Published November 18, 2013

Genetic and Genomic Analysis of the Tree Legume *Pongamia pinnata* as a Feedstock for Biofuels

Bandana Biswas, Stephen H. Kazakoff, Qunyi Jiang, Sharon Samuel, Peter M. Gresshoff, and Paul T. Scott*

Abstract

The tree legume Pongamia {*Pongamia pinnata* (L.) Pierre [syn. *Millettia pinnata* (L.) Panigrahi]} is emerging as an important biofuels feedstock. It produces about 30 kg per tree per year of seeds, containing up to 55% oil (w/v), of which approximately 50% is oleic acid ($C_{18:1}$). The capacity for biological N fixation places Pongamia in a more sustainable position than current nonlegume biofuel feedstocks. Also due to its drought and salinity tolerance, Pongamia can grow on marginal land not destined for production of food. As part of the effort to domesticate Pongamia our research group at The University of Queensland has started to develop specific genetic and genomic tools. Much of the preliminary work to date has focused on characterizing the genetic diversity of wild populations. This diversity is reflective of the outcrossing reproductive biology of Pongamia and necessitates the requirement to develop clonal propagation protocols. Both the chloroplast and mitochondrial genomes of Pongamia have been sequenced and annotated (152,968 and 425,718 bp, respectively), with similarities to previously characterized legume organelle genomes. Many nuclear genes associated with oil biosynthesis and nodulation in Pongamia have been characterized. The continued application of genetic and genomic tools will support the deployment of Pongamia as a sustainable biofuel feedstock.

DEDICATED bioenergy crops as feedstocks for biofuel production and as alternatives to fossil fuels have been at the forefront of discussions regarding future sustainability of energy and resources for many years. The arguments for the exploitation of biomass to meet an increasing demand for energy are many, any one of which is substantive. First, it is widely accepted that the global status of peak oil, where the demand for fossil fuels is in excess of the capacity to meet this demand, has either been passed, is current, or is soon to be reached. Of particular immediate concern is the demand for liquid fuels, which makes up the predominant component (70 to 80%) of energy consumption (BP, 2012). While the currently predicted production levels of biofuels are unlikely to completely replace all sources of fossil fuels, there is certainly an expectation that biofuels will be able to meet a significant proportion of the foreseeable demand for liquid fuels. With respect to the potential of a future biofuels industry in Australia that includes Pongamia as one of the feedstock species, it is expected that seed-borne Pongamia oil will be able to meet a substantial proportion of the domestic diesel fuel demand. Based on an annual production of 3 to 5 t of oil ha^{-1} and the expectation that initial formulations of biodiesel will include 80% mineral diesel, approximately 7000 to

ARC Centre of Excellence for Integrative Legume Research, School of Agriculture and Food Sciences, The University of Queensland, St Lucia, QLD, Australia 4072. Received 20 May 2013. *Corresponding author (p.scott@uq.edu.au).

Abbreviations: ACP, acyl carrier protein; CoA, coenzyme A; cpDNA, chloroplast DNA; EC, electrical conductivity; FA, fatty acid; FAME, fatty acid methyl ester; GFP, green fluorescent protein; GHG, greenhouse gas; IR, inverted repeat; ISSR, inter-simple sequence repeat; LAP, low agriculturally productive; mtDNA, mitochondrial DNA; NEB, net energy balance; PISSR, Pongamia inter-simple sequence repeat; PUFA, polyunsaturated fatty acid; RNA, ribonucleic acid; RNAi, ribonucleic acid interference; rRNA, ribosomal ribonucleic acid; RuBisCO, ribulose-1,5-bisphosphate carboxylase oxygenase; tRNA, transfer ribonucleic acid; TAG, triacylglyceride.

Published in The Plant Genome 6
doi: 10.3835/plantgenome2013.05.0015

5585 Guilford Rd., Madison, WI 53711 USA
An open-access publication

11,500 km^2 of Pongamia plantations will be required to meet Australia's demands. This area fits well within the 1 to 2 million km^2 of so-called marginal, low agriculturally productive (LAP) land in Australia that could be suitable for cultivation of Pongamia. The abundance of marginal land in Australia presents both a challenge and an opportunity: a challenge to establish plantations of resilient species such as Pongamia and an opportunity to develop both a domestic and export biofuels industry.

Second, and perhaps more controversially, the environmental impact of biofuels is generally regarded as being much less than that of fossil fuels (Bessou et al., 2011). Simplistically, the generation of CO_2 and other greenhouse gases (GHGs) (e.g., CH_4) during the combustion of fuel is compensated by C fixation during photosynthesis and growth of the biofuel feedstock. However, recent more in-depth life cycle assessments have indicated that the capacity for GHG mitigation of some proposed biofuel feedstock species is not as significant as initially suggested (Crutzen et al., 2008; Erisman et al., 2010; Smith and Searchinger, 2012). Of particular relevance to Pongamia (and other legume biofuel feedstocks) (e.g., soybean [*Glycine max* (L.) Merr.]) is the contribution that biological N fixation makes to N cycling during plant growth. For nonlegumes the requirement for energetically and environmentally costly N fertilizers is detrimental to the net energy balance (NEB) of biofuel production (Hill et al., 2006). In addition, the application of N fertilizers leads to production of the highly potent class of GHGs, N_2O (nitrous oxide). In general the importance of N in addition to C in the accounting of NEB and GHG emissions and the positive environmental impact that legumes (including Pongamia) have in this regard is neglected when considering potential biofuel feedstock species.

Pongamia pinnata is a medium-sized tree legume that can grow to between 10 and 15 m in height (Biswas et al., 2011; Scott et al., 2008; Fig. 1). It is also sometimes known as *Millettia pinnata* in the scientific literature and has numerous common names in countries throughout southern and southeast Asia (e.g., in India it has the colloquial names Pongam and Karanj). Although Pongamia is yet to be domesticated as an established tree crop there is a long history of using various components of the tree for fuel, green manure, insecticides, and traditional medicines in India and elsewhere in southeast Asia. Today, of particular agronomic interest is the annual production of large oil-rich seeds. This oil is suitable as feedstock for biodiesel and aviation jet fuel production. These seeds range in mass from 1 to 3 g and contain on average 40 to 50% oil (v/v). The composition of the oil is dominated by oleic acid at 45 to 55%. There are many reports showing that biodiesel quality depends on the fatty acid (FA) composition of the corresponding feedstock (Azam et al., 2005; Imahara et al., 2006; Srivastava and Prasad, 2000). With respect to the FA composition of plant oils, the monounsaturated oleic acid is preferred over other FAs for biodiesel production because of its propensity to give the oil qualities of low temperature cloud and pour points. Our analysis of Pongamia oil, shown in Table 1, confirms the presence of a high proportion of oleic acid and detects the lesser presence of some undesirable saturated and polyunsaturated FAs (PUFAs). The oil can be easily converted to biodiesel (fatty acid methyl esters [FAMEs]) by transesterification with CH_3OH (methanol) in the presence of KOH (potassium hydroxide) catalyst to meet current industry standards: European EN 14214 and U.S. ASTM D 6751-08 (Bora and Baruah, 2012).

The pour and cloud points of Pongamia FAMEs are 2.1 and 8.3°C, respectively, consistent with the presence of saturated oils such as palmitic acid ($C_{16:0}$) and stearic acid ($C_{18:0}$) (Table 1). The pour point of a fuel indicates the lowest temperature at which the oil can still flow whereas the cloud point determines the temperature at which the dissolved solids in the oil will precipitate from the liquid (Joshi and Pegg, 2007). Lower cloud points are more desirable for engine performance and although the cloud point of Pongamia FAMEs compares favorably with biodiesel derived from sources such as palm (*Elaeis guineensis* Jacq.) oil (10°C) and beef (*Bos primigenius*) tallow (13°C), biodiesels made from soybean (–1°C), rapeseed (*Brassica napus* L.) (–7°C), and sunflower (*Helianthus annuus* L.) (1°C) are lower. Engine performance tests performed with Pongamia FAMEs concluded that blends of up to 40% (v/v) with mineral diesel were successful in reducing exhaust emissions together with increases in torque, thermal efficiency, and brake power and a reduction in brake-specific fuel consumption (Raheman and Phadatare, 2004). However, as the concentration of Pongamia FAMEs in the blend was increased, deterioration in viscosity, cloud, and pour points (important for cold weather performance) of the fuel was detected. Therefore, a major focus of the research program in our laboratory is improving oil composition of Pongamia so that the biodiesel derived from this tree can see a market in all climatic regions of the world. Optimization of growth and improvements in oil-seed productivity are other major requirements and towards this end research targeted at life cycle analysis, nodulation and N fixation, salinity and drought tolerance, disease and pest resistance, and flowering time are being pursued. This review highlights the current status of our research and lays out the priorities and direction of future research initiatives.

Growth and Life Cycle Assessment of Pongamia

While it is thought that the center of origin for Pongamia is most likely India there are wild stands of trees found throughout southeast Asia, Indonesia, Papua New Guinea, northern Australia, and southern China. In Australia, Pongamia can be found in many of the northern tropical regions and the subtropical east coast ranging from Darwin to as far south as northern New

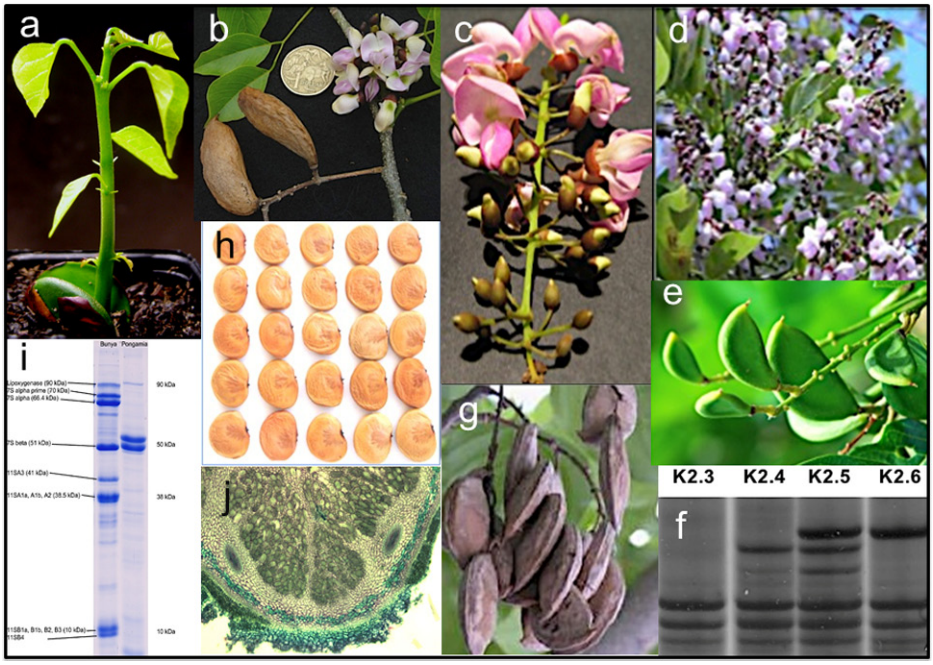

Figure 1. General *Pongamia pinnata* (syn. *Millettia pinnata*) biology. (a) Seedling about 1 to 3 wk after planting for germination. Emergence depends on seed age and drops rapidly after 6 to 9 mo. (b) Flowers and mature pods. (c) Close-up of multiple (35–45) flowers as a florescence. Fertilization is variable depending on pollen availability and environmental conditions. (d) Abundant flowering about 6 to 10 wk after the spring equinox. (e) Green immature pods (2–3 cm in length) with small seed (usually single seed [5 mm at this stage]). (f) Example of DNA profiling using the DNA amplification fingerprinting (arbitrary primer polymerase chain reaction) technique (Caetano-Anollés et al., 1991). Common and variable amplification bands are clearly seen. (g) Mature, dry seedpods with large dry seeds (1.3–2.9 g per seed were detected in the Brisbane area). (h) Freshly harvested seed showing yellow-to-light brown seed coat (storage darkens seed coat to dark red-brown). (i) Seed protein separation comparing soybean (left lane) to *Pongamia pinnata* seed. Note the absence of abundant storage protein classes in Pongamia with two 7S-β-conglycinin (molecular weight about 50,000 and 52,000 Da) being noted. (j) Stained section of a N-fixing Pongamia nodule illustrating key symbiotic tissues, such as the infected zone (central), nodule vascular bundles (dispersed), and nodule parenchyma and cortex (peripheral) (diameter of this nodule was about 3 mm).

Table 1. Major components of oil of several plants currently used as feedstock for biofuel production.

Plant	Oil yield (L ha^{-1} per annum)	Percent oleic acid ($C_{18:1}$)	Percent palmitic acid ($C_{16:0}$)	Percent stearic acid ($C_{18:0}$)	Source or reference[†]
Corn	172	30.5–43.0	7.0–13.0	2.5–3.0	Dantas et al., 2007
Soybean	446	22.0–30.8	2.3–11.0	2.4–6.0	Hildebrand et al., 2008
Canola	1,196	55.0–63.0	4.0–5.0	1.0–2.0	Mosser, 2009
Jatropha curcas L.	1,892	34.3–45.8	13.4–15.3	3.7–9.8	Becker and Makkar, 2008
Palm oil	5,950	38.2–43.5	41.0–47.0	3.7–5.6	Sarin et al., 2007
Algae[‡]	59,000	1.7–14.3	3.7–40.0	0.6–6.0	Hu et al., 2008
Tallow	NA[§]	26.0–50.0	25.0–37.0	14.0–29.0	Canakci and Sanli, 2008
Pongamia	3,600–4,800	25.4–68.3	5.41–9.49	2.15–8.00	Centre for Integrative Legume Research, The University of Queensland

[†]These references are representatives of an extensive list in the publicly available scientific literature.

[‡]Algal yield represents extrapolations from smaller volume trials with multiple species. These projected yields are yet to be demonstrated on a commercial scale.

[§]NA, not applicable.

South Wales. In Queensland, it is commonly grown as an ornamental street tree and many streets of Brisbane and smaller towns and cities on the coast are lined with these beautiful trees. Pongamia can tolerate a range of conditions from minimum temperatures of 0 to 16°C, maximum temperatures of 27 to 50°C, and mean annual rainfall of 500 to 2500 mm (Daniel, 1997; Orwa et al., 2009). However, Pongamia prefers humid tropical and subtropical climates interspersed with a dry and hot summer period of 2 to 6 mo.

Pongamia is a fast growing species reaching adult height in 4 to 5 yr. The initial crop of seeds generally occurs after 4 to 7 yr although we have identified early flowering varieties where flowering occurred as early as 16 mo after germination (Jiang et al., 2012). Flowering occurs in late spring and summer in Queensland but can

occur throughout the year in some parts of the world (Orwa et al., 2009; Scott et al., 2008). Seeds mature about 10 to 11 mo after flowering. Preliminary harvests from our own field trial sites suggest that Pongamia trees are capable of producing from 15,000 to 20,000 seeds yr^{-1}. With an average mass of 1.5 g per seed this equates to between 22.5 and 30 kg of seeds per tree per year. At a conservative estimate of 40% (v/v) oil per seed that equates to between 9 and 12 L of oil per tree per year, and with a planting density of 400 trees ha^{-1} this would yield between 3600 and 4800 L of oil ha^{-1} yr^{-1}.

Trial and commercial plantations of Pongamia have been set up in several regions of Australia, India, and the southwestern United States. In Australia, a number of trial plantations have been established throughout Queensland, in the Northern Territory, and in Western Australia. In Queensland, three climatically distinct sites have been allocated by our research group for the establishment of trial plantations. The first trial site planted by our research group is located at the Gatton Campus of The University of Queensland (27.54°S, 152.34°E), where the subtropical climatic conditions are very similar to the city of Brisbane (the location of the parent plants), including a distinct winter dormancy period. The second site at Walkamin in Far North Queensland (17.13°S, 145.43°E) constitutes the dry tropics and experiences 4 to 5 mo of little to no rainfall. In sharp contrast, the conditions at the field site at South Johnstone (17.61°S, 146.00°E) are wet tropics with rainfalls in excess of 5 m yr^{-1}. In addition to seed-derived material, clonally propagated plants derived from the same parent tree (clonal propagation methods are described in the later part of this review) have been planted in these locations. Preliminary results will provide useful insights into the effect of plant growth and nodulation under different climatic conditions.

Results from the Gatton Campus trial site of the University of Queensland indicate rapid increases for both root and aboveground biomass (Fig. 2). After 4 yr of growth, the aboveground biomass has increased from a starting point at planting of approximately 3 g per sapling to more than 13 kg per maturing tree. The roots appeared to have reached deeper soil layers and the trees were able to tolerate long periods of water deficit without signs of wilting or stress. The importance of the Gatton campus field trial lies not only in the growth of Pongamia as a biofuel feedstock. In Australia and other developed countries the so-called carbon economy is beginning to have an impact on agriculture through the planting of trees and the associated C sequestration. Therefore, the Gatton campus field trial is enabling quantification of Pongamia biomass production over time. The C content of aboveground Pongamia is 44%, which over the initial 4-yr period of the trial (Fig. 2) equates to 5.72 kg C per tree. At a plantation density of 450 trees ha^{-1} this equates to approximately 9.4 t of CO_2 sequestered per hectare. In the future, calculating the belowground biomass will require industrial scale earthmoving equipment, but nonetheless it is expected that the root system will at least equal that of the aboveground biomass.

Nodulation and Nitrogen Fixation

Most biofuel feedstocks including sugarcane (*Saccharum officinarum* L.), canola (*Brassica napus*), sweet sorghum [*Sorghum bicolor* (L.) Moench], maize (*Zea mays* L.), and woody trees such as eucalypts (*Eucalyptus globulus* Labill.) and willows (*Salix* spp.) require N fertilizers. As described above, the implications of this are enormous in economic and environmental terms (Jensen et al., 2011; Murphy et al., 2012). In contrast, legumes are capable of forming symbiotic relationships with N-fixing bacteria (collectively called rhizobia), which are housed in specialized root organs called nodules (Ferguson et al., 2010). Here rhizobia convert atmospheric N into NH_3 (ammonia) that is then used by the host plant, thus reducing the dependence on N fertilizers.

Most legume plants nodulate effectively with one specific or a select few species of rhizobia. However, Pongamia has been reported to be quite promiscuous, establishing symbioses with several species of both *Bradyrhizobium* and *Rhizobium* (Arpiwi et al., 2013; Kesari et al., 2013; Rasul et al., 2012). However, the capacity of Pongamia to fix N with these species of rhizobia has not been reported and the establishment of superior inoculants was not possible (Rasul et al., 2012). The selection of superior rhizobial strains for Pongamia is of utmost importance as it will help to promote growth and potentially increase yields of oil-rich seeds. Towards this end, our lab (Samuel et al., 2013) tested a wide range of bacterial strains from Australia and India and established *Bradyrhizobium japonicum* strains CB1809 and USDA 110 as the best inocula tested. The nodules produced by these strains were larger with more uniformly and extensively filled infected zones (Fig. 3). Nitrogen fixation activities within the nodules was demonstrated using the acetylene reduction assay where C_2H_2 (acetylene) served as a substrate for bacterially encoded nitrogenase and its reduction to C_2H_4 (ethylene) was quantified by gas chromatography. The nodules produced by the less effective inocula had several lobed infection zones with variable bacterial concentration (Samuel et al., 2013).

Legume nodules can either be determinate with a spherical morphology due to lack of persistent meristems or indeterminate where retention of meristems produces more cylindrical nodules. Pongamia nodules have been reported to be determinate in nature (Kazakoff et al., 2011; Scott et al., 2008); however, our later studies established that the determinate nodules progressed to indeterminate in older plants giving rise to larger coralloid nodules (Samuel et al., 2013; Fig. 3). Older Pongamia trees will therefore exhibit a combination of spherical and coralloid nodules. This observation is consistent with previous reports that most tree legumes do tend to have woody indeterminate nodules (Baird et al., 1985; Sprent and Parsons, 2000).

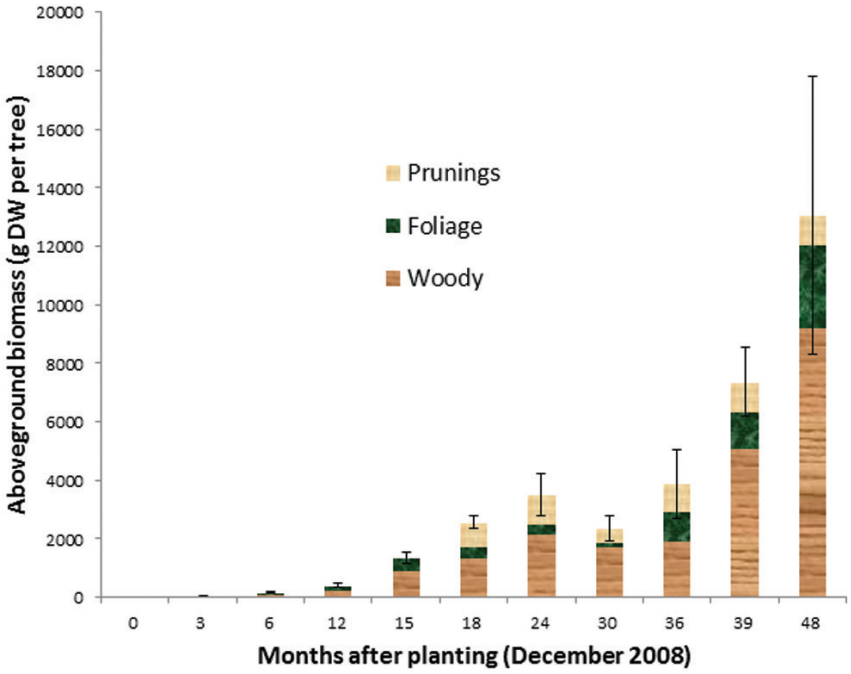

Figure 2. Aboveground biomass production of *Pongamia pinnata* trees from The University of Queensland Gatton campus field trial site. A field trial of 296 trees was established in December 2008 and at subsequent intervals four to six trees were sacrificed and the mass of woody, leaf, and pruned foliage was determined after drying. DW, dry weight.

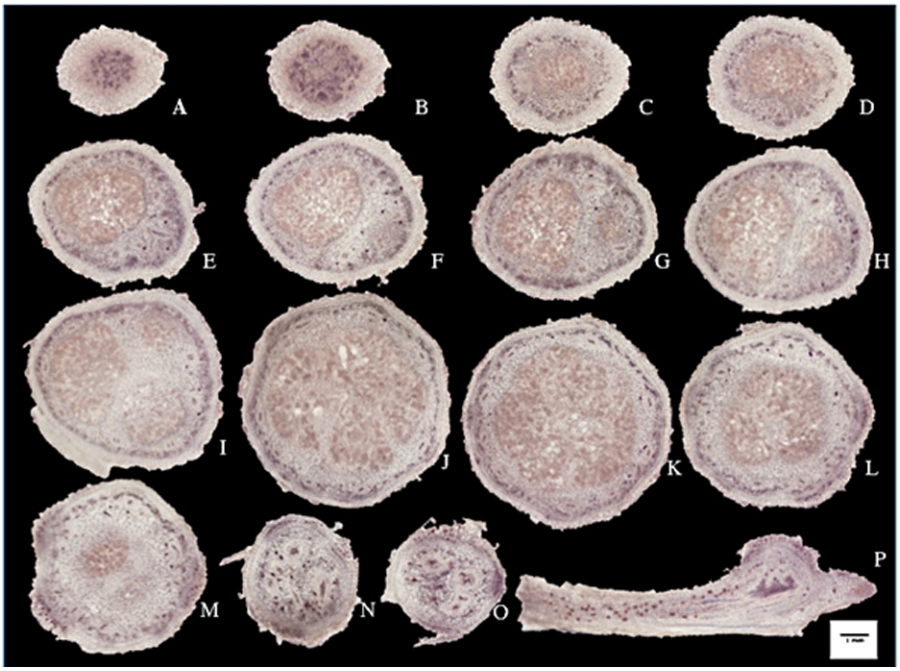

Figure 3. Cross-sections of a Pongamia nodule. A sequential set of sections were prepared of a single nodule from the outermost region (A) to the site of attachment of the nodule to the root (P).

We performed split root experiments to demonstrate that NO_3^- (nitrate) inhibition of nodulation and autoregulation of nodulation, common in legumes, was also observed in Pongamia (Samuel et al., 2013). Therefore, as a tree, Pongamia shares both qualitatively and quantitatively major characteristics found in other better-characterized annual crop legumes, such as soybean and pea (*Pisum sativum* L.).

Salinity and Drought Tolerance

The first generation biofuel feedstock plants such as corn, soybean, wheat (*Triticum aestivum* L.), sugarcane, and rapeseed are all food crops, giving rise to ethical questions regarding their use as biofuel crops when hunger and malnutrition are still major problems in many parts of the developing world. In addition, the

rising commodity prices have been attributed largely to redirecting of crops and scarce crop producing land and water for biofuel production. This has given rise to the development of the second-generation biofuel crops, which are not food crops and can grow on marginal land, unsuitable for food production. This makes Pongamia highly desirable as, due to its drought- and salinity-tolerant nature, it can grow on globally abundant marginal land (LAP land), which is not economically viable for food production. In Australia, there are over 1 million km^2 of marginal land, 15% of the total land area (Bureau of Rural Sciences, 2002). In addition, land clearing in areas such as the Murray-Darling River Basin has resulted in dry land salinity problems. Affected lands are currently being reclaimed by planting salt-tolerant tree legumes such as *Acacia* spp. (Zou et al., 1995). Such marginal lands are excellent options for planting salt-tolerant biofuel crops, such as Pongamia (Farine et al., 2012; Odeh et al., 2011).

Salinity, measured by the electrical conductivity (EC) of a solution or saturation extract of soil, can negatively affect plant growth by osmotic effects and ion toxicity. Soils are considered saline when their EC exceeds 4 dS m^{-1} and water exceeding 4.7 dS m^{-1} is unsuitable for the irrigation of most crop species (Myers et al., 1995). However, 62% of Pongamia trees survived in soil with EC values varying from 10 to 12 dS m^{-1} (Patil et al., 1996) and 13% could survive salinity values as high as 19 dS m^{-1} (Tomar and Gupta, 1985). Since none of the studies reported how salinity affected nodulation and symbiotic N fixation in Pongamia, we investigated the effects of salinity on plant growth as well as nodulation and N gain. Our study showed no direct effect of relatively high levels of salinity (Table 2) on growth of 12-wk-old Pongamia seedlings over an 8 wk period of exposure. However, nodulation (nodule number and nodule mass per plant) and N tissue concentration of nodulated plants decreased with increasing salinity (P. Scott, unpublished data, 2008). Efforts are required to improve nodulation and N fixation of Pongamia if it is to be a successful biofuel feedstock crop. Salt-tolerant legumes, such as salt wattle (*Acacia ampliceps* Maslin), have been shown to improve nodulation and N fixation in saline environments when inoculated with salt-tolerant strains of rhizobia (Bala et al., 1990; Zou et al., 1995). As such, Pongamia grown in saline conditions may too benefit from inoculation with salt-tolerant strains of rhizobia.

In recent times there has been an increase in both frequency and intensity of drought in Australia and other parts of the world (Hennesy et al., 2008), presumably caused by global climate change. In 2006 drought had affected more than three-quarters of Australia with 38% of agricultural land eligible for government assistance, amounting to billions of dollars. According to the Commonwealth Scientific and Industrial Research Organization, the outlook is bleak and by 2030 rainfall in major capitals could drop by 15%. Pongamia has repeatedly been reported as drought tolerant, possibly due to its dense network of lateral roots and thick, long taproot. In Australia it was anecdotally reported to have survived months of no rain during the 2007 through 2008 droughts. Preliminary studies in our lab, where water was withheld from seedlings grown from a mixed batch of seeds, showed a wide range of response (from no noticeable effect to complete wilting and drying out) indicating that genetic diversity was manifested in the drought tolerance level between different trees (P. Scott, unpublished data, 2010).

Genetic Diversity in Pongamia

Pongamia exhibits an outcrossing reproductive strategy. This results in wide genetic diversity between trees, which manifests itself in almost all physiological characteristics of the plant. Phenotypic differences in height, shape, leaf morphology and size, and seed size and shape as well as differences in oil composition have been demonstrated (Jiang et al., 2012; Kaushik et al., 2007; Kesari et al., 2008). This provides an opportunity to identify and select elite trees displaying superior qualities of many desirable traits. For example, analysis of seeds from trees in Brisbane, Australia, showed variation in oil content and composition between trees and between progeny seeds of a single parent tree. Seed oil content ranged from 19.7 to 52.3% in Pongamia seeds derived from a variety of sources in Queensland and Malaysia and variation from 40.3 to 52.3% was observed in the oil content of progeny seeds of the same parent tree (Arpiwi et al., 2013; Jiang et al., 2012). In addition, oleic acid content of seed oil ranged from 25.4 to 68.3% (Jiang et al., 2012). Therefore, a breeding program based on selection and clonal propagation of elite varieties with the most favorable oil content and composition seems feasible. Densities of 400 to 600 trees ha^{-1} have been proposed as the standard for Pongamia plantations. Densities of 1000 to 1200 ha^{-1} of high-yielding semidwarf varieties through repeated pruning have also been suggested. However, future projections of yield based on data from individual street trees need to be treated with caution, as the impact of plantation style growth on yield is yet to be assessed. Research is needed to establish how yield is affected by plantation density and arrangement and managed tree architecture.

Several attempts have been made to evaluate the genetic diversity in Pongamia using DNA markers. This is essential so that elite plants can be selected at the seedling stage, especially as many traits are only expressed when the trees are mature. Reports of the use of markers such as simple sequence repeats, inter-simple sequence repeats (ISSRs), random amplification of polymorphic DNA, and amplified fragment length polymorphism have been made (Kesari et al., 2010; Sahoo et al., 2010; Sharma et al., 2010). However, even though valuable information has been generated from these studies, pooling of the samples of populations have prevented the drawing of conclusions regarding individual trees. Therefore, we analyzed individual

DNA samples from 29 Pongamia trees located in and around the Brisbane area, using specifically anchored Pongamia "deep sequence"-derived ISSR markers (Jiang et al., 2012). The Pongamia inter-simple sequence repeat (PISSR) primers were designed using a Pongamia 75 bp paired-end read DNA database constructed using Illumina Solexa GAIIx deep DNA sequencing (Marshall et al., 2010) from one Brisbane source tree. The special feature of PISSR primers is that the number of repeats of nucleotide core units and the anchored 5′ and 3′ nucleotide residues of PISSR primers were all based on the above database, thus representing actual sequences of the Pongamia genome. The separation of the polymerase chain reaction products amplified by the PISSR primers was accomplished with high resolution by polyacrylamide gel electrophoresis and visualized using silver staining methods (Bassam et al., 1991; Bassam and Gresshoff, 2007). Genetic similarity analysis was done by binomial scoring for the presence or absence of 105 polymorphic markers for the 29 randomly selected trees.

Through analysis of individual trees, we were able to identify correlations between genotype and phenotype. For example, the PISSR amplification profile of the Brisbane tree GC2 reveals polymorphic markers in concert with its unique phenotypic traits (Fig. 4; Jiang et al., 2012). This opens the possibility of crop improvement programs based on breeding by hybridization and association mapping through development of molecular linkage maps for both single gene traits and quantitative trait loci.

Table 2. Response of nodulated and nonnodulated Pongamia seedlings to salt stress. Four weeks after germination Pongamia seedlings were exposed to NaCl treatments of 4, 10, or 20 dS m^{-1} for a period of 8 wk.

	Electrical conductivity (dS m^{-1})	12-wk-old seedlings (8 wk after inoculation)	
		Nonnodulated	Nodulated
Shoot height, cM	0	14.9 ± 1.1†	20.0 ± 0.8†
	4	15.7 ± 1.0†	20.5 ± 1.0†
	10	15.7 ± 1.2	18.5 ± 1.4
	20	17.5 ± 1.1	18.4 ± 0.8
Root length, cM	0	37.9 ± 3.6	42.2 ± 2.0
	4	40.8 ± 3.2	46.0 ± 2.6
	10	38.9 ± 3.7	45.4 ± 2.4
	20	37.5 ± 1.8	36.7 ± 2.4
Shoot dry weight, g	0	0.62 ± 0.10†	1.01 ± 0.07†
	4	0.72 ± 0.12†	1.33 ± 0.16†
	10	0.69 ± 0.09	0.96 ± 0.16
	20	0.76 ± 0.08	0.89 ± 0.10
Root dry weight, g	0	0.43 ± 0.11	0.71 ± 0.07
	4	0.49 ± 0.06†	0.89 ± 0.10†
	10	0.39 ± 0.11	0.67 ± 0.08
	20	0.42 ± 0.06	0.64 ± 0.09
Nodule number	0	Not applicable	21.4 ± 2.2
	4	Not applicable	29.0 ± 4.4
	10	Not applicable	19.7 ± 3.2
	20	Not applicable	9.2 ± 2.5

†Significant difference between nodulated and nonnodulated plants for the same treatment.

Clonal Propagation, Tissue Culture, and Transformation

In spite of many beneficial characteristics, establishment of Pongamia plantations with elite phenotypes has been limited to date because of its outcrossing reproductive nature and resulting genetic diversity between seed-derived progeny trees. Therefore, once elite germplasm has been identified, there is a requirement for efficient vegetative propagation methods such as rooted cuttings, grafted saplings, and tissue culture regeneration.

Tissue culture has the potential to produce large numbers of genetically identical propagules (so-called clones) from a small amount of source tissue. Commonly used tissue for regeneration of this type has been buds, meristems, and leaves. Regeneration follows either through organogenesis or somatic embryogenesis. In organogenesis, the explant is placed in medium supplemented with plant hormones to induce multiple shoot formation followed by transfer of individual shoots to root induction medium to allow root formation. Somatic embryogenesis involves production of structures similar to zygotic embryos with root and shoot meristems, which then develop into plantlets. The most common way to induce somatic embryogenesis is to apply explants to a medium high in auxin, most commonly (2,4-dichlorophenoxy)acetic acid.

Regeneration by organogenesis from nearly mature green cotyledons has been demonstrated using the plant hormone thidiazuron [1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea] (Biswas et al., 2011; Sujatha et al., 2008) and a regeneration frequency of 66% has been reported (Fig. 5). However, success of this procedure is highly dependent on genetic background of each individual tree as demonstrated by differing responses of explants from different candidate trees (Brisbane street trees) under identical tissue culture conditions. Whereas some plants were able to be regenerated quite efficiently (up to 100%) from cotyledonary explants, others lacked even basic callus formation (B. Biswas, unpublished data, 2011).

For genetic transformation somatic embryogenesis is more desirable as most of the embryos originate from single cells and result in uniform transgenic plants without chimeric sectors. Like woody fruit trees, transformation protocols in Pongamia using *Agrobacterium tumefaciens* and particle bombardment should be possible, and manipulation of genes in the FA biosynthesis pathway should be the target of future genetic manipulation. In our lab transformation protocols using *Agrobacterium rhizogenes* (hairy root transformation) and *A. tumefaciens* (stable transformation) are currently being developed.

Hairy root transformation produces composite plants containing transgenic roots through the expression of the *Rol* genes present in the Ri (Root inducing) plasmid of *A. rhizogenes*, forming transgenic hairy roots and nontransgenic shoots. Even though the

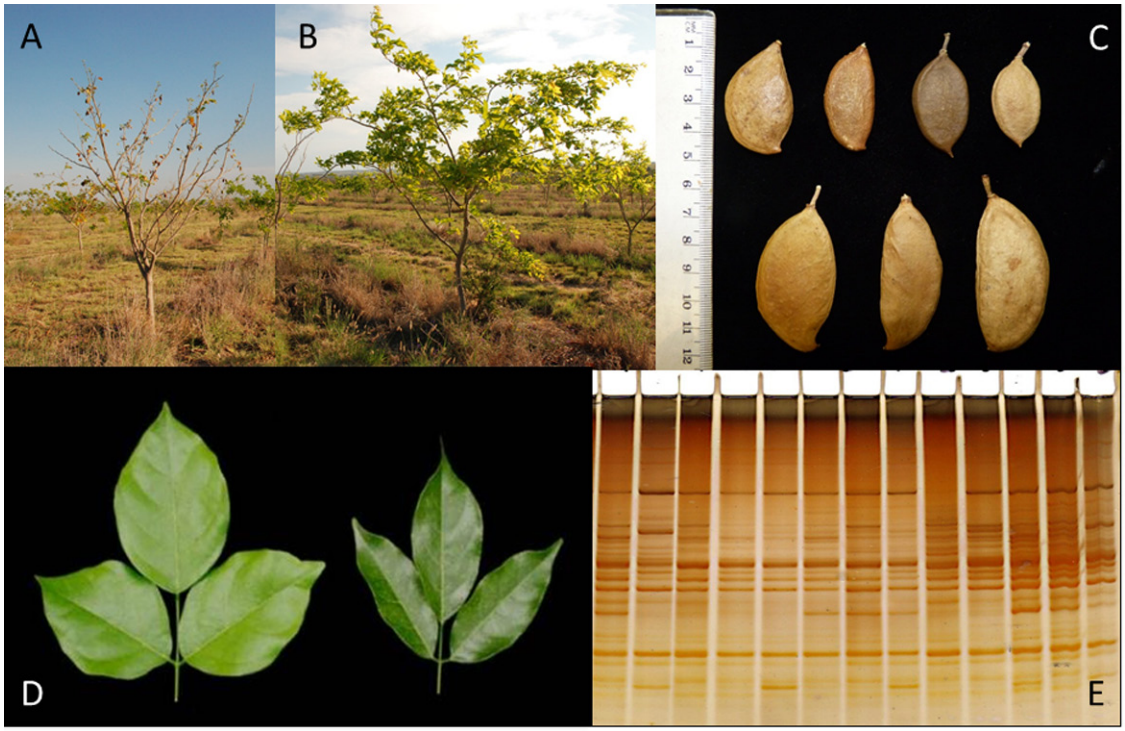

Figure 4. Phenotypic and genotypic diversity of Pongamia. (A) and (B) show the diversity in spring foliage regrowth in trees from a field site near Roma, Queensland. (C) A selection of Pongamia seedpods sourced from trees in and around the Brisbane area. (D) Variation in morphology of leaves from two Brisbane street trees. (E) Silver stained polyacrylamide gel of polymerase chain reaction products amplified with Pongamia inter-simple sequence repeat markers.

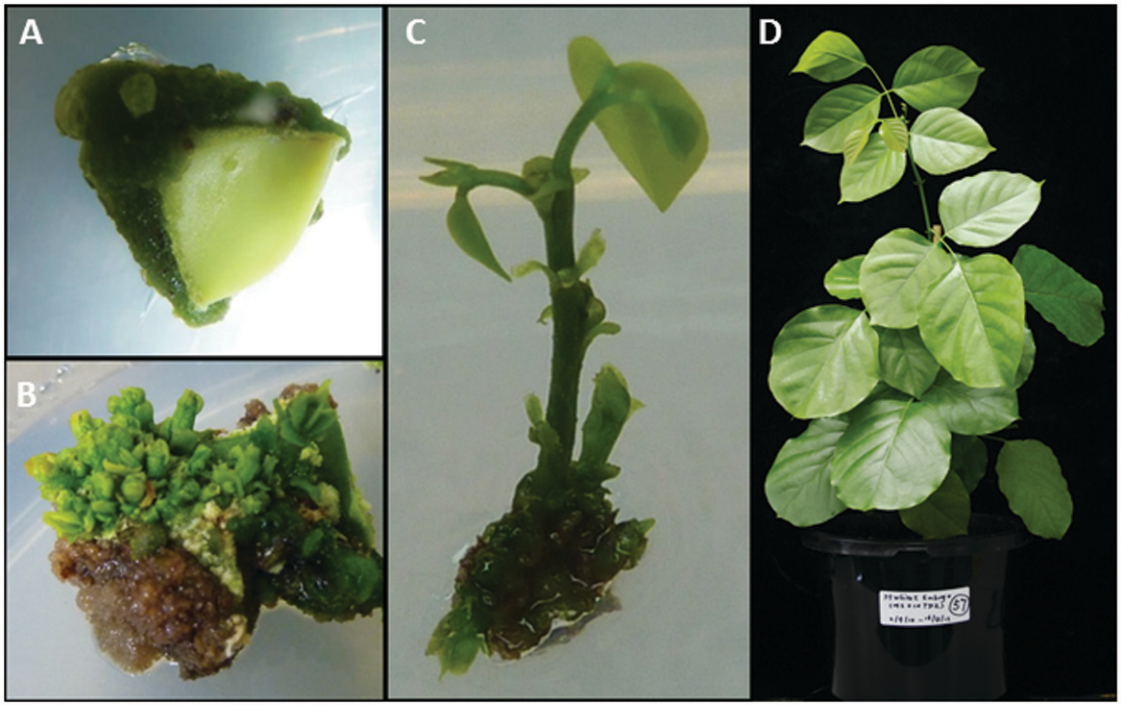

Figure 5. Regeneration of Pongamia through tissue culture from immature cotyledons (A) through subsequent developmental stages (B) and (C) to fully regenerated plant (D).

transgenic nature of the plants cannot be maintained through the generations, it is a quick method to check gene expression, promoter analysis, and root related phenotypes. We tested 11 strains of *A. rhizogenes* and identified two, MSU 440 and A4, that successfully produced transgenic hairy roots expressing β-glucuronidase (GUS) and green fluorescent protein (GFP) reporter genes (Fig. 6; B. Biswas, unpublished

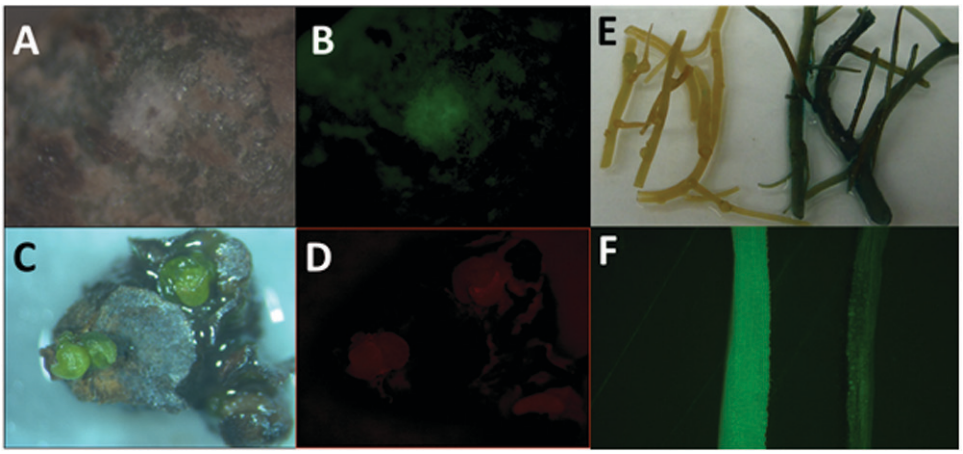

Figure 6. Green fluorescent protein (GFP) (B) and (F), red fluorescent protein (DsRED) (D), and β-glucuronidase (GUS) (E) reporter gene expression in transgenic Pongamia. A and B show the same callus tissue under bright field and epifluorescence microscopy, respectively, as do C and D. A, B, C and D were cotyledons transformed with *Agrobacterium tumefaciens*, and E and F were hairy roots generated by transformation with *Agrobacterium rhizogenes*.

data, 2012). The method that worked for us was removing the main roots in young seedlings and rubbing the cut ends in a paste of the desired strains of bacteria followed by replanting. Pongamia has a propensity to produce secondary roots under these conditions and expression of reporter genes could be detected even in roots where *Rol* gene expression was absent. However, we found some trees more susceptible to transformation whereas others were highly resistant, likely due to genotypic variation (B. Biswas, unpublished data, 2012). Attempts at stable transformation using *A. tumefaciens* strains EHA105 and AGL1 look promising, showing callus expressing GFP and red fluorescent protein (DsRED) reporter genes (Fig. 6). Once these methods have been optimized and fully developed, transgenic plants are likely to be produced on a routine basis and a plant improvement program based on targeting key genes will be started in our lab in the near future. However, since very little is known about the genomics of Pongamia and only a very few genes have been identified, our immediate focus is on gene identification and cloning.

Genomics and Gene Discovery

The ability to sequence whole genomes quickly, accurately, and inexpensively has advanced considerably in recent years with the development of the next generation sequencing methodologies. We created two data sets of Pongamia genome consisting of millions of copies of short paired-end "reads" of 36 and 75 bp using the Illumina Solexa Genome Analyzer (GAII), which uses a technique in which the sequencing templates are immobilized on a flow cell surface followed by solid phase amplification to create clusters of identical copies of DNA molecules. These reads can be quickly and efficiently assembled to create large contigs, often with the support of a fully sequenced and annotated reference genome (e.g., soybean).

Using the above datasets, a gene discovery program in Pongamia targeted at identifying and sequencing key genes needed for tree improvement has been started. With this aim in mind, three complete FA biosynthesis genes have been identified and sequenced from Pongamia leaf tissue (B. Verwaaijen and S. Kazakoff, personal communication, 2009). Two of these genes, *PpKASII* and *PpSAD*, are responsible of the production of stearic and oleic acid in the plastids and the third, *PpPDAT*, transfers an acyl group to diacylglycerol to produce triacylglycerides (TAGs) (Fig. 7).

A thorough understanding of the FA biosynthetic pathway and the genes involved is the crucial first step towards any program directed towards improvement of oil composition and content. The biosynthesis process starts during seed development when plants accumulate proteins, oil, and/or starch to provide N, carbohydrates, and energy for seedling establishment. Oilseed plants such as soybean, canola, and Pongamia accumulate TAGs (a major class of glycerolipid) in the seeds instead of starch and therefore are called "oil-seeds" crops (Hildebrand et al., 2008). Sucrose, the product of photosynthesis, provides the precursors for TAG accumulation. Cleavage of sucrose and the glycolysis reactions mark the start of the process. The later glycolytic reactions relevant to FA biosynthesis proceed in the plastid. The first committed step of FA biosynthesis is the formation of malonyl-coenzyme A (CoA) from acetyl-CoA followed by formation of malonyl-acyl carrier protein (ACP) by malonyl-CoA acyltransferase. Malonyl-ACP is elongated in cycles adding two C units at a time. Each cycle starts with a condensation followed by a series of reduction, dehydration, and final reduction reactions synthesizing acyl chains containing up to 18 carbons. Several enzymes are involved in these reactions and are well described in multiple plant species (Dyer et al., 2008). The major FAs of plants have a chain length of 16 or 18 carbons and contain from zero to three *cis*-double bonds. The ideal composition of plant oil for biodiesel production would

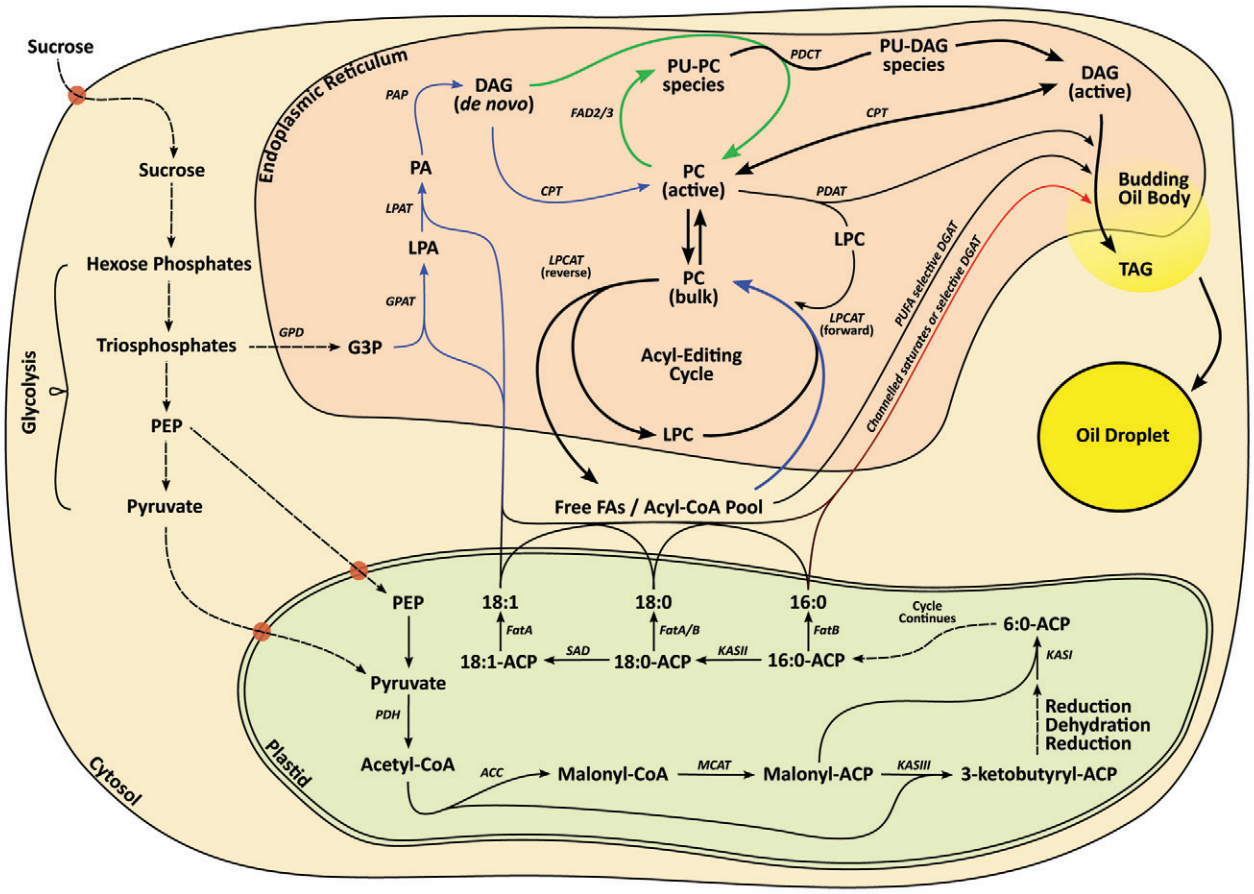

Figure 7. Integrated model of the fatty acid and oil biosynthesis pathway in plants. The key genes of interest for future Pongamia research include ACC (acetyl-coenzyme A [CoA] carboxylase), CPT (cholinephosphotransferase), DGAT (diacylglycerol [DAG] acyltransferase), FAD2 (oleate desaturase), FAD3 (linoleate desaturase), FatA (thioesterase Fat A), FatB (thioesterase Fat B), GPAT (glycerol-3-phosphate [G3P] acyltransferase), GPD (G3P dehydrogenase), KASI (keto-acyl-acyl carrier protein [ACP] synthase I), KASII (keto-acyl-ACP synthase II), KASIII (keto-acyl-ACP synthase III), LPAT (lysophosphatidic acid [LPA] acyltransferase), MCAT (malonyl-CoA acyltransferase), PAP (phosphatidic acid phosphatase), PDAT (phospholipid:DGAT), PDH (pyruvate dehydrogenase), and SAD (stearoyl-Δ9 desaturase). FA, fatty acid; LPC, lysophosphatidylcholine; LPCAT, lysophosphatidylcholine acyltransferase; PA, phosphatidic acid; PC, phosphatidylcholine; PDCT, phosphatidylcholine:diacylglycerol cholinephosphotransferase; PEP, phosphoenolpyruvate; PU-DAG, polyunsaturated diacylglycerides; PUFA, polyunsaturated fatty acid; PU-PC, polyunsaturated phosphatidylcholine; TAG, triacylglyceride.

be one high in monounsaturated FAs and low in PUFAs and saturated FA content. Figure 7 shows the model of FA synthesis and key genes to be considered for genetic transformation (Kazakoff et al., 2011).

While oil biosynthesis is the priority, metabolic pathways such as photosynthetic C assimilation, which determines the yields of plant biomass and hence oil, need to be investigated. Photosynthesis consists of the light reactions where small carbohydrates are manufactured from CO_2 using the adenosine triphosphate and nicotinamide adenine dinucleotide phosphate produced in the chloroplast and the Calvin-Benson cycle where CO_2 is fixed by the enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO). Ribulose-1,5-bisphosphate carboxylase oxygenase activity is the rate-limiting step in the C-assimilating photosynthetic pathway and correlations between RuBisCO activity and total plant growth has been documented (Spreitzer and Salvucci, 2002; Suzuki et al., 2009).

In higher plants, RuBisCO is a hetero-16-mer consisting of a core of four large subunits (RbcL) (50–55 kDa) encoded by a single chloroplast gene (*RbcL*) and four small subunits (RbcS) (12–18 kDa) at each end encoded by a light-regulated multigene family in the nuclear genome (Gutteridge and Gatenby, 1995; Spreitzer and Salvucci, 2002). The assembled RuBisCO is activated by carbamylation of an active site lysine residue and binding of a Mg ion cofactor before it is fully functional in its primary catalytic roles. Using the above-mentioned Illumina datasets we sequenced and annotated the complete coding sequences for *RbcL* and a copy of one *RbcS* in Pongamia by sequence similarity to characterized *G. max RbcL* and *RbcS* orthologues (J. Vogt, personal communication, 2010).

Our interest in nodulation and N fixation in Pongamia extends to identification of genes involved in the process. For many legumes the products of fixation are the ureides, such as allantoin and allantoic acid, which

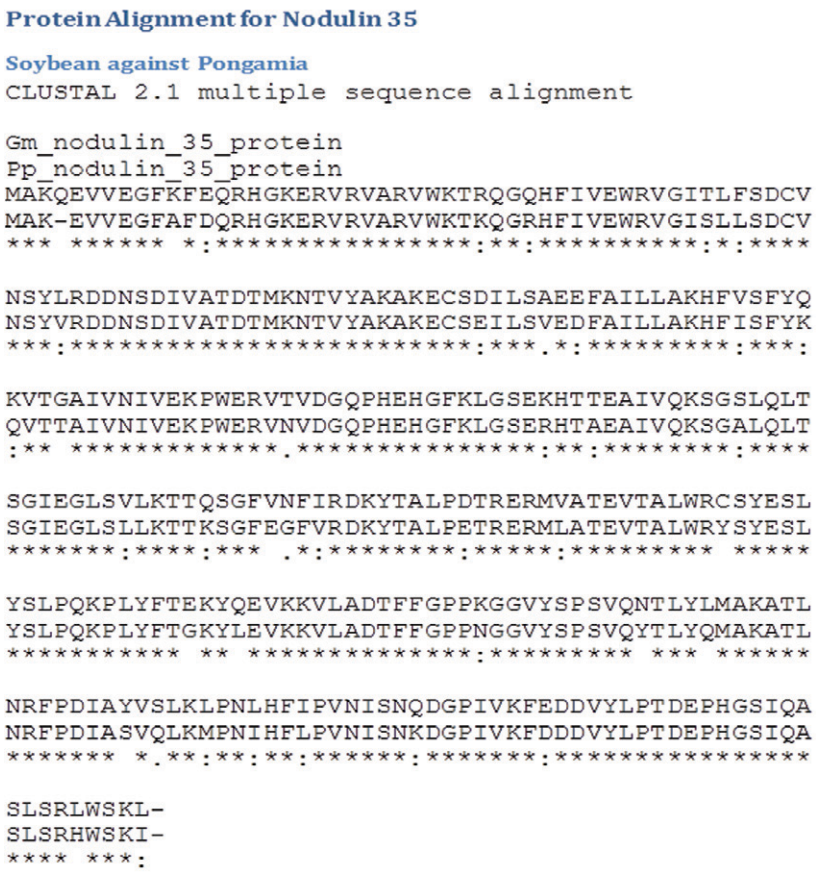

Figure 8. Alignment of amino acid sequences for nodulin 35 from Pongamia and soybean.

are also the dominant long-distance transport forms of N from nodules to the shoot. Using known soybean genes as the reference sequence, we have been able to identify several Pongamia genes involved in this process that encode for allantoate amidohyrolase, allantoinase, xanthine dehydrogenase, and nodulin 35 (Fig. 8).

Organellar Genomes of Pongamia

The chloroplasts and mitochondria of Pongamia are directly involved in C sequestration (photosynthesis) and energy metabolism (respiration) and therefore represent the well-head and powerhouse equivalents of the bioenergy economy of crude oil production. In the biofuel context, leaf mesophyll cells produce sugars (glucose and sucrose) that are stored in the plant either directly or converted to starch, biomass (cellulose), oils, and other metabolites. Upon seed maturation, sucrose translocated to the developing seed is metabolized into components that represent the feedstock for biofuel and co-product synthesis (Fig. 9). We used the Illumina Pongamia dataset in conjunction with a new short-read de novo assembler, called SaSSY (short-read assembly), to assemble and annotate the Pongamia chloroplast DNA (cpDNA) (152,968 bp) and mitochondrial DNA (mtDNA) (425,718 bp) genomes (Kazakoff et al., 2012). These were then compared with the chloroplast and mitochondrial genomes of two other legume species, *Lotus corniculatus* L. var. *japonicus* Regel [syn. *Lotus japonicus* (Regel) K. Larsen] and mung bean [Vig*na radiata* (L.) R. Wilczek] (Fig. 10).

The cpDNA and mtDNA contained 77 and 33 unique protein-coding genes, respectively, making the cpDNA gene rich in comparison to the mtDNA. The cpDNA includes a pair of inverted repeats (IRA and IRB) of 25,528 bp, a large single-copy region of 83,282 bp, and a small single-copy region of 18,511 bp. With a total of eight ribosomal ribonucleic acid (rRNA) genes, 37 transfer ribonucleic acid (tRNA) genes, and 83 protein coding genes, the genome consists of 51.1% protein-coding, 5.9% rRNA, and 1.8% tRNA sequences. As with cpDNA from other species, Pongamia cpDNA was found to be AT rich (i.e., the GC content was 34.8%). Alignment with *L. corniculatus* var. *japonicus* cpDNA showed a pairwise identity of 82.4%. However, the Pongamia cpDNA also contained a small inversion of approximately 6.5 kb, which appears to be unique when compared with other species of flowering plants (Kazakoff et al., 2012).

The Pongamia mtDNA was much more complex and contained two sets of IRs and two sets of direct repeats. The first set of IRs, were each 6229 bp whilst the second set were each 2274 bp. There were 37 protein-coding genes, three rRNA genes, 24 tRNA genes, and seven pseudogenes, gene density of 8.0% protein-coding, 1.2% rRNA, and 0.4% tRNA gene sequences, and a GC content of 45.0%. Both *L. corniculatus* var. *japonicus*

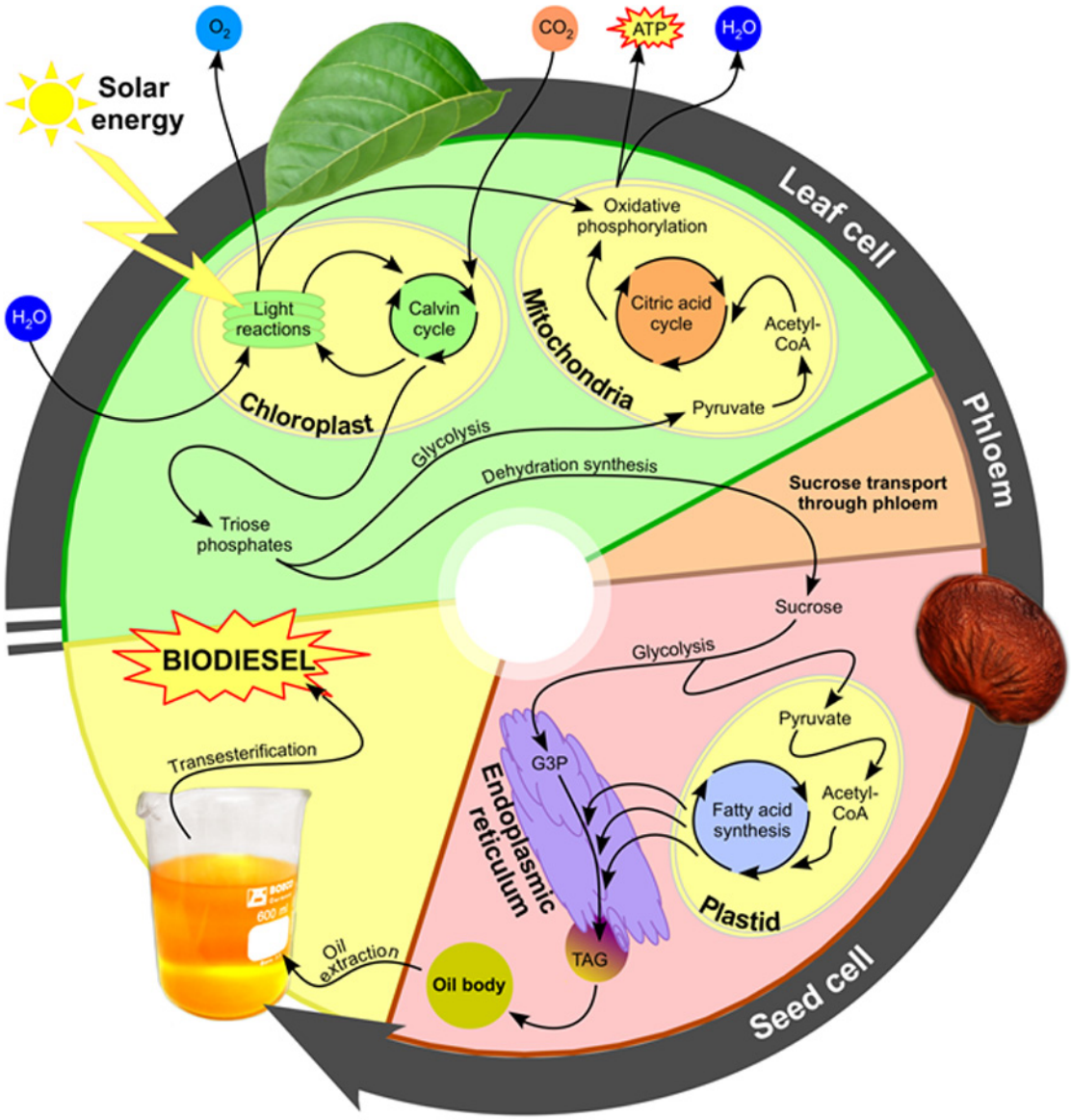

Figure 9. Schematic diagram of oil biosynthesis in Pongamia from the capture of solar energy in the leaves (chloroplasts) through the metabolism of intermediates in the various organelles (plastids, mitochondria, and endoplasmic reticulum) to the storage of oil in the seed. ATP, adenosine triphosphate; CoA, coenzyme A; G3P, glycerol-3-phosphate; TAG, triacylglyceride.

and Pongamia mtDNA contained duplicated genes that resided on repeat regions. However, the genes that were found on the *L. corniculatus* var. *japonicus* mtDNA were different to those found on the repeat regions of the Pongamia mtDNA (Kazakoff et al., 2012).

Pongamia Improvement Program

In addition to the selection and detection of naturally occurring variants, other methods such as induced mutagenesis and M2 and M3 selection (long term) or targeted gene transfer using overexpression or ribonucleic acid (RNA) suppression (e.g., RNA interference [RNAi] or virus-induced gene silencing [VIGS]) need to be considered for genetic improvement of Pongamia germplasm. Even though Pongamia has many desirable characteristics, further improvement is important if this tree is to be domesticated for commercial production of biodiesel and aviation biofuel. Unlike most of today's crop plants, which have gone through thousands of years of selection, breeding, and domestication to achieve superior varieties, Pongamia lacks this sort of time frame, because of the impending peak oil crisis, and has to rely in part on genetic transformation for quick results in the area of plant improvement (Gressel, 2008). Moreover, as a tree with a long life cycle Pongamia breeding programs are more difficult and time consuming. Genetic transformation, a tool that was previously considered challenging, is now routinely used for improvement of all major plants of interest, including woody trees such as fruit trees (Gambino and Griboudo, 2012). In Pongamia genetic transformation is predicted to accelerate the process of domestication and germplasm improvement by either

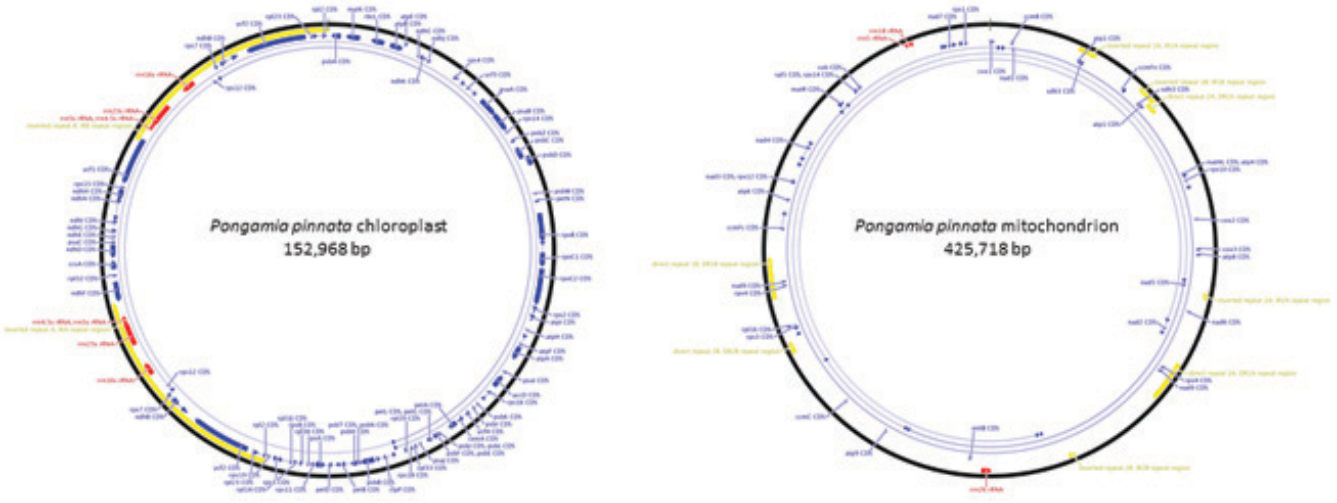

Figure 10. Maps of the Pongamia chloroplast (left) and mitochondrial (right) genomes with the respective locations of the annotated genes.

introducing and expressing a foreign gene into the plant or manipulating its existing genome.

For biodiesel production, the monounsaturated FA oleic acid ($C_{18:1}$) is most desirable as it is capable of producing a low cloud point biofuel with high oxidative stability. At the same time a reduction in the PUFAs such as linoleic acid ($C_{18:2}$), which has a large effect on the auto-oxidation of biodiesel, will ensure complete combustion during ignition. Reducing the saturated FA content is essential for improving cold weather performance properties of biodiesel. Pongamia oil is naturally high in oleic acid and low in PUFAs and saturated FAs (Table 1). However, with a pour point of –2°C and a cloud point of just 5°C, the low temperature flow properties of Pongamia FAMEs need improvement to find a market in colder regions (Joshi and Pegg, 2007). The long chain saturated FA molecules such as palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), and behemic acid ($C_{22:0}$) are less mobile and a reduction in these would significantly improve the pour and cloud points of Pongamia FAMEs, achieving a more desirable cold weather fuel.

Modifications of the FA profile can be achieved by either up- or downregulating expression of genes involved in FA biosynthesis. Upregulating the expression of a gene usually involves the incorporation of an extra copy of a gene of interest under the regulation of either its cognate promoter or a promoter of a different expression pattern (Fang et al., 1989). The repression of gene expression can be accomplished by a number of methods (e.g., RNAi). Here, Pongamia can benefit from work done on other oilseed crops where large changes to the FA composition have been made through targeted gene repression. In many plant species inhibition of the synthesis of linoleic acid has been successfully achieved by hairpin RNA mediated gene silencing (e.g., seed specific silencing of *FAD2*) (Thelen and Ohlrogge, 2002). In soybean the expression of *FAD2* and *FatB*, responsible for the production of palmitic and stearic acids, were simultaneously downregulated in a seed-specific fashion to generate superior seed oil for biodiesel production (Buhr et al., 2002; Duffield et al., 1998). So far, attempts at changes in oil composition of the seeds through targeted gene manipulations have produced far greater success than attempts at increasing oil content. Increases in oil content, however, can be achieved through other methods such as improved plant growth and vigor, early and biannual flowering, and disease and pest controls, allowing plants to produce larger number and bigger seeds.

On a more practical note, the storage of Pongamia seeds for extended periods of time raises the question of whether the quantity and composition of oil will be adversely impacted. Preliminary experiments in our laboratory suggest that this is unlikely to be an issue for Pongamia. In 2012 the protein and oil contents were determined from seeds that were harvested from a single tree in 2009, 2010, and 2011. The oil content was 27.2, 25.7, and 33.0%, respectively, for these 3 yr and similarly the protein content was 27.1, 27.2, and 32.4. In any event it is not expected that Pongamia seed will be stored for lengthy periods before they are processed for fuel production. In addition, it is expected that future Pongamia plantations will include germplasm that will flower and mature seeds over an extended period to enable harvesting and production to occur throughout the year.

Conclusions

The demand for food, fuel, and fiber continues to increase in parallel with an increasing world population while at the same time set against a backdrop of predicted decreasing access to readily available fossil fuels. To meet this demand the race is on to secure future fuel security through the development of an economically viable and environmentally sustainable biofuels industry. At the present time the limiting factor for the successful deployment of a global biofuels industry is the identification and commercialization of appropriate feedstock species. These feedstocks should yield commercially significant amounts of biomass capable of conversion

to relevant biofuels (e.g., ethanol, biodiesel, aviation jet fuel) and be capable of cultivation on land that does not adversely affect food crop production.

Pongamia pinnata, an as yet to be fully domesticated tree legume, has been identified as a strong candidate species for development as a feedstock for biodiesel and aviation fuel. In addition to the good quality oil that can be harvested from the abundant seeds that are produced on an annual basis, it has the potential to yield a number of commercial viable by-products that result from the biofuel production process. These products include an animal feed supplement arising from the residual seed cake following oil extraction, a source of combustible energy from the waste seedpods, and biochar that could be produced from any or all the components of the waste biomass. The challenge that will see Pongamia established as a sustainable bioenergy crop in the tropics and subtropics is a research and development program that will take this unimproved wild species and produce a tree that will be a reliable and predictable source of oil and other valuable by-products (Murphy et al., 2012). Genetic and genomics tools will play a substantial part in extending our current knowledge of the biology of Pongamia as well as the genetic improvement. For example, genetic marker and DNA sequencing technologies should be used to characterize and describe more fully the extent of genetic diversity amongst the wild populations and link markers with associated phenotypes of interest (e.g., oil content and composition). Similar technologies should also be used to clarify the taxonomic status of Pongamia and determine its comparative genetic relatedness to other legume species. Collectively, functional genomics approaches and technologies in combination with more traditional plant breeding should lay a strong scientific foundation for the future deployment of Pongamia as a productive bioenergy crop.

Acknowledgments

We wish to acknowledge the financial support of the Australian Research Council, Bioenergy Plantations Australia Pty Ltd., Stanwell Corporation, Origin Energy Ltd., Brisbane City Council, and The University of Queensland. The authors gratefully acknowledge the contributions of Cherie Wilkinson, Jessica Vogt, Jasper Koehorst, Zurianti Idris, Eileen Sim, and Bart Verwaaijen.

References

Arpiwi, N.L., G. Yan, E.L. Barbour, and J.A. Plummer. 2013. Genetic diversity, seed traits and salinity tolerance of *Millettia pinnata* (L.) Panigrahi, a biodiesel tree. Genet. Resour. Crop Evol. 60:677–692. doi:10.1007/s10722-012-9866-y

Azam, M.M., A. Waris, and N.M. Nahar. 2005. Prospects and potential of fatty acid methyl esters of some non-traditional seed oils for use as biodiesel in India. Biomass Bioenergy 29:293–302. doi:10.1016/j.biombioe.2005.05.001

Baird, L.M., R.A. Virginia, and B.D. Webster. 1985. Development of root nodules in a woody legume, *Prosopis glandulosa*. Torr. Bot. Gaz. 146:39–43. doi:10.1086/337498

Bala, N., P.K. Sharma, and Lakshminarayana. 1990. Nodulation and nitrogen fixation by salinity-tolerant rhizobia in symbiosis with tree legumes. Agric. Ecosyst. Environ. 33:33–46. doi:10.1016/0167-8809(90)90142-Z

Bassam, B.J., G. Caetano-Anollés, and P.M. Gresshoff. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal. Biochem. 196:80–83. doi:10.1016/0003-2697(91)90120-I

Bassam, B.J., and P.M. Gresshoff. 2007. Silver staining DNA in polyacrylamide gels. Nat. Protoc. 2:2649–2654. doi:10.1038/nprot.2007.330

Becker, K., and H.P.S. Makkar. 2008. *Jatropha curcas*: A potential source for tomorrow's oil and biodiesel. Lipid Technol. 20:104–107. doi:10.1002/lite.200800023

Bessou, C., F. Ferchaud, B. Gabrielle, and B. Mary. 2011. Biofuels, greenhouse gases and climate change. A review. Agron. Sustain. Dev. 31:1–79. doi:10.1051/agro/2009039

Biswas, B., P.T. Scott, and P.M. Gresshoff. 2011. Tree legumes as feedstock for sustainable biofuel production: Opportunities and challenges. J. Plant Physiol. 168:1877–1884. doi:10.1016/j.jplph.2011.05.015

Bora, D.K., and D.C. Baruah. 2012. Assessment of tree seed oil biodiesel: A comparative review based on biodiesel of a locally available tree seed. Renew. Sustain. Energy Rev. 16:1616–1629. doi:10.1016/j.rser.2011.11.033

BP. 2012. BP statistical review of world energy. June 2012. BP plc, London, UK. http://www.bp.com/content/dam/bp/pdf/Statistical-Review-2012/statistical_review_of_world_energy_2012.pdf (accessed 1 Oct. 2012).

Buhr, T., S. Sato, F. Ebrahim, A. Xing, Y. Zhou, M. Mathiesen, B. Schweiger, A.J. Kinney, P. Staswick, and T. Clemente. 2002. Ribozyme termination of RNA transcripts down-regulate seed fatty acid genes in transgenic soybean. Plant J. 30:155–163. doi:10.1046/j.1365-313X.2002.01283.x

Bureau of Rural Sciences. 2002. Land use mapping at catchment scale – Principles, procedures and definitions. Edition 2. Department of Agriculture, Food and Fisheries, Canberra, Australia.

Caetano-Anollés, G., B.J. Bassam, and P.M. Gresshoff. 1991. DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. Nat. Biotechnol. 9:553–557. doi:10.1038/nbt0691-553

Canakci, M., and H. Sanli. 2008. Biodiesel production from various feedstocks and their effects on the fuel properties. J. Ind. Microbiol. Biotechnol. 35:431–441. doi:10.1007/s10295-008-0337-6

Crutzen, P.J., A.R. Mosier, K.A. Smith, and W. Winiwarter. 2008. N_2O release from agro-biofuel production negates global warming reduction by replacing fossil fuels. Atmos. Chem. Phys. Discuss. 8:389–395. doi:10.5194/acp-8-389-2008

Daniel, J.N. 1997. Pongamia pinnata – A nitrogen fixing tree for oilseed, NFT Highlights, Nitrogen Fixing Tree Association (NFTA) 97-03. Fact Net, Winrock International, Morrilton, AR.

Dantas, M.B., M.M. Conceição, V.J. Fernandes Jr., N.A. Santos, R. Rosenhaim, A.L.B. Marques, I.M.G. Santos, and A.G. Sousa. 2007. Thermal and kinetic study of corn biodiesel obtained by the methanol and ethanol routes. J. Therm. Anal. Calorim. 87:835–839. doi:10.1007/s10973-006-7780-2

Duffield, J., H. Shapouri, M. Graboski, R. McCormick, and R. Wilson. 1998. US biodiesel development: New markets for conventional and genetically modified agricultural products. Economic Research Service, USDA, Washington, DC.

Dyer, J.M., S. Stymne, A.G. Green, and A.S. Carlsson. 2008. High-value oils from plants. Plant J. 54:640–655. doi:10.1111/j.1365-313X.2008.03430.x

Erisman, J.W., H. van Grinsven, A. Leip, A. Mosier, and A. Bleeker. 2010. Nitrogen and biofuels; An overview of the current state of knowledge. Nutr. Cycling Agroecosyst. 86:211–223. doi:10.1007/s10705-009-9285-4

Fang, R.X., F. Nagy, S. Sivasubramania, and N.H. Chua. 1989. Multiple cis regulatory elements for maximal expression of the cauliflower mosaic virus 35S promoter in transgenic plants. Plant Cell 1:141–150. doi:10.1105/tpc.1.1.141

Farine, D.R., D.A. O'Connell, J.R. Raison, B.H. May, M.H. O'Connor, D.F. Crawford, A. Herr, J.A. Taylor, T. Jovanovic, P.K. Campbell, M.I.A. Dunlop, L.C. Rodriguez, M.L. Poole, A.L. Braid, and D. Kriticos. 2012. An assessment of biomass for electricity and biofuel, and for greenhouse gas emission reduction in Australia. Global Change Biol. Bioenergy 4:148–175. doi:10.1111/j.1757-1707.2011.01115.x

Ferguson, B.J., A. Indrasumunar, S. Hayashi, M.-H. Lin, Y.-H. Lin, D.E. Reid, and P.M. Gresshoff. 2010. Molecular analysis of legume nodule

development and autoregulation. J. Integr. Plant Biol. 52:61–76. doi:10.1111/j.1744-7909.2010.00899.x
Gambino, G., and I. Griboudo. 2012. Genetic transformation of fruit trees: Current status and remaining challenges. Transgenic Res. 21:1163–1181. doi:10.1007/s11248-012-9602-6
Gressel, J. 2008. Transgenics are imperative for biofuel crops. Plant Sci. 174:246–263. doi:10.1016/j.plantsci.2007.11.009
Gutteridge, S., and A.A. Gatenby. 1995. Rubisco synthesis, assembly, mechanism, and regulation. Plant Cell 7:809–819. doi:10.1105/tpc.7.7.809
Hennesy, K., R. Fawcett, D. Kirono, F. Mpelasoka, D. Jones, J. Bathols, P. Whetton, M. Stafford Smith, M. Howden, C. Mitchell, and N. Plummer. 2008. Commonwealth Scientific and Industrial Research Organization and Bureau of Meteorology Report (July 2008). Australian Government, Bureau of Meteorology, Canberra, Australia.
Hildebrand, D.F., R. Li, and T. Hatanaka. 2008. Genomics of soybean oil traits. In: G. Stacey, editor, Genetics and genomics of soybean. Springer, New York, NY. p. 185–209.
Hill, J., E. Nelson, D. Tilman, S. Polasky, and D. Tiffany. 2006. Environmental, economic and energetic costs and benefits of biodiesel and ethanol biofuels. Proc. Natl. Acad. Sci. USA 103:11206–11210. doi:10.1073/pnas.0604600103
Hu, Q., M. Sommerfeld, E. Jarvis, E. Ghirardi, M. Posewitz, M. Seibert, and A. Darzins. 2008. Microalgal triacylglyerols as feedstocks for biofuel production: Perspectives and advances. Plant J. 54:621–639. doi:10.1111/j.1365-313X.2008.03492.x
Imahara, H., E. Minami, and S. Saka. 2006. Thermodynamic study on cloud point of biodiesel with its fatty acid composition. Fuel 85:1666–1670. doi:10.1016/j.fuel.2006.03.003
Jensen, E.S., M.B. Peoples, R.M. Boddey, P.M. Gresshoff, H. Hauggaard-Nielsen, B.J.R. Alves, and M.J. Morrison. 2011. Legumes for mitigation of climate change and feedstock in a bio-based economy – A review. Agron. Sustain. Dev. 32:329–364. doi:10.1007/s13593-011-0056-7
Jiang, Q., S.-H. Yen, J. Stiller, D. Edwards, P.T. Scott, and P.M. Gresshoff. 2012. Genetic, biochemical, and morphological diversity of the legume biofuel tree *Pongamia pinnata*. J. Plant Genome Sci. 1:54–67. doi:10.5147/jpgs.2012.0084
Joshi, R.M., and M.J. Pegg. 2007. Flow properties of biodiesel fuel blends at low temperatures. Fuel 86:143–151. doi:10.1016/j.fuel.2006.06.005
Kaushik, N., S. Kumar, K. Kumar, R.S. Beniwal, N. Kaushik, and S. Roy. 2007. Genetic variability and association studies in pods and seeds of *Pongamia pinnata* (L.) Pierre in Haryana, India. Genet. Resour. Crop Evol. 54:1827–1832. doi:10.1007/s10722-006-9204-3
Kazakoff, S.H., P.M. Gresshoff, and P.T. Scott. 2011. *Pongamia pinnata*, a sustainable feedstock for biodiesel production. In: N.G. Halford and A. Karp, editors, Energy crops. Royal Society for Chemistry, Cambridge, UK. p. 233–258.
Kazakoff, S.H., M. Imelfort, D. Edwards, J. Koehorst, B. Biswas, J. Batley, P.T. Scott, and P.M. Gresshoff. 2012. Capturing the biofuel wellhead and powerhouse: The chloroplast and mitochondrial genomes of the leguminous feedstock tree *Pongamia pinnata*. PLoS ONE 7:1687–1699. doi:10.1371/journal.pone.0051687
Kesari, V., A. Krishnamachari, and L. Rangan. 2008. Systemic characterisation and oil seed analysis in candidate plus trees of biodiesel plant, *Pongamia pinnata*. Ann. Appl. Biol. 152:397–404. doi:10.1111/j.1744-7348.2008.00231.x
Kesari, V., A.M. Ramesh, and L. Rangan. 2013. *Rhizobium pongamiae* sp. nov. from root nodules of *Pongamia pinnata*. Biomed. Res. Int. doi:10.1155/2013/165198
Kesari, V., V.M. Sathyanarayana, A. Parida, and L. Rangan. 2010. Molecular marker-based characterization in candidate plus trees of *Pongamia pinnata*, a potential biodiesel legume. AoB Plants 2010:plq017. doi:10.1093/aobpla/plq017
Marshall, D.J., A. Hayward, D. Eales, M. Imelfort, J. Stiller, P.J. Berkman, T. Clark, M. McKenzie, K. Lai, C. Duran, J. Batley, and D. Edwards. 2010. Targeted identification of genomic regions using TAGdb. Plant Methods 6:1–6. doi:10.1186/1746-4811-6-19
Mosser, B.R. 2009. Biodiesel production, properties and feedstocks. In Vitro Cell. Dev. Biol. Plant 45:229–266. doi:10.1007/s11627-009-9204-z
Murphy, H.T., D.A. O'Connell, G. Seaton, R.J. Raison, L.C. Rodriguez, A.L. Braid, D.J. Kriticos, T. Jovanovic, A. Abadi, M. Betar, H. Brodie, M. Lamont, M. McKay, G. Muirhead, J. Plummer, N.L. Arpiwi, B. Ruddle, P.T. Scott, C. Stucley, B. Thistlethwaite, B. Wheaton, P. Wylie, and P.M. Gresshoff. 2012. A common view of the opportunities, challenges and research actions for Pongamia in Australia. Bioenergy Res. 5:778–800. doi:10.1007/s12155-012-9190-6
Myers, B.A., D.W. West, L. Callinan, and C.C. Hunter. 1995. Long term effects of saline irrigation on the yield and growth of mature Williams pear trees. Irrig. Sci. 16:35–46. doi:10.1007/BF00208394
Odeh, I., D. Tan, and T. Ancev. 2011. Potential suitability and viability of selected biodiesel crops in Australian marginal agricultural lands under current and future climates. Bioenergy Res. 4:165–179. doi:10.1007/s12155-010-9110-6
Orwa, C., A. Mutua, R. Kindt, R. Jamnadass, and A. Simons. 2009. Agroforestry database: A tree reference and selection guide. Version 4.0. World Agroforestry Centre, Nairobi, Kenya. http://www.worldagroforestry.org/af/treedb/ (accessed 8 Mar. 2010).
Patil, S.G., M. Hebbara, and S.B. Devarnavadagi. 1996. Screening of multipurpose trees for saline vertisols and their bioameliorative effects. Ann. Arid Zone 35:57–60.
Raheman, H., and A.G. Phadatare. 2004. Diesel engine emissions and performance from blends of Karanja methyl ester and diesel. Biomass Bioenergy 27:393–397. doi:10.1016/j.biombioe.2004.03.002
Rasul, A., E.L.D. Amalraj, G.P. Kumar, M. Grover, and B. Venkateswarlu. 2012. Characterisation of rhizobial isolates nodulating *Milletia pinnata* in India. FEMS Microbiol. Lett. 336:148–158. doi:10.1111/1574-6968.12001
Sahoo, D.P., S. Aparajita, and G.R. Rout. 2010. Inter and intra-population variability of *Pongamia pinnata*: A bioenergy legume tree. Plant Syst. Evol. 285:121–125. doi:10.1007/s00606-009-0254-9
Samuel, S., P.T. Scott, and P.M. Gresshoff. 2013. Nodulation in the legume biofuel feedstock tree *Pongamia pinnata*. Agric. Res. 2:207–214. doi:10.1007/s40003-013-0074-6
Sarin, R., M. Sharma, S. Sinharay, and R.K. Malhotra. 2007. Jatropha-Palm biodiesel blends: An optimum mix for Asia. Fuel 86:1365–1371. doi:10.1016/j.fuel.2006.11.040
Scott, P.T., L. Pregelj, N. Chen, J.S. Hadler, M.A. Djordjevic, and P.M. Gresshoff. 2008. *Pongamia pinnata*: An untapped resource for the biofuels industry of the future. Bioenergy Res. 1:2–11. doi:10.1007/s12155-008-9003-0
Sharma, S.S., M.S. Negi, P. Sinha, K. Kumar, and S.B. Tripathi. 2010. Assessment of genetic diversity of biodiesel species *Pongamia pinnata* accessions using AFLP and three endonuclease-AFLP. Plant Mol. Biol. Rep. 29:12–18. doi:10.1007/s11105-010-0204-2
Spreitzer, R.J., and M.E. Salvucci. 2002. Rubisco: Structure, regulatory interactions, and possibilities for a better enzyme. Annu. Rev. Plant Biol. 53:449–475. doi:10.1146/annurev.arplant.53.100301.135233
Sprent, J.I., and R. Parsons. 2000. Nitrogen fixation in legume and non-legume trees. Field Crops Res. 65:183–196.
Smith, K.A., and T.D. Searchinger. 2012. Crop-based biofuels and associated environmental concerns. Glob. Change Biol. Bioenergy 4:479–484. doi:10.1111/j.1757-1707.2012.01182.x
Srivastava, A., and R. Prasad. 2000. Triglycerides-based diesel fuels. Renew. Sustain. Energy Rev. 4:111–128.
Sujatha, K., B.M. Panda, and S. Hazra. 2008. De novo organogenesis and plant regeneration in *Pongamia pinnata*, oil producing tree legume. Trees (Berl.) 22:711–716. doi:10.1007/s00468-008-0230-y
Suzuki, Y., T. Miyamoto, R. Yoshizawa, T. Mae, and A. Makino. 2009. Rubisco content and photosynthesis of leaves at different positions in transgenic rice with an overexpression of *RBCS*. Plant Cell Environ. 32:417–427. doi:10.1111/j.1365-3040.2009.01937.x
Thelen, J.J., and J.B. Ohlrogge. 2002. Metabolic engineering of fatty acid biosynthesis in plants. Metab. Eng. 4:12–21. doi:10.1006/mben.2001.0204
Tomar, O.S., and R.K. Gupta. 1985. Performance of some forest tree species in saline soils under shallow and saline water-table conditions. Plant Soil 87:329–335. doi:10.1007/BF02181900
Zou, N., P.J. Dart, and N.E. Marcar. 1995. Interaction of salinity and rhizobial strain on growth and N2-fixation by *Acacia ampliceps*. Soil Biol. Biochem. 27:409–413. doi:10.1016/0038-0717(95)98611-Q