1a; **3** – 3/25 2b; **4** – 4/23 1a; **5** – 4/23 2b; **6** – 4/24 1; **7** – 4/24 4a.

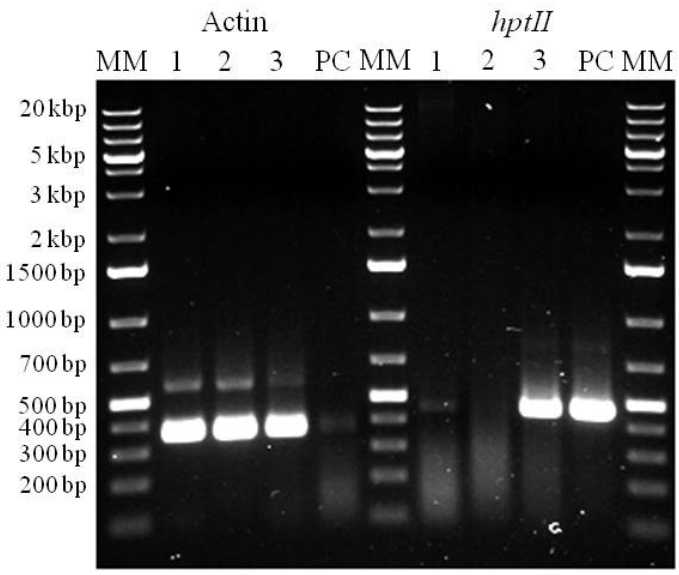

Figure 4.6 PCR amplification of two '4/24 1' mature leaf DNA using actin and *hptII* primers

Each PCR amplification included 100 ng of genomic DNA for the samples or 10 ng of pC1-RKO plasmid DNA for the positive control (PC). Amplifications performed with the actin primer pair with an expected 360 bp amplicon, and the *hptII* primer pair, with an expected 465 bp amplicon. **MM** - GeneRuler 1kb plus molecular marker (Fermentas; Burlington, Canada); **PC** – pC1-RKO positive control; **1** – non-transformed control castor; **2 & 3** – two independent isolations of '4/24 1' DNA.

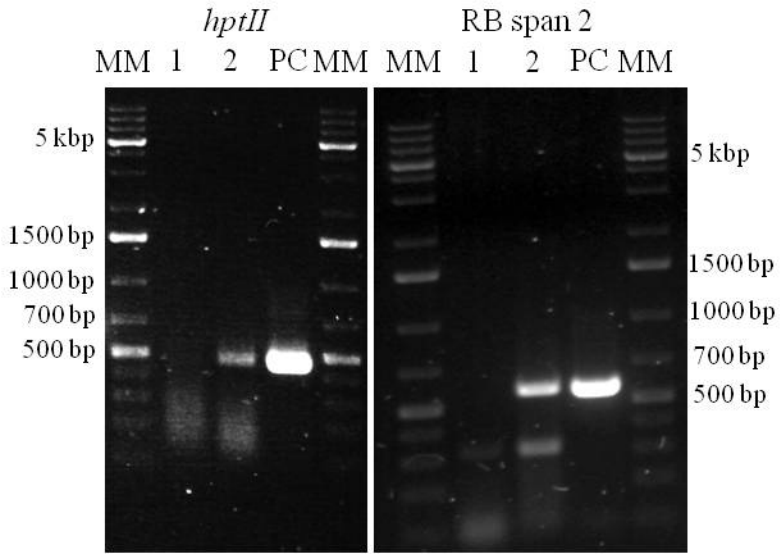

Figure 4.7 PCR amplification of mature leaf DNA using *hptII* and region spanning the right border sequence of pC1-RKO (RB span 2) primers

Each PCR amplification included 100 ng of genomic DNA for the samples or 10 ng of pC1-RKO plasmid DNA for the positive control (PC). Amplifications performed with the *hptII* primer pair, with an expected 465 bp amplicon, and the RB span 2 primer pair, with an expected 572 bp amplicon. **MM** - GeneRuler 1kb plus molecular marker (Fermentas; Burlington, Canada); **PC** – pC1-RKO positive control; **1** – non-transformed control castor; **2** – '4/24 1' DNA.

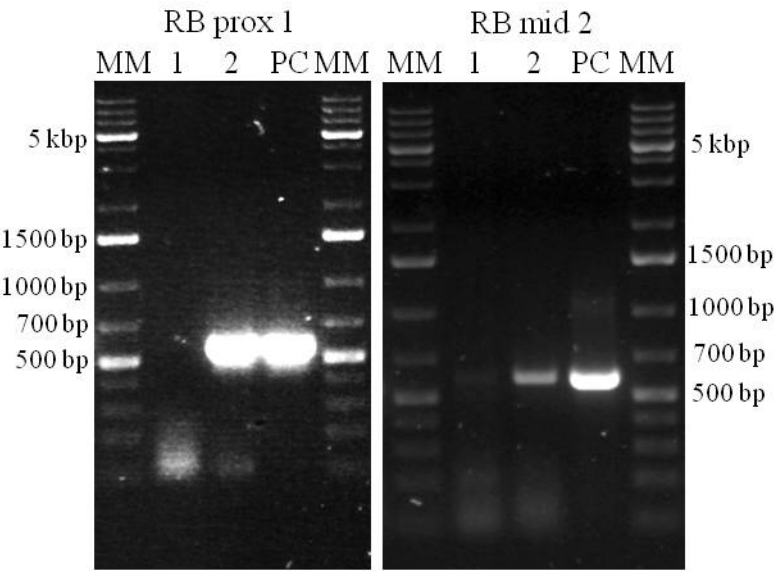

Figure 4.8 PCR amplification of mature leaf DNA using primers specific to regions within the non-T-DNA vector backbone

Each PCR amplification included 100 ng of genomic DNA for the samples or 10 ng of pC1-RKO plasmid DNA for the positive control (PC). Amplifications performed, with the RB prox 1 primer pair with an expected 558 bp amplicon, and the RB mid 2 primer pair, with an expected 588 bp amplicon. **MM** - GeneRuler 1kb plus molecular marker (Fermentas; Burlington, Canada); **PC** – positive control; **1** – non-transformed control castor; **2** – '4/24 1' DNA.

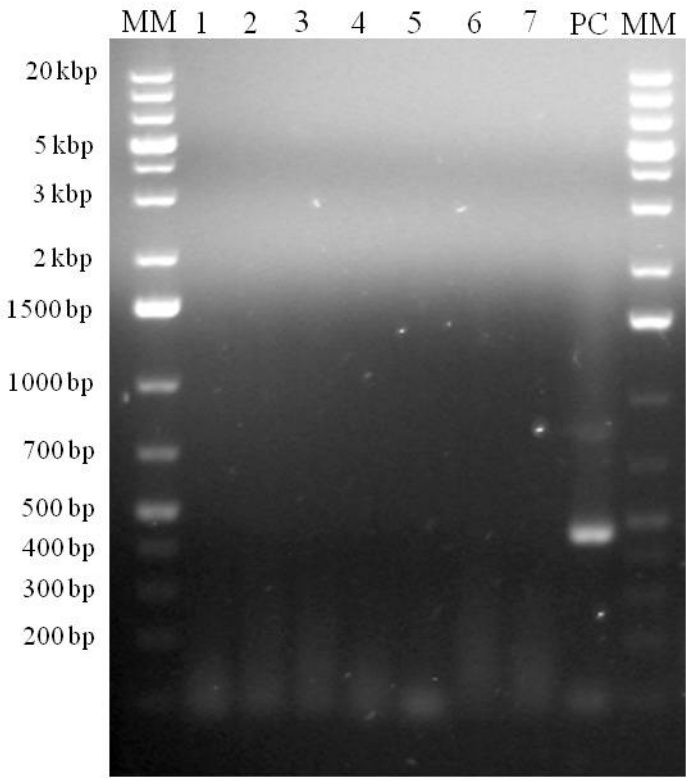

Figure 4.9 End-point RT-PCR of cDNA generated from mature leaf RNA from pC1-RKO-treated individuals using *hptII* mRNA primers

Each PCR amplification included 10 µL of cDNA or 10 ng of pC1-RKO plasmid DNA for the positive control (PC). Amplifications performed with the *hptII* mRNA primer pair, with an expected 465 bp amplicon. **MM** - GeneRuler 1kb plus molecular marker (Fermentas; Burlington, Canada); **PC** – pC1-RKO positive control; **1** – non-transformed control castor; **2** – 3/25 1a; **3** – 3/25 2b; **4** – 4/23 1a; **5** – 4/23 2b; **6** – 4/24 1; **7** – 4/24 4a.

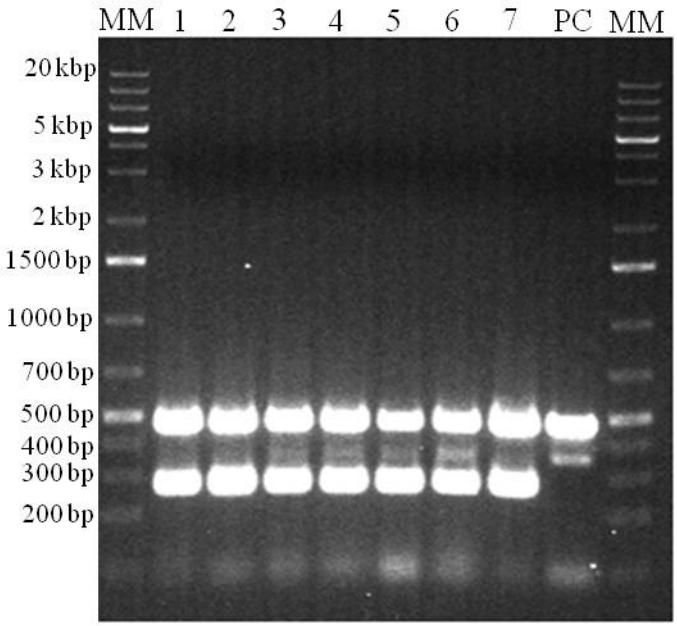

Figure 4.10 End-point RT-PCR of cDNA generated from mature leaf RNA from pC1-RKO-treated individuals using actin and RKO primers

Each PCR amplification included 10 μL of cDNA or 10 ng of pC1-RKO plasmid DNA for the positive control (PC). Amplifications performed with the actin primer pair, with an expected 280 bp amplicon and the RKO primer pair with an expected 473 bp amplicon. **MM** - GeneRuler 1kb plus molecular marker (Fermentas; Burlington, Canada); **PC** – pC1-RKO positive control; **1** – non-transformed control castor; **2** – 3/25 1a; **3** – 3/25 2b; **4** – 4/23 1a; **5** – 4/23 2b; **6** – 4/24 1; **7** – 4/24 4a.

While PCR was being optimized and performed, three individuals (4/23 2b, 4/24 1, and 4/24 4a) produced seed. Western blots and dot-blots using RCA_{60} A-chain specific antibodies were performed on protein extracted from their seed (Figures 4.11 & 4.12). The appearance of bands at 30 kDa corresponding to ricin A-chain in protein extracted from all samples on the Western blot in Figure 4.11 indicates that ricin expression was not disrupted. Furthermore, the dilution series performed via dot-blot in Figure 4.12 indicates that there is no obvious decrease in the amount of ricin present in the seed.

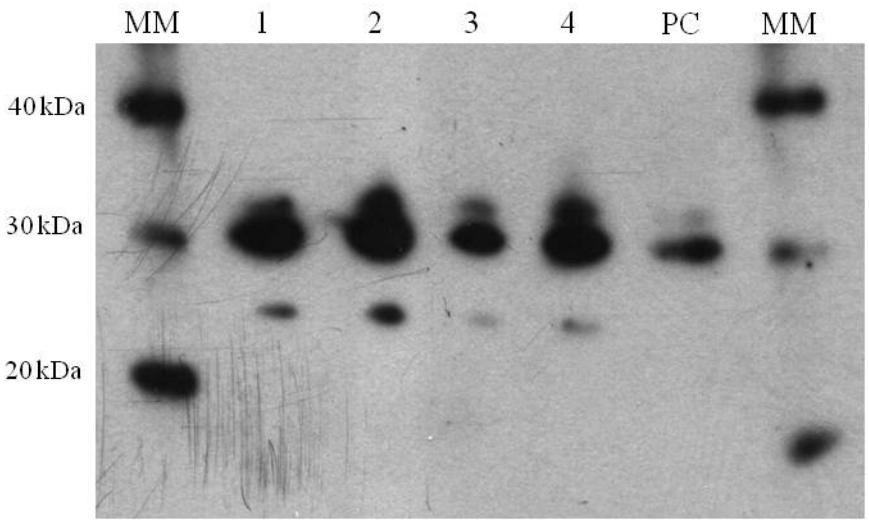

Figure 4.11 Western blot with ricin A-chain specific primary antibodies on protein samples collected from seed of pC1-RKO-treated individuals

All samples were loaded at 50 ng per well. Positive control (PC) loaded at 5 ng per well. Blot probed with 1:1,000 dilution of RCA-52B ricin A-chain specific primary antibody (US Biological; Swampscott, MA) and 1:100,000 dilution of HRP-bound Fc specific secondary antibody (Sigma-Aldrich; St. Louis, MO). Signal was generated with SuperSignal© West Femto detection reagent (Pierce; Rockford, IL) and exposed to X-ray film. MM – MagicMark XP Western standard (Invitrogen; Carlsbad, CA); PC – RCA_{60} positive control (Vector Labs; Burlingame, CA); 1 – non-transformed control castor; 2 – 4/23 2b; 3 – 4/24 1; 4 – 4/24 4a.

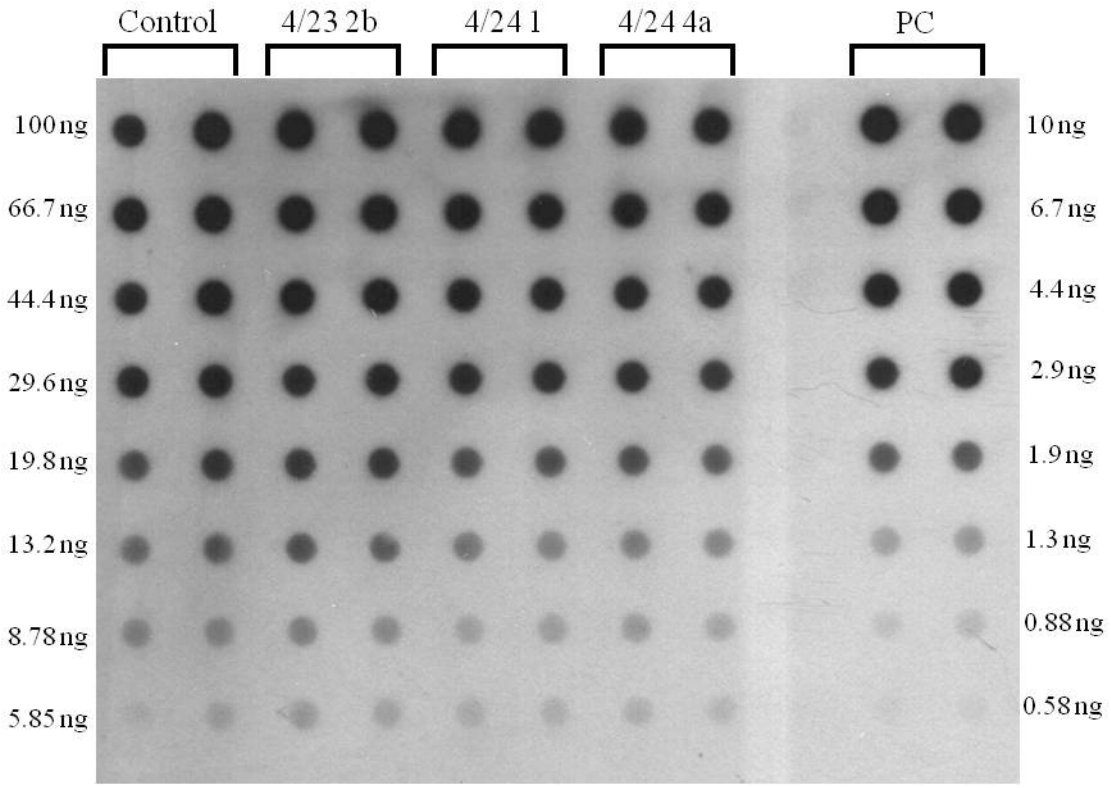

Figure 4.12 Dot-blot with ricin A-chain specific primary antibodies on protein samples collected from seed of pC1-RKO-treated individuals

Samples loaded in a 1.5x serial dilution scheme with duplicate columns as indicated on the image. Blot probed with 1:1,000 dilution of RCA-52B ricin A-chain specific primary antibody (US Biological; Swampscott, MA) and 1:100,000 dilution of HRP-bound Fc specific secondary antibody (Sigma-Aldrich; St. Louis, MO). Signal was generated with SuperSignal© West Femto detection reagent (Pierce; Rockford, IL) and exposed to X-ray film. The intensity of the 'dot' corresponding to each sample indicates the relative amount of ricin contained in that sample. PC – RCA_{60} positive control (Vector Labs; Burlingame, CA)

Discussion

The protocol used in this research did not produce confirmed transformants or ricin-free castor. However, this research has highlighted some interesting points of discussion. This project mainly followed the protocol of Sujatha and Sailaja (2005); however, the modifications that were imposed may have led to a reduction in transformation efficiency. The explants used in this research were only pre-cultured for 2

days compared to the 7 days in the published protocol. This was the case because it was seen that, after *A. tumefaciens* co-cultivation, the older explants did not survive as well. This has been noted in *Jatropha* as well, where younger explants responded better to transformation and were more easily recovered (Mazumdar et al. 2010). One possible reason for this is the accumulation of PGR in the tissues during pre-culture, which may cause the explant to react unfavorably during co-cultivation (Yadav et al. 2012). It may be possible to alleviate this issue by co-cultivation for a shorter period of time. A co-cultivation period of 2-3 days has been shown to be optimal for a variety of species (Gelvin 2003; Yadav et al. 2012). Therefore, a study of shorter co-cultivation periods with explants of varying ages may provide a more optimal transformation protocol. Additionally, it has been shown that cooler temperatures at the beginning stages of co-culture may increase transformation rates. This is because the pillus of some *Agrobacterium* strains is more stable at lower temperatures (Gelvin 2003). The Sujatha and Sailaja (2005) protocol stated a resting period between co-cultivation and selection of 20 days compared to the 28 days used in this protocol. Typically a pre-selection period of only 7-10 days is used (Negrotto et al. 2000; Khan et al. 2003; Chaudhry and Rashid 2010). This elongated resting period may have contributed to the 'escapes' produced by this protocol as the explants were more developed when exposed to the selection medium and possibly more apt to survive selection without the *hptII* gene. Activated charcoal and silver nitrate were added to the recovery medium for this protocol. This was performed to enhance rooting and may explain why Sujatha and Sailaja (2005) had to use 10.7 µM NAA for root induction as opposed to the 2 µM NAA used in this research. However, it may have had unknown negative effects on the transformation efficiency when used in

the recovery media, although silver nitrate has been shown to have positive effects on *Agrobacterium*-mediated transformation when used during co-culture (Opabode 2006).

The false positives generated in this research are worth examining. Initial GUS assays showed positive blue coloration indicating *gusA* expression. However, later analysis showed no GUS activity. This may be explained by the interaction of phenolic compounds in the sample with the azocoupling reaction that produces the blue coloration (Vitha et al. 1995). The young leaf samples that exhibited GUS activity may have been generating a different range of phenolic compounds leading to a false positive. The PCR false positive is more troublesome; however, there are some reasonable theories as to what caused it. It is not uncommon for non-T-DNA vector backbone sequence to be incorporated into the host genome (Gelvin 2003). However, the fact that the right border sequence-spanning amplicon was intact indicates that the vector providing the false positive result was intact and not integrated into the host genome. One explanation for the false positive is that intact pC1-RKO vector somehow cross-contaminated the PCR reaction. This is unlikely since DNA was extracted from the individual on several separate occasions and fresh dilutions were performed completely separate from the positive control prior to PCR. Additionally, filtered pipet tips were used to prevent pipet-barrel contamination, and samples were prepared in randomized order to prevent bias should sample handling be the issue. Another explanation is that *A. tumefaciens* harboring the pC1-RKO plasmid is still present in the plant despite cefotaxime treatment. This has been reported even at concentrations of 500 mg/L cefotaxime as used in this research (Barrette et al. 1997). In fact, this is a major concern for the plant transformation

industry as *A. tumefaciens* harboring a gene or interest represents a risk for gene escape into the environment.

Summary

The final stage of this project examined a putative protocol for *Agrobacterium tumefaciens*-mediated transformation of Ultradwarf castor. The test plasmid pCambia1304 was used to troubleshoot issues with the existing protocol described in the literature. It was found that exmbryo axis explants pre-cultured for 2 days, co-cultivated for 5 days, and decontaminated with 500 mg/L cefotaxime produced the most successful conditions for the recovery of explants after transformation. Using these modifications, 2 of 870 embryo axis explants treated with pCambia1304 showed GUS activity. This protocol with slight modification was used with the RNAi vector pC1-RKO on 2,500 explants, generating 6 putative transformants. Further analysis showed these to be non-transformed and ricin content in the seed unaffected.

CHAPTER V

CONCLUSION

Summary

The goal of this project was to develop transgenic castor in which the ricin gene had been genetically silenced for the purpose of encouraging castor cultivation within the United States. The primary objectives set in place to reach this goal were: 1.) the construction of an RNA interference vector, 2.) the successful generation of a protocol for castor tissue culture, and 3.) the genetic transformation of castor with the RNA interference vector. The RNA interference vector was designed and successfully constructed based on the framework of the pCambia1301 vector using the *chsA* intron from pFGC5941 flanked by inverted repeats of an element designed using a multiple sequence alignment of several putative ricin genes. Tissue culture protocols were examined to produce a reliable procedure for shoot generation from castor seed explants. Challenges to this process were the presence of fungal contaminants within the explants, the non-uniformity of the source seed, and the senescence of explants in culture before roots were established. These challenges were resolved by the use of 0.1% mercuric chloride (w/v) for seed disinfestation, the use of genetically homogenous breeder's seed as the source for the explants, and the incorporation of activated charcoal into the medium prior to rooting. The transformation protocol for this project was adapted from work reported by Sujatha and Sailaja (2005). Embryo axis explants pre-cultured for 2

days responded better after inoculation with *A. tumefaciens* compared to explants pre-cultured for 5 and 7 days. Inoculation with concentrations of *A. tumefaciens* exceeding an $O.D._{600}$ of 0.5 resulted in downstream contamination. Likewise, 500 mg/L cefotaxime successfully decontaminated the explants after co-cultivation. Explants surviving hygromycin screening recovered more quickly when placed on MS medium supplemented with 0.02% activated charcoal (w/v) and 10 mg/L silver nitrate. Two of the 870 explants treated with *A. tumefaciens* containing the pCambia1304 test vector survived transformation and hygromycin screening. Six explants treated with *A. tumefaciens* containing the pC1-RKO RNAi vector survived hygromycin screening. All 6 have been successfully rooted though only three specimens remain healthy. Initial PCR results indicated a positive transformant, '4/24 1'. However, further PCR, RT-PCR, and Western and dot-blotting indicate that there was no transformation and no ricin knockout.

Future Work

This research has developed a potential RNAi vector for ricin knockout in castor. Further work is needed to successfully transform this plasmid into castor in order to classify its effect on ricin expression. The first step should be refinement a more efficient protocol for castor micropropagation. The work of Kumari et al. (2008) may provide a valuable tool for the induction of somatic embryogenesis in castor. This may lead to more homogeneous and rapidly accessible castor tissue culture lines, allowing for more rapid protocol development for castor transformation. In addition to ricin knockout, there are several other potential uses for castor transformation. The most obvious may be the production of different compositions of oils from castor seed. Castor seed produces large amounts of oil, and its principal oil, ricinoleic acid, is valuable. However, there may be a

market for the production of more unique oil compositions in the large amounts that castor is already capable of producing. Additionally, the removal of the allergenic components of castor seed is of interest.

Further research into ricin knockout via RNAi is warranted; however, multiple knockout elements should be tested, and control under different promoters should be analyzed. This work did not seek to use a random RNAi sequence control to adjust for any off-type effects due to the efforts required to get even one plasmid transformed into castor. However, this research should be performed in order to assess the effect of the insertion of an RNAi element itself. As mentioned previously, insertion of DNA into the host genome often occurs at transcriptionally active sites, which may disrupt the function of important genetic elements. These effects should be accounted for with proper controls.

Commercial Outlook

Though castor oil is already a multi-million dollar market in the United States and a multi-billion dollar market globally, domestic production has failed to reinitiate. India, China, and Brazil produce most of the world's castor oil and, as the industry grows, prices grow and are controlled by these countries (FAOSTAT 2014). In order to encourage castor cultivation in the United States, the market needs to be stabilized. Currently soy (*Glycine max* (L.) Merr.) is the major biodiesel crop with corn (*Zea mays* L.) leading the bioethanol feedstocks (Demirbas 2008). Castor will need to compete with these crops and others such as rapeseed (*Brassica napus* L.) and cotton (*Gossypium hirsutum* L.) in order to find a place in United States production. The major market pressures against castor reintroduction are capital costs and the existence of strong,

established competition in the soy and corn markets. One of the primary stimuli for the reintroduction of U.S. castor production would be the suppression of ricin within the seed. This would potentially lead to a value-added by-product as the meal could be used as animal feed (Bris and Algeo 1970; Vilhjalmsdottir and Fisher 1971). The elimination of ricin would also potentially reduce public perception of castor as a hazardous crop. Backing by agricultural groups would likely follow, precipitating an influx of available field space and cultivation equipment. Once castor is seen as a viable income to farmers, cultivation would expand and take its place in the domestic market. Current pressure from the competitive markets could potentially suppress cultivation. However, castor does not compete with edible oilseed crops, and it produces high quantities of chemically valuable oil. These facts may help to establish castor in the market. When castor cultivation has been reintroduced, research will be needed in order to upgrade the technology used to extract oil. This could potentially allow castor oil to enter the domestic biofuels market as a stronger source than it presently is. With these factors combined, castor could find its place in domestic agriculture and help alleviate some of the country's dependence on foreign markets.

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