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Cocoa

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1. INTRODUCTION

Cacao, *Theobroma cacao* L., is a small understory tree believed to have evolved in the lowland rainforests of the Amazon basin (Wood and Lass, 1985; Bartley, 2005). Today, cacao is grown throughout the humid tropics, often in agroforestry ecosystems with other fruits and commodity crops. The production of dried seeds of cacao (cocoa beans) supports an international market of approximately 3 million tons, with two-thirds being processed into cocoa powder and cocoa butter, and the remaining one-third used for cocoa liquor (the flavor and color component of chocolate) (Wood and Lass, 1985). Cocoa is the major export commodity of several countries in West Africa (68% of world production), including Ivory Coast, Cameroon, Nigeria, and Ghana. Other major cocoa exporters include Ecuador, Venezuela, Brazil, Panama, Costa Rica, Dominican Republic, Malaysia, and Indonesia. Worldwide, approximately 5–6 million small-holder farmers grow 95% of the worlds' production providing an important source of cash to otherwise primarily subsistence farmers. World cocoa export commerce is $5 to $6 billion/year and in the United States alone, the use of cocoa and cocoa butter in chocolate manufacturing, cosmetics, and other products drives an approximately $70 billion dollar market providing over 60 000 jobs (Morais, 2005). In addition, US chocolate production also uses large amounts of sugar, nuts, and milk valued at approximately $3 billion per year in receipts to American farmers.

Cacao is grown in some of the world's most important biodiversity hotspots (Piasentin and Klare-Repnik, 2004). With a long life cycle, predominantly cultivated under shade trees, there are many environmental benefits of cacao cultivation such as watershed and soil conservation, provision of habitat for birds and other animals, and provision of buffering habitat for protected wild rainforest regions (Rice and Greenberg, 2003; Ruf and Zadi, 2003). With these benefits in mind development agencies such as United States Agency for International Development (USAID), Conservation International (CI), The World Wildlife Federation (WWF), and the World Cocoa Foundation have formed alliances to promote sustainable cacao production (Guyton *et al.*, 2003).

Overwhelmingly, plant pathogens present the major challenge to sustainable cacao farming throughout the world. It is estimated that pests and diseases reduce the potential crop by an estimated 810 000 t annually (30% of world production) and individual farm losses can approach 100% (Keane, 1992; Bowers *et al.*, 2001). In one example, Witches' broom disease resulted in a decrease of production in Southern Bahia, Brazil, from 300 000 t in 1989 to 130 000 t 10 years later, for an estimated loss of $220 million each year (Pereira *et al.*, 1990). This caused a cascade of widespread social disruption among small-holder growers

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potassium nitrate and 2.5 μM the amino acids arginine, glycine, leucine, lysine, and tryptophan increased the conversion rate to 92%. In addition to the media composition, it was observed that the morphological characteristics of the mature embryos are important factors for the success of the conversion. Two different classes of embryos (I and II) were first described by Li *et al.* (1998) based on morphological appearance. Type I embryos displayed yellow translucent color and larger cotyledons compared to type II embryos that had ivory white color and small cotyledons. The conversion of type II embryos resulted in higher conversion rate to plants. Plants produced by this method were transplanted to soil in a greenhouse with 100% humidity and 70% shade (approximately 100 $\mu mol\, m^{-2}\, s^{-1}$). Humidity was maintained by misting for 10 s every 15 min. After 4 weeks the misting was omitted and plants were successfully maintained to maturity at 60–65% humidity with 50% shade (250–500 $\mu mol\, m^{-2}\, s^{-1}$) (Maximova *et al.*, 2005). Plants produced by different genotypes are in the process of field evaluation in Saint Lucia, Ecuador, and Puerto Rico.

2.2 SE Research and Genetic Transformation Studies of Cacao at the Malaysian Cocoa Board, COCOA Biotech Center

Various explant sources (leaf, stem, young shoots, seed, flowers, etc.) from different commercial cocoa clones planted in Malaysia were evaluated for their potential for SE using previously published protocols (Aguilar *et al.*, 1992; Figueira and Janick, 1995; Alemanno *et al.*, 1996). From these, only seed, cotyledon tissue, and flower organs regenerated somatic embryos (T.C. Lock, unpublished data). Young cotyledon tissues (white to pale purple color) were more reactive compared to older cotyledon tissues (T.C. Lock, unpublished data). Although somatic embryos were produced from cotyledons, this source was not suitable for clonal propagation due to the fact that cotyledon tissue is a product of fertilization and is genetically different from the source materials. Further experiments were conducted with flower parts—anther filaments, staminodes, and petals (Tan and Furtek, 2003). Similar to the results reported by Li *et al.* (1998), the best regeneration was recorded for staminode tissues (Figure 1). The genotype of cocoa was also an important factor for the success of embryogenesis as genotype-specific variations in the response were observed. Additionally, the time of the year when flowers were collected from the field and introduced in culture affects the embryo production (T.C. Lock, unpublished data). It was observed that after occurrence of a distinct dry period followed by a wet season, floral tissues performed better during SE. However, there is no actual data recorded that correlate the climatic conditions with embryogenesis rate and future experiments need to be conducted to verify these observations.

For induction of PSE, unopened flower buds (4–6 mm in length) were collected from the field and rinsed with tap water. The flowers were then sterilized in 70% ethanol for 5 min followed by 20 min in 10% commercial bleach (5.25% (v/v) sodium hypochlorite) and 0.10% sodium dodecylsulfate (SDS) solution, and 20 min in 1% bleach and 0.10% SDS solution. Buds were rinsed three times for 5 min in sterile reverse osmosis water. Staminodes were dissected in sterile conditions and cultured on somatic embryo induction medium (MIM41n). MIM41n consisted of DKW basal salt (Duchefa, Biochemie BV, The Netherlands), 20 $g\, l^{-1}$ glucose, MS vitamins, 2 $mg\, l^{-1}$ of 2,4-D, 0.1 $g\, l^{-1}$ N^6-[2-isopentenyl]adenine (2-iP), 500 $mg\, l^{-1}$ glutamine, and 2 $g\, l^{-1}$ Phytagel (Sigma), pH 5.8. Calli were formed after 2–3 weeks of culture on MIM41n media (Figure 1b). After 4–8 weeks, the calli were transferred to embryo differentiation media (MEM22a) consisting of DKW basal salts, MS vitamins, 50 $mg\, l^{-1}$ and 2 $g\, l^{-1}$ Phytagel, pH 5.5. Newly formed somatic embryos (Figure 1c) were separated from the calli and subcultured onto differentiation media for another 4–6 weeks. Secondary embryos (Figure 1d) were formed spontaneously on MEM22a medium and after culture on MEM22a supplemented with 0.01 $mg\, l^{-1}$ 2-iP and 0.01 $mg\, l^{-1}$ kinetin. The mature somatic embryos produced were approximately 40 mm long with distinct shoot and root apices. These were introduced into conversion media (MGM) consisting of one-fifth strength DKW basal salts, 5 $g\, l^{-1}$ sucrose, 10 $g\, l^{-1}$ glucose, 100 $mg\, l^{-1}$ myo-inositol, 2 $mg\, l^{-1}$ glycine, 2.2 $g\, l^{-1}$ Phytagel (Sigma), pH 5.8. MGM medium was dispensed onto 250 ml glass jars

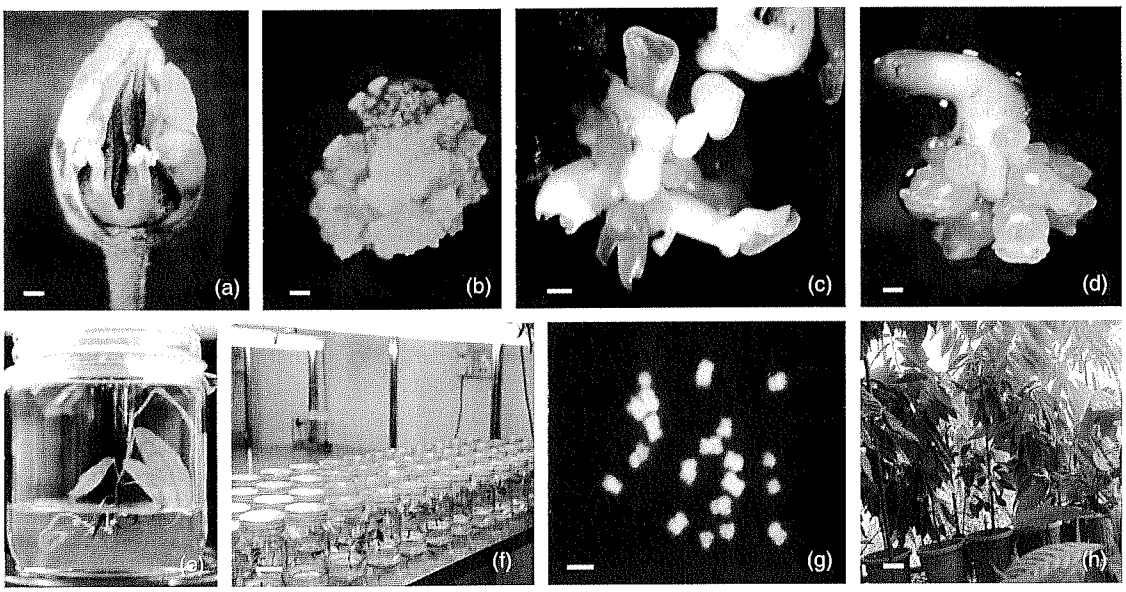

Figure 1 Somatic embryogenesis of *Theobroma cacao*. (a) Unopened flower bud, scale bar = 1.6 mm, (b) callus from staminode, sale bar = 1 mm, (c) primary somatic embryos from callus, scale bar = 2 mm, (d) secondary somatic embryos from primary embryo, scale bar = 0.9 mm, (e) plantlet from somatic embryo, scale bar = 10 mm, (f) plantlets in light culture room, scale bar = 5 cm, (g) cytological analysis of root tips from plantlet, scale bar = 10 mm, (h) somatic embryo derived plants established under *ex vitro* conditions, scale bar = 21 cm

(Biocraft, Singapore) and covered with Suncaps (Sigma) for ventilation. The conversion cultures were kept under light (16 h photoperiod, daylight fluorescent tubes, 25 mmol m^{-2} s^{-1}) at 25 ± 2 °C (Tan and Furtek, 2003). Roots usually appear after 3 weeks on conversion media followed by leaf development (approximately 4 to 8 weeks on conversion media). Once a healthy root system and dark green leaves were developed, the plantlets were transferred to *ex vitro* conditions (Figures 1e and f). Embryogenesis was most successful for genotypes QH1560, KKM19, PBC137, and KKM4 (commercial clones in Malaysia). The percentages of embryos producing explants (0% to 50%) varied greatly with the season and age of the source plants, and the genotypes. Between 5% and 15% of the somatic embryos were converted to plantlets.

To test for mutations and somaclonal variation as a result of the SE, SE-derived plants were analyzed and compared to the explant source plants by molecular fingerprinting and morphological characterization. Two- to three-year-old somatic embryo-derived plants and their mother plants were fingerprinted using 39 different microsatellite markers (T.C. Lock, unpublished data). Genomic DNA was isolated from young, fully expanded leaves from somatic embryo-derived plants and mother plants using DNeasy 96 Plant kit (Qiagen). Polymerase chain reaction (PCR) amplification of the target sequences was performed using Applied Biosystems 96-Well GeneAmp® PCR System 9700. Each reaction contained 1U of *Taq* polymerase, 10 ng cocoa DNA, 0.2 mM dNTP mix, 2 mM $MgCl_2$, 1× reaction buffer, and 2 pmol of fluorescently labeled primers in total volume of 20 µl. The PCR conditions applied were: 94 °C for 4 min. followed by 30 cycles of 30 s at 94 °C, 30 s at 50 °C, and 1 min at 72 °C. One to 10 dilutions were made from each PCR product and 1 µl from each sample was mixed with 1 µl ROX in an individual tube. The samples were denatured at 95 °C for 3–5 min, cooled on ice, and fractionated by capillary electrophoresis using Amersham MegaBACE 500 analysis system. The SSR profiles were analyzed using GENOTYPER software (Applied Biosystems, Foster City, CA). The genotyping analysis demonstrated no differences between the somatic embryo-derived plants and the flower source plants. Additional cytological

analysis of root tips from the somatic embryo-derived plants and the source plants revealed an equal chromosome count of $2n = 20$ (Figure 1g) (T.C. Lock, unpublished data). Preliminary morphological examination of leaves and flowers also showed no differences from the mother plants (Figure 1h).

Agrobacterium-mediated transformation experiments were conducted with untreated staminode tissue, 3-week-old calli from staminodes, zygotic and somatic embryo pieces. The *Agrobacterium* strain used in the transformation was AGL1 carrying vector pGPTV-Kan: contains neomycin Phosphotransferase II (*npt*II) (kanamycin resistance) gene and β-glucuronidase (*GUS*) reporter gene (Becker *et al.*, 1992). The *Agrobacterium* was incubated for 48 h at room temperature (approximately 28 °C) in 523 medium with 50 mg l^{-1} rifampicin, 50 mg l^{-1} tetracycline, 50 mg l^{-1} carbenicillin, and 100 μM acetosyringone. The calli were inoculated with the *Agrobacterium* suspension (1.0 OD at 600 nm) for 30 s, blotted on sterile paper towels, and were then cultured on MEM22a medium with mg l^{-1} cefotaxime and 250 mg l^{-1} augmentin for 7 days. Subsequently, the calli were transferred to MEM22a medium with 250 mg l^{-1} cefotaxime, 250 mg l^{-1} augmentin, and 100 mg l^{-1} paromomycin for selection. After approximately 5 weeks, the calli and newly developed somatic embryos were histochemically stained for GUS expression. The blue color observed indicated the development of putatively transformed calli (Figure 2a). Additionally, the formation of chimeric somatic embryos was observed (Figure 2b) (Tan *et al.*, 2001), which could be due to the possible multicellular origin of the primary embryos (Maximova *et al.*, 2002). Similar results were observed after transformation of callus derived from zygotic and somatic embryo pieces (data not shown). Additionally, biolistics transformation was carried out using pVDH65 and 3-week-old staminode calli using BioRad PDS-1000/He system. The vector construct used included a triple cauliflower mosaic virus (CaMV) 35S promoter fused to a *GUS* gene (Tan *et al.*, 1999). The GUS histochemical staining resulted in the formation of approximately 40–50 blue spots per 3 mm^2 of callus as an indicator of transient expression. The number of blue spots observed decreased over time and no differentiation of transgenic somatic embryos was recorded.

2.3 *Agrobacterium*-Mediated Cacao Transformation: Protocol Development and Expression of Genes with Potential Agronomic Importance

2.3.1 Genetic transformation protocol development

Prior to year 2000 only one research publication could be found reporting attempts at cacao transformation. In this study, cacao leaf strips were inoculated with *Agrobacterium tumefaciens* carrying a vector with GUS and *npt*II genes (Sain *et al.*, 1994). The experiment resulted in the formation of transformed callus cells, but no transgenic plants were regenerated. In 1996, the Pennsylvania State University Cacao group began a program dedicated to the development of a reliable genetic transformation protocol based on the recently established SE tissue culture system described above. The development and optimization studies included evaluations of different vectors and reporter genes, various selective antibiotics, different somatic embryo tissues,

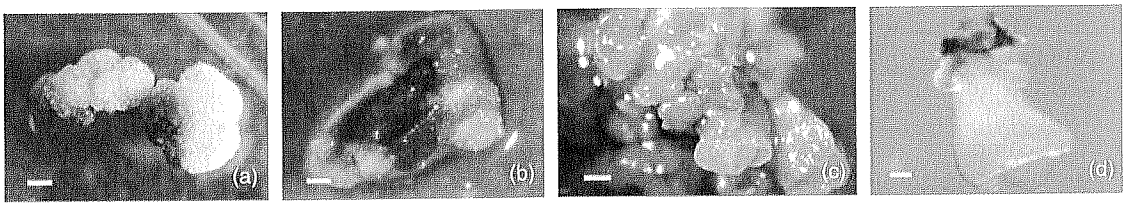

Figure 2 Genetic transformation of *Theobroma cacao* L. (a) Partially transformed callus, scale bar = 0.9 mm, (b) putative transformed callus, scale bar = 0.9 mm, (c) putatively transformed somatic embryos, scale bar = 0.8 mm, (d) partially transformed somatic embryo, scale bar = 0.7 mm

and the potentials of particle bombardment and *Agrobacterium*-mediated methods (Guiltinan and Maximova, 2000; Traore, 2000; Antunez de Mayolo *et al.*, 2003; Maximova *et al.*, 2003). To assess the potential of the biolistic method, cells grown in suspension cultures and somatic embryo cotyledon tissues were bombarded with 1 μm gold particles in a helium-driven PDS-100/He system (Traore, 2000). The gold particles were coated with pBI221 DNA containing *GUS* and *npt*II genes (De Block *et al.*, 1984). Three days after treatment GUS expression was evaluated and large numbers of blue spots were observed in the callus and the cotyledon tissue. However, no embryos were ever regenerated from these experiments (Traore, 2000). This method is considered a useful and quick method to check for construct expression in cacao cells prior to use in further attempts at stable transformation, but highlighted the need for improvement of cacao regeneration systems.

Thus, after several years of optimization of the SE system, attempts were made for *Agrobacterium*-mediated transformations using somatic embryos as explant sources. A major contribution to the development of the protocol was the utilization of the green fluorescent protein (GFP) reporter gene (Chalfie *et al.*, 1994). The ability to observe green fluorescence as an indication of transformation events within 48 h after *Agrobacterium* inoculation greatly increased the early screening capabilities (Maximova *et al.*, 2003). Additionally, the incorporation of GFP allowed the nondestructive observation of the explants throughout the entire culture period including the detection of the transgenic somatic embryos that were regenerated in low frequencies. This permitted relatively rapid optimizations of the many variables in the protocol.

Another important variable optimized was the selective antibiotic used in the regeneration medium. To select the best antibiotic for *npt*II resistance of transgenic cacao cells, the effect of different types of aminoglycoside antibiotics including paromomycin, neomycin, kanamycin, and geneticin at concentrations of 125, 150, 175, and 200 mg l^{-1} was evaluated. The number of GFP expressing explants and the total number of GFP expressing areas were recorded at 1, 2, and 3 weeks after bacterial inoculation. The highest percentage of expressing explants (49%) and the highest total number of GFP areas (64) was observed after application of geneticin at 125 mg l^{-1} (S.M. Maximova and M.J. Guiltinan, unpublished data). Thus, this antibiotic was selected for further experiments subsequently adjusted to a concentration of 50 mg l^{-1} for 2 weeks after inoculation (Maximova *et al.*, 2003). A similar approach was exploited for selection of the *Agrobacterium* counter-selection antibiotic (Antunez de Mayolo *et al.*, 2003). For this study, it was necessary to evaluate the effect of the applied antibiotics on SE and in eliminating *Agrobacterium* in the regeneration culture after the initial co-cultivation period of 48 h. Different antibiotics were supplemented to the regeneration medium in four different concentrations (100, 150, 200, and 300 mg l^{-1}). The antibiotics included: from the cephalosporin class—cefotaxime and moxalactam, and from the penicillin class—amoxicillin and carbenicillin. The percentage of explants lost to *Agrobacterium* overgrowth was evaluated in addition to the effect of the antibiotic type and concentration to the average number of embryos produced in the absence of *Agrobacterium* inoculation. As a result moxalactam at 200 mg l^{-1} was selected as the most promising antibiotic that assured that no explants were lost to bacterial overgrowth and in fact increased the average number of primary and secondary embryos produced per explant by threefold compared to no antibiotic controls (Antunez de Mayolo *et al.*, 2003). This increase of regeneration efficiency could be due to a possible auxin response of the tissue to one of the several breakdown components of moxalactam, phenylacetic acid, which is a naturally occurring auxin (Webber and Yoshida, 1979).

As a result of combining high efficiency SE with *Agrobacterium*-mediated transformation using a GFP reporter gene, transgenic *T. cacao* plants were obtained and grown to maturity (Maximova *et al.*, 2003). With optimization of the protocol, the numbers of transgenic plants recovered from a single experiment increased from one in early experiments to a high of 10 independent transgenic plants. All binary vectors utilized included the Bin19 backbone (Bevan, 1984), *npt*II (De Block *et al.*, 1984) and EGFP (Clontech, Palo Alto, CA) genes both under the control of E12-Ω constitutive promoter, a very highly expressed derivative of CaMV 35S promoter (Mitsuhara *et al.*, 1996). Additionally, pGH00.0131 included

Tobacco Matrix Attachment regions (MARs) (Spiker and Thompson, 1996) flanking the transfer DNA (T-DNA) region. The presence of the MARs contributed to increased GFP fluorescence in primary transgenics and stable and uniform GFP expression among the tertiary somatic embryos propagated from the cotyledons of primary transgenics (Maximova *et al.*, 2003). The physiological and morphological observations of the transgenic cacao plants produced indicated that genetic transformation events and transgene expression did not cause any detectable pleiotropic phenotypic changes. On occasion gene silencing was observed during reiterative embryogenic multiplication (S.M. Maximova and M.J. Guiltinan, unpublished data). Many mature somatic embryos were converted to plants and transferred to greenhouse conditions for further evaluation. Ten transgenic plants from the first established transgenic lines were closely observed during a period of 5 months and their growth parameters were recorded and compared to ten nontransgenic somatic embryo-derived control plants (Maximova *et al.*, 2003). The parameters measured were height, stem diameter, number of leaves, and total leaf area. No significant differences were recorded among the plants for any of the parameters. After approximately 1.5 years of growth in the greenhouse, the transgenic plants developed flowers exhibiting bright GFP expression. The flowers were pollinated with pollen from nontransgenic *T. cacao* genotype Catongo. The pods produced from this crosses exhibited high levels of GFP fluorescence of all maternal pod tissues including pod surface, exocarp, placental tissues, and seed coat. Very high levels of GFP fluorescence were observed in the cotyledons and the embryos of the transgenic seeds. The GFP gene in the T_1 generation from several of the transgenic plants segregated as a single locus insertion (1:1 segregation ratio).

2.3.2 Expression of cacao *TcChi1* gene in transgenic cacao

As discussed in the introduction, cacao pests and diseases are major constraints to sustainable cacao production, and many of these are fungal pathogens. With this in mind, the first gene with potential agronomic importance that was introduced into cacao was a class I chitinase gene (*TcChi1*), encoding a potential antifungal protein (Maximova *et al.*, 2006). The *TcChi1* gene isolated from *T. cacao* encodes an endochitinase protein (NCBI accession U30324 (Snyder, 1994; Snyder-Leiby and Furtek, 1995)). The gene/protein belongs to the pathogenesis-related (PR) protein family and it was demonstrated to be expressed in cacao fruit in response to fungal elicitor treatment (Snyder-Leiby and Furtek, 1995). As a test of its putative function in pathogen resistance, it was hypothesized that the ectopic overexpression of this gene in cacao would contribute to increased resistance to fungal pathogens. The coding sequence of the chitinase gene was placed under the control of the highly expressed constitutive E12-Ω promoter (Mitsuhara *et al.*, 1996), the same used to drive the EGFP and *npt*II genes as described above. The gene was introduced into cacao via the SE co-cultivation system described above. Transgenic embryos were produced and converted to plants (Maximova *et al.*, 2003). Clonally propagated plants from nine transgenic lines were characterized for transgene integration and expression, and *in vitro* and *in vivo* chitinase activities were measured (Maximova *et al.*, 2006).

Southern genomic DNA blot analysis revealed multiple transgene insertion sites in eight of the lines, and one line with a single insertion. Expression of the *TcChi1* transgene was verified by Northern blot (Maximova *et al.*, 2006) and by quantitative real-time reverse transcription polymerase chain reaction (Q-PCR) analysis. For Q-PCR total leaf RNA was extracted from control PSU-Sca6 plants, three chitinase transgenic lines (T44.1, T56.2, and T61.7), and from one GFP transgenic control line (T-31-GFP) (Maximova *et al.*, 2006). RNA (400 ng) from each sample (three individual samples per transgenic line) was treated with DNAse 1 (0.2 units) (#2222, Ambion, Austin, TX) in 20 µl total reaction volume with 60 ng of yeast tRNA (Invitrogen, Carlsbad, CA) by the manufacturer's instructions. The reactions were incubated for 30 min at 37 °C, then for 5 min at 65 °C, and immediately placed on ice. The DNAse treated RNA (50 ng) was added to High Capacity cDNA (complementary DNA) Archive Kit mix (#4322171, Applied Biosystems, Foster City, CA) to a final volume of 20 µl and the reverse transcription reaction was performed by the manufacturer's instructions.

Gene specific primers were synthesized at the Penn State Nucleic Acid Facility with a MerMade12 automated DNA synthesizer (Bioautomation, Plano TX). Gene specific fluorescent probes were synthesized by Biosearch Technologies (Novato, CA). The fluorescent label used at the 5′ end of all cacao gene probes was 6-carboxyfluorescein (6-FAM) and quencher at the 3′ end of all gene probes was BHQ1 (Biosearch). The total volume of the PCR reaction was 25 µl and the mix included: 5 µl of cDNA (~12.5 ng), 12.5 µl 2× TaqMan® Universal Master Mix (#4304437, Applied Biosystems, Foster City, CA), 400 nmoles of each primer, and 200 nmoles of probe. The PCR reactions were run in 96 well thin-walled PCR plates in an Applied Biosystems 7300 Q-PCR system (Foster City, CA) with the following reaction conditions: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Each sample was amplified in triplicate and the results were averaged. In addition to the *TcChi1* transgene, the expression of four endogenous pathogenesis related (PR) genes in the control and transgenic lines were also evaluated. These genes were selected based on previous results that indicated their involvement in defense responses in cacao plants induced with chemical elicitors (Verica *et al.*, 2004). The genes included: two additional PR3 chitinases genes—Class 4 and Class 7; PR4-Osmotin; PR6-Type I proteinase inhibitorlike protein; and NPR1—a potential key regulator in the signal transduction pathway that leads to systemic acquired resistance. The mean expression of two cacao house keeping control genes, *actin* and *ubiquitin*, were used to normalize the data. Amplification efficiencies of all target and reference genes were calculated from the slopes of the dilution curves for each sample ($E = 10^{(-1/\text{slope})} - 1$) (Bustin, 2004). Average efficiency for each gene was then calculated and used for efficiency data correction. The data normalization, efficiency correction, statistical randomization test, and relative transgenic/control nontransgenic expression ratios were computed using REST software (Pfaffl *et al.*, 2002). Ratios (fold difference) with *p*-values less than 0.05 were considered significant (Table 1).

The results from the Q-PCR analysis indicated a large increase in *TcChi1* expression in all lines containing the transgene (Table 1). The fold difference compared to the nontransgenic samples varied from 8.8-fold for line T61.7 to 5053.6-fold for the highest expressing line T56.2. Line T56.2 also demonstrated a 2.2-fold increase in *TcChi4*. Interestingly, a down-regulation of between 1.5- to 1.9-fold was observed for the *TcChi7* gene in all lines overexpressing *TcChi1*. The *in vitro* endochitinase protein activity assay also detected significant increases of chitinase activity in all *TcChi1* transgenic lines compared to the control T31-GFP and nontransgenic lines (Maximova *et al.*, 2002). The levels of chitinase activity in the three transgenic lines correlated well with the expression levels of the transgene: T61 had the lowest protein activity, followed by T44 with intermediate activity and T56 with the highest activity (Maximova *et al.*, 2006). The level of chitinase activity also corresponded well with the *in vivo* antifungal activity against *Colletotrichum gloeosporioides,* a pathogen of economic importance for cacao in India and Venezuela. To test the antifungal activity *in vivo,* excised cacao leaves were challenged with spores from *C. gloeosporioides* (Maximova *et al.*, 2006). The lines with the highest mRNA levels and the highest *in vitro* chitinase activity were better able to suppress the development of the fungus and formed smaller necrotic lesions compared to the lines with lower *in vitro* chitinase activity and the control nontransgenic plants. This study

Table 1 Quantitative real-time reverse transcription polymerase chain reaction analysis (Q-PCR) of gene expression of *TcChi1* transgene and selected pathogenesis related genes. Total RNA was isolated from control PSU-Sca6 plants, transgenic line T31-GFP (expressing *EGFP* and *npt*II only), and three different transgenic lines containing the *TcChi1* gene. Differences in gene expression between the transgenic and nontransgenic tissues are represented as relative expression ratios (fold difference). The ratios were calculated using REST software (Pfaffl *et al.*, 2002) based on Q-PCR efficiencies and the mean crossing point (CP) values of the samples from individual transgenic line versus the mean CP values for PSU-Sca6 control nontransgenic samples. Ratios with *p*-values less than 0.05 were considered significant and listed in the table. When the transgenic samples were not significantly different, they are recorded as not significant (Ns)

Gene name	T31-GFP	T44.1	T56.2	T61.7
TcChi1	Ns	1,555.5	5,053.6	8.8
TcChi4	Ns	Ns	2.2	Ns
TcChi7	Ns	−1.7	−1.5	−1.9
TcNPR1	Ns	Ns	Ns	Ns
TcPR6	Ns	Ns	Ns	Ns

demonstrated the utility of the transformation system as a tool for gene functional analysis and supports a functional role of the cacao *TcChi1* gene in pathogen resistance in cacao. While by itself, overexpression of this gene is unlikely to confer high level fungal pathogen resistance, it is possible that combined with other resistance genes via breeding, and pest management strategies, it could contribute to an integrated approach for enhanced pathogen resistance.

2.3.3 Biotechnology for CPB resistance in cocoa

Conopomorpha cramerella, the CPB is one of the most important pests of cacao. Currently CPB affects almost all cocoa producing provinces in Indonesia. By 2000, CPB had infested 60 000 ha, inflicting losses of $40 million per year (ICCO, 2006). Losses in cocoa yield depend on the age of the infested pods and the degree of infestation. The youngest pods are most susceptible to infection, and with increase in the insect pressure it was estimated that the losses could reach 93% on the individual plantations (Lumsden, 2003). In 2006, an outbreak of CPB was also reported in Papua New Guinea where according to a US State Department report, cacao represents the second largest export product (Merrett, 2006). From January to May 2006, CPB caused a 21% decrease in cacao export of Papua New Guinea compared to 2005. Methods for CPB control include: cultural practices, application of pesticides, biocontrol, use of resistant/tolerant planting materials, and use of pheromones. The effectiveness of any of these methods is hindered due to the fact that the CPB larvae are protected inside the cocoa pod almost half of their life cycle. The selection and breeding efforts for resistance to CPB are mainly directed toward plants with improved physical characteristics of the pod. These include hardiness and thickness of sclerotic layer, husk thickness and smoothness of pod surface (Lumsden, 2003; Teh *et al.*, 2006). Although a few clones have been identified as moderately resistant to CPB, in general cacao breeding is a long and slow process and the introduction of new varieties is especially difficult considering the small-holder production systems in the developing countries. Thus, engineering resistance to CPB in transgenic plants could potentially benefit the cacao producers.

The potential candidate genes are those encoding for the production of *Bacillus thuringiensis* toxins (*Bt*). *Bt*-toxins are natural proteins with insecticidal activity against lepidopteran insects and are also considered harmless to humans and most higher organisms (Schnepf *et al.*, 1998). Different *Bt* toxins have been evaluated for their activity against CPB larvae (Santoso *et al.*, 2004). Twelve different purified Cry1 class *Bt* toxins were added to CPB artificial diet and fed to CPB larvae. The larvae mortality was observed every day for 3 days. Application of a single dose of 100 ng cm^{-2} of four different *Bt* toxins resulted in approximately 20–50% larvae mortality.

With the goal to produce transgenic cacao including *Bt* toxin genes with potential resistance to CPB, a collaborative research project was established between the Plant Research International, Wageningen, The Netherlands and the Indonesian Biotechnology Research Institute of Estate Crops, Bogor, Indonesia (Chaidamsari, 2005). The *Bt* toxin gene selected was SN19 hybrid gene encoding a protein consisting of domains I and III of *Cry1Ba* and domain II of *Cry1Ia* (Naimov *et al.*, 2001). The sequences of domains I and III of the gene were modified by PCR site directed mutagenesis to increase its expression in plants (Chaidamsari, 2005). Binary vectors pSN48, pTC4, pTC9, and pTC12 contained different versions of the gene containing modifications of one, two, or all three domains of the gene. Vector pTC13 included a control nonmodified gene *SN19*. The constructs were first introduced in transgenic *Arabidopsis thaliana* ecotype WS as a model system to rapidly evaluate the expression and effectiveness of the *Bt* transgenes. Larvae of two insect species, *Pieris rapae* and *Plutella xylostella*, were fed with leaves from transgenic plants containing the various constructs and larvae mortality was observed. Depending on the vector, between 25% and 86% of the plants evaluated caused 100% larvae mortality for both the insect species. The largest percentage of plants causing 100% mortality was recorded for pSN48 containing synthetic domains I and III. The analysis of protein content of these plants indicated that SN48 constituted 0.01–0.1% of the total soluble protein.

Transformation vectors pMH58 and pMH59 were constructed for transformation of cacao.

Vector pMH58 contained the *SN19* gene with synthetic domains I and III encoding fragments (comparable with pSN48) and vector pMH59 contained modifications of all the three domains of *SN19*. Both genes were placed under the control of the CaMV 35S promoter. *Agrobacterium* transformations were performed with somatic embryos derived from staminodes of clone Sca6. After recovery of transgenic somatic embryos, secondary embryos were transferred to PCG medium (Li *et al.*, 1998) to allow callus formation. Calli were maintained on the same medium for 5 months and *Bt* protein levels evaluated by immunoassay. Preliminary data demonstrated that calli transformed with pMH58 showed the highest protein content/expression levels, ranging from 0% to 1.4% of total soluble protein, while pMH59 ranged from 0% to 0.2% (Chaidamsari, 2005).

Because CPB primarily affects the pods and specifically enters through the pod wall, it would be advantageous to utilize promoters to drive *Bt* expression only in the pod wall. Thus as part of this study, Chaidamsari (2005) also identified the *TcLea5* gene as primarily expressed in the inner pod wall and somewhat in the outer pod wall but not in pulp, beans, and leaves. The putative *TcLea5* promoter was characterized and the promoter activity was verified in tomato as a model species. Further production and evaluation of transgenic plants producing *Bt* toxins under tissue-specific promoters are necessary to demonstrate the success and potential application of this technology for cacao.

3. FUTURE ROAD MAP

The future of transgenic cacao is currently uncertain. The use of this system for studying gene expression and protein function has been demonstrated but with the current protocols, the efficiency and expense of this method is prohibitive to most laboratories. Heterologous model plant systems such as *Arabidopsis* and tomato may prove to be much better species for the study of cacao gene function. However, precise investigations of molecular interactions may be limited in heterologous expression systems due to subtle differences between species. Therefore in the future, advanced studies of cacao gene function will ultimately require improved and more efficient cacao transformation methods. Higher transformation efficiencies, faster regeneration methods, and tissue-specific and regulated promoters are all required.

In addition to its utility as a method to study gene function in cacao, transformation holds a huge potential to contribute to the improvement of insect- and disease-resistant lines of cacao or for development of other desirable traits such as drought, cold, and herbicide resistance, etc. However, before commercial lines of cacao with desired traits are even considered, the societal, legal, and ethical issues involved must be carefully thought-out. By far, the largest impediment to the development of these technologies and to the application of genetic engineering for cacao improvement is the public perception of the potential ethical and biosafety issues surrounding this field. The perception of chocolate as a luxury food, and its association with children makes cacao biotechnology especially sensitive to public scrutiny. Because of this, research to develop this technology for cacao has suffered from lack of funding, and thus has lagged well behind other plant species such as corn, rice, and soybean. Furthermore, because cacao is grown in the developing countries, issues surrounding IP rights, farmer rights, and biosafety protocols in these countries are currently highly variable and not clear in many cases.

However, fact remains that cacao germplasm does not contain resistance genes for many of the opportunistic pathogens that inflict huge losses to farmers in many countries where cacao has been introduced. Integrated pest management approaches are being employed to address these problems; however, implementation of these methods requires educating millions of poor farmers who may not have the time or resources to apply them. Clearly, genetically encoded traits such as those made possible with transgenesis would be a powerful tool to help solve the problems facing cacao farmers today. The paradox between public hesitance and farmers' needs will play out in the future as cacao pathogens continue to spread from country to country and new pathogens invade the cacao fields.

In the future, native gene transformation (transformation with gene sequences originating only from the same plant species) and gene targeting methods need to be developed which

will eliminate many of the concerns regarding the precision and safety of transgenic technologies. These breakthroughs will be as important for cacao as for any other plant species. Manipulation of the key transcription factors that regulate plant growth and development as well as responses to biotic and abiotic stress will lead to novel approaches to crop improvement, but a much deeper understanding of the basic mechanisms of plant molecular biology is required before this can be adapted to cacao.

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