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Development of Transgenic Teak (*Tectona grandis*) Expressing a *cry*1Ab Gene for Control of the Skeletoniser

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Abstract. Teak (*Tectona grandis*) is one of the exotic forest species that has been planted in Malaysia. Teak skeletoniser, *Paliga damastesalis*, causes minor to considerable damage to teak trees in plantations. We have utilised and modified a cloned *cry*1Ab gene to construct a chimeric gene capable of expression in plant cells. Using the Biolistic® particle delivery system, we then transferred the *cry*1Ab gene into teak cells and regenerated transgenic plants. The presence and integration of the introduced genes was confirmed by growth of tissue on selection media (MS-10Hm), and by molecular analyses, i.e. Polymerase Chain Reaction (PCR) and PCR-Southern Blotting. PCR analyses for the *cry*1A(b) gene was carried out on 90 putative transformed teak plants. The expected internal fragment of 746bp for *cry*1Ab gene was successfully amplified from some of the transformants. The PCR-Southern analyses confirmed the presence of this gene, and RT-PCR analyses confirmed the expression of this transgene in the positive transformants. A bioassay result showed that about 57 cm^2 - 66 cm^2 of the non-transgenic leaves area showed skeletonisation, whereas only 3 cm^2 - 6 cm^2 of the transgenic leaves were skeletonised. The size of the larvae feeding on the control teak leaves increased about 13 to 18 mm. In contrast, larvae feeding on transgenic teak leaves were significantly smaller, only 5 to 8 mm. Currently the transgenic teak trees have been successfully planted in a greenhouse at the Forest Research Institute of Malaysia (FRIM)'s nursery.

Keywords: Biolistic® system, *cry*1Ab gene, Skeletoniser, *Tectona grandis*, Transgenic teak.

INTRODUCTION

Teak (*Tectona grandis*) is indigenous to peninsular and central India, Myanmar, Indonesia, Thailand, and Laos, and has also emerged as a major hardwood plantation species in a number of other countries in Africa, West Indies, and Sri Lanka (Mohanan *et al*., 1997). Teak is a slow growing species, and tree improvement is therefore a long-term investment but the species is easily established in plantation. The rotation age is traditionally high (60-100 years) (Kjaer and Foster, 1996). Application of improved material would reduce the rotation age, because of an improved growth rate. The rotation age of teak plantings grown for traditional products could be reduced to 40-50 years by the use of improved planting stock. Still, 10-15 years will be required from the initiation of an improvement program before the first improved seeds are available, and another 40-50 years will be required before the timber from the first rotation of improved planting stock is harvested (Kjaer and Foster, 1996).

Teak has been planted in Malaysia for a considerable number of years and many systematic trials have been carried out in the north of West Malaysia and East Malaysia (Tee, 1995). However, teak plantations in Malaysia are confronted with problems including susceptibility of the species to various pests and diseases (Chey, 2000). Among them is the teak skeletoniser *Paliga damastesalis* Walter, which is a different species from the teak skeletonisers found in India and Myanmar (Intachat, 1998).

Paliga damastesalis is the most common teak defoliator species in Malaysia. Low levels of defoliation are usually observed throughout the year although *P. damastesalis* has been reported to cause heavy defoliation on teak trees in the plantations in Malaysia (Chey, 1996). Heavy defoliation, as high as 100%, has been recorded during serious outbreaks, however these outbreak have been observed to be seasonal, occurring more during wetter periods (Chey, 2000). Skeletonisation of leaves caused by the teak *P. damastesalis* larvae is a serious problem in teak plantations, recurring yearly during the flushing of new leaves (Intachat, 1999).

Biotechnological advancements in crop improvement through genetic transformation have attracted great attention from both the scientific and lay communities. This is as true for forestry as it is for agriculture. Compared with the advances made in agricultural biotechnology, which can now be seen looking back at more than twenty years of successful commercial application, genetic transformation for forestry has lagged behind. This is mainly due to the availability of far fewer resources, longer rotation times of the trees and significant hurdles to overcome with regards to efficient plant

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tissue culture and propagation techniques. The more recent development of efficient plant tissue culture techniques has allowed forestry to emulate what has been achieved for agricultural and horticultural species. While there have been major advances with the conventional breeding, there are some desirable traits that are not available in the tree species of choice. Possible traits of interest include herbicide and insect resistance, and modified lignin and cellulose content (Hu *et al.*, 1999; Bishop-Hurley *et al.*, 2001; Pilate at el., 2002; Grace *et al.*, 2005).

Bacillus thuringiensis (Bt) has proved to be a remarkable source of the insecticidal protein genes used for transgenic expression in crop species (Kumar, 2003). During the sporulation phase, Bt produces crystalline inclusions that consist of proteins about 130kDa in size known as δ-endotoxins or *cry*stal (Bt/Cry) toxins. The incorporation of Bt/*cry* genes that code for the production of insecticidal toxin into plants reduces many problems associated with the use of chemical pesticides, as the toxins are produced continuously within the plants. A gene encoding the Cry toxin (*cry* gene) has been successfully introduced into various trees including poplars (McCown *et al.*, 1991), conifer (*Picea glauca*) (Ellis *et al.*, 1993) and pine (*Pinus radiata*) (Walter *et al.*, 1994).

Trees expressing Bt transgenes may be preferable to the use of spray applications for several reasons. First, vegetation, soil and water surrounding the crop are not exposed to spray drift. Susceptible, non-target, insects in areas adjacent to the transgenic crop would not be exposed, reducing the potential for development of Bt resistance. Second, spray applications quickly degrade, persisting on leaves for, at most, a few days (Thompson *et al.*, 1995; James *et al.*, 1999). Genetically engineered trees, however, can produce the toxin continuously, thereby avoiding sensitivities to application timing and the costs associated with repeated application. Finally, because transgenic trees produce the toxin within plant tissues, it is possible to affect insects which reside within the plant, such as shoot borers and leaf folders.

At Forest Research Institute Malaysia (FRIM) we have successfully developed a transformation system for teak, using *gus* as a marker gene (Norwati *et al.*, 2007). We report the production of a transgenic teak plant expressing the *cry*1Ab gene encoding the Bt-toxin and its effect on a Lepidopteran pest, *P. damastesalis* larvae. We hope that good planting materials can thus be produced for teak plantation programmes.

MATERIALS AND METHODS

Plant materials Nodal segments, the target tissues used in this study, were provided by the Tissue Culture Laboratory, FRIM. Cultures were subcultured at monthly intervals by transferring nodal segments onto hormone-free MS medium (Murashige and Skoog, 1962). Nodal segments were maintained in a growth chamber for 2 weeks prior to bombardment (Norwati *et al.*, 2007).

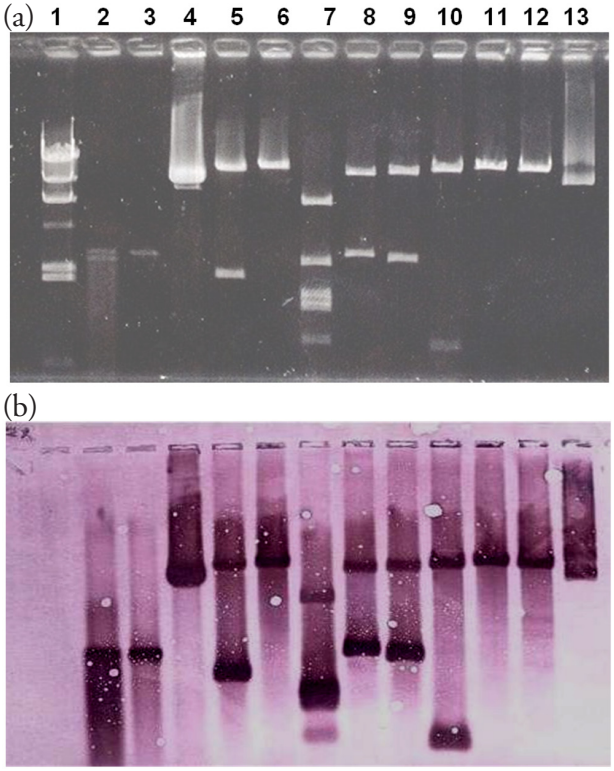

Figure 1. Agarose gel electrophoresis (b) and Southern Blot hybridization (b) of pCAMSB1 plasmid digested with different restriction enzymes to prove the presence of the cloned *cry*1AB gene. Lane 1: λ /*Hind*III marker, Lane 2: pSB/*Hind*III, Lane 3: Insert DNA, Lane 4: pCAMBIA1301 uncut, Lane 5: pCAMSB1/*BamH*I, Lane 6: pCAMSB1/*Eco*RI, Lane 7: pCAMSB1/*Eco*RV, Lane 8: pCAMSB1/*Hind*III, Lane 9: pCAMSB1/*Pst*I, Lane 10: pCAMSB1/*Sac*I, Lane 11: pCAMSB1/*Sal*I, Lane 12: pCAMSB1/*Sma*I, Lane 13: pCAMSB1 uncut.

Plasmid DNA isolation The pCAMSB1 plasmid (Norwati and Abdullah, 1998) carrying the *cry*1Ab gene was used in all transformation experiments. This plasmid also carries β*–glucuronidase* (*uidA*) and *hygromycin phosphotransferase* (*hpt*) as a reporter and a selectable marker gene, respectively. The pCAMSB1 plasmid was isolated from *Escherichia coli* HB101 using a Plasmid Preparation Kit (Pharmacia, USA).

Biolistic transformation DNA for bombardment experiments was isolated using the Plasmid Preparation Kit (Pharmacia, USA). The biolistic particle delivery device (PDS 1000He, BioRad) was used for all transformation experiments. Gold particles (1.6 μm) were coated with DNA (Sanford *et al.*, 1993; Walter *et al.*, 1994) and were bombarded into target tissues using the following conditions: rupture disc-pressure, 1100 psi; gap-distance from rupture disc to macrocarrier, 6 mm; macrocarrier travel distance, 16 mm; microcarrier travel distance, 6 cm.

Isolation of DNA and RNA Genomic DNA was isolated from leaves of regenerated bombarded plants (Doyle and Doyle, 1987), purified using the High Pure PCR Template Preparation Kit (Roche Diagnostics, GmbH, Mannheim,

Figure 2. Selection and regeneration of putative transformed teak. **(a)** Selection of putative transformants on hygromycin-containing media. **(b)** Plantlets undergoing rooting on rooting media. **(c)** Rooted plants acclimatized on sand bed. **(d)** Plants hardened in polybags in a sheltered nursery.

extracted using the Plant RNA Extraction Maxi s-Prep Kit (GeneTACG, BioScience, USA) and was either used directly for RT-PCR or stored at -80°C until use.

PCR analysis PCR reactions were carried out in 1xPCR buffer containing 1.0 mM dNTPs, 2 U *Taq* DNA polymerase (Roche Diagnostics), 50 pmoles primer and 100 ng of template DNA. PCR analysis to show stable transformation was based on the amplification of 746 bp fragments of the *cry*1Ab gene. The primers for detecting *cry*1Ab have a sequence of 5'-GCG AGT TCG TGC CAG GTG CTG GG-3' and 5'-CGC TGA ATC CAA CTG GAG AGG CC-3'. The reaction consisted of 30 cycles of 1 minute annealing at 63°C, 2 minute elongation at 72°C and 1 minute denaturation at 95°C. The programme was started and ended with 5 minutes at 95°C and 5 miutes at 72°C. For a positive control, 0.1 ng pCAMSB1 DNA was used, and for negative control, DNA extracted from non-transgenic plant material was used. PCR products were separated on 1.0% (w/v) agarose gel, electrophoresed at 100 V for 30 minutes and later stained with ethidium bromide (0.5 µg/ml) before being photographed using Image Analyzer, FluorChem™ 8800, USA.

Reverse transcriptase-polymerase chain reaction (RT-PCR) cDNAs were synthesised using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). For the construction of the first-strand cDNA from RNA, the desired amount of RNA (normally 1 – 5 µg) was first added to an oligo-dT primer (0.5 µg/µl) or a specific primer for *cry*1Ab gene (15 – 20 pmol) and adjusted with nuclease free-deionised water, to a final volume of 12 µl. The mixture was then denatured at 70°C for 5 minutes and then instantly cooled on ice. Subsequently, other reaction components were added: 4 µl 5x reaction buffer; 1 µl RNAse inhibitor (20 U/µl), and 2 µl dNTP mix (10 mM each). The reaction mix was then incubated at 37°C for 5 minutes. 1 µl RevertAid™ M-MulV reverse transcriptase (200 U/µl) was then added and the reaction mixture incubated at 42°C for 1 hour. Subsequently, the reaction was stopped by heating the tubes at 72°C for 10 minutes and the product directly used for amplification through PCR.

PCR-Southern Blotting The presence of the *cry*1Ab gene

fragment in the PCR product was confirmed by a Southern hybridization technique (Southern, 1975). The PCR product for the *cry*1Ab gene was run through a 1.0% agarose gel and transferred onto a nylon membrane. Hybridization was carried out using the DIG-labelled *cry*1Ab gene fragment as a probe. The procedure of labelling and detection were conducted according to the procedure outlined by the supplier (Roche Diagnostics).

Laboratory bioassay The *P. damastesalis* larvae used in this study were provided by FRIM's Entomology Laboratory, and the insect toxicity assay was carried out as described by Barton *et al.* (1987). Leaves from transgenic plants were collected and leaves of untransformed plants were taken as a negative control. Leaf pieces were washed with distilled water, dried for 10 minutes, and the edge of the leaf surrounded with wet cotton and aluminium foil. Then the leaves were placed in a PVC chamber (35cm (W) X 43cm (L) X 46cm (H)), two leaves per chamber. The newly hatched larvae of *P. damastesalis* were placed directly on leaves (15 larvae per chamber) and incubated at 25 ± 2°C for 5 days. Two replicates were conducted. The consumed area of leaf tissue and the larval characteristics and mortality were observed after two days and recorded on the fifth day after inoculation.

RESULTS AND DISCUSSION

Plant transformation and regeneration In this study, we dealt with the production of transgenic teak resistant to *Paliga damastesalis* larvae. Nodal segments of teak were bombarded with a recombinant plasmid named pCAMSB1. This pCAMSB1 plasmid carried a CaMV 35S 5'/*cry*1Ab/nosT 3' expression cassette associated with the *hpt* gene for hygromycin selection and the *gus* gene as a reporter gene (Norwati and Abdullah,1998). To prove the presence of the *cry*1Ab gene, this recombinant plasmid was digested with different restriction enzymes, electrophoresed on 0.8% (w/v) agarose gel and finally Southern-blotted onto a membrane. The results from the Southern blot analysis revealed that the recombinant plasmid was bearing the *cry*1AB gene and was stable in *E. coli* HB101 (Figure 1) and subsequently can be transferred into teak using a biolistic approach.

The biolistic approach to transferring the *cry*1Ab gene into teak tissues is always influenced by a combination of important factors including vacuum, acoustic shock, particle penetration, impact and toxicity, and cell death (Hunold *et al.*, 1994). Many of these factors are specific for the particular experimental set-up, while others appear to be of more general importance. Other general parameters resulting in improved performance of genetic transformation include the reduction of the size of particle aggregates by using post-launch baffles (Russel *et al.*, 1992a). Biolistic techniques have been developed to stably transform species that are difficult to transform using *A. tumefaciens* (Walter *et*

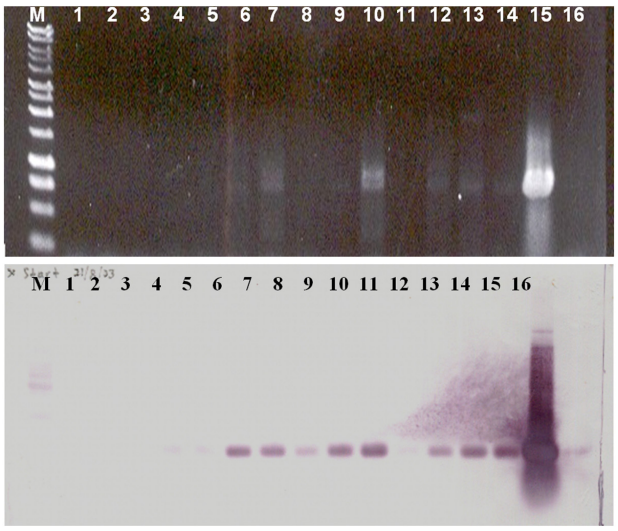

Figure 3. Detection of *cry*1AB gene in putative transformed teak plantlets. **(a)** PCR analysis of genomic DNA from plant tissues derived from bombarded nodal segments using primers specific to the *cry*1Ab gene. **M:** Marker; **Lanes 1-14:** transgenic teak tissues; **Lane 15:** pCAMSB1 DNA (positive control); **Lane 16:** non-transgenic teak tissues (negative control). **(b)** PCR-Southern analysis of transgenic teak plants using DIG labelled probe for *cry*1Ab gene. **M:** Marker; **Lanes 1-14:** transgenic teak tissues; **Lane 15:** pCAMSB1 DNA (positive control); **Lane 16:** non-transgenic teak tissues (negative control).

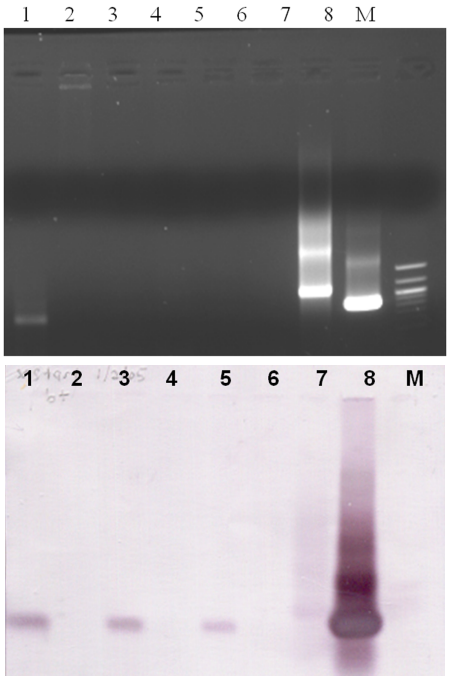

Figure 4. Gene expression analysis of cry1Ab in two selected transgenic teak using RT-PCR (a) and RTPCR-Southern (b). Different concentrations of cDNA template were used in PCR: 1000ng (**Lanes 1, 3 and 5**) and 100ng (**Lane 2, 4 and 6**). **Lanes 1-2:** cDNA from transgenic teak A; primer cry1Ab, **Lanes 3-4:** cDNA from transgenic teak B; primer cry1Ab, **Lanes 5-6:** cDNA from transgenic teak A; primer oligo (dT), **Lane 7:** cDNA from control RNA provided in the Kit, **Lane 8:** pCAMSB1 plasmid (positive control), **M:** DNA size marker.

Table 1. Larval mortality after feeding with transgenic and non-transgenic teak leaves.

Source of the tested leaves for larvae feeding	No. larvae that grazed on the tested leaves	No. dead larvae after feeding on the tested leaves	% dead
Non-transgenic teak	15	5	33
Transgenic teak (A)	15	13	87
	15	12	80
Transgenic teak (B)	15	12	80
	15	11	73

al., 1998, 1999; Find *et al*., 2005; Henderson and Walter, 2006; Trontin *et al*., 2007).

During the regeneration of putative transformed teak, MS media containing 10mg/ml of hygromycin was used as the selection medium (Figure 2a). Screening was carried out twice, since it has been demonstrated that a two step selection protocol, can improved genetic transformation of *Ruta graveolens* L. (Lièvre *et al*., 2005), and surviving putative transformed plants were subcultured on fresh hormone-free MS media for rooting (Figure 2b) prior to transfer onto soil. On MS-10Hm, most explants stopped their growth and development, and turned brown within the first two subcultures. Only about 2/3 of the initially green shoots retained their colour and later produced additional green shoots on MS-10Hm. About 9.6% of the plants acclimatized, hardened and were maintained in the nursery. Overall, only 8% of the plants derived from bombarded nodal segments reached the nursery stage. Putative transformed teak plants showed similar morphology and growth rate to those of non-transformed teak (Figure 2c-2d).

PCR analysis and regeneration of putatively transformed plantlets A direct check for the presence of transferred *cry*1Ab gene in the putatively transformed plantlets was performed using the PCR technique. Isolated genomic DNA from the putative transformed plantlets was used as a PCR template. Primers for the *cry*1Ab gene were designed to amplify an internal 746bp fragment of the *cry*1Ab gene. Following PCR amplification and agarose gel electrophoresis, only 64% of the analysed transgenic teak were positive for the expected 746bp band, whilst the rest showed negative results (Figure 3a). Southern blot analysis carried out using *cry*1Ab DNA probe further confirmed that the PCR-amplified 746bp fragments were the *cry*1Ab gene (Figure 3b).

Several techniques are available to detect gene expression. These include the Northern blot analysis, *in situ* hybridisation and the Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) (William and Jeffrey, 1995). In the RT-PCR analysis, fewer than 10 copies of target DNA are required, and it has been successful when RNA was isolated from a single cell (Razin *et al*., 1991). Because of this high sensitivity, RT-PCR is increasingly being used to quantify small but physiologically relevant changes in genes expression that would otherwise be undetectable.

In this study, the expression of the *cry*1Ab gene in the transformed plants was analyzed using RT-PCR followed by Southern blot. Total RNA was isolated from two putative transformed plants from the nursery. Total RNA (1 – 5µg) was used for cDNA synthesis and were later used as the template for subsequent PCR amplification of the *cry*1Ab gene fragment with different concentrations of cDNA template (1000ng and 100ng). The amplified 746bp *cry*1Ab gene fragment was detected in the agarose gel (Figure 4A). The identity of the PCR product was further confirmed by Southern hybridisation using a *cry*1Ab DNA probe, as shown in Figure 4B. Positive results were shown in Southern hybridization when 1000 ng cDNA was used as template (Figure 4B) although the PCR products were not visible after agarose gel electrophoresis (Lanes 1, 3 and 5, Figure 4A). This result confirmed that the Southern hybridization is sufficiently sensitive to detect less than 0.1 pg of DNA that is complimentary to a probe, as seen by Sambrook *et al*. (1989), whilst, negative results were observed when 100 ng cDNA was used as a template (Lanes 2, 4 and 6). According to Rocher *et al*., (1998), the *cry* gene was poorly expressed in some plants, because of the rapid degradation of the mRNA. Some A/T rich sequences in the *cry* gene have been reported to contribute to mRNA instability in plants (Chen and Shyu, 1995).

Insect bioassay In the insect bioassay, fifteen newly hatched larvae of *P. damastesalis* were released each onto transgenic and non-transgenic teak leaves. The transgenic leaves caused 73 – 80% larval mortality and the larval growth was severely inhibited on all of these leaves, and most of the larvae died by 5 days post-infestation. In contrast, more than 60% of the larvae that were fed the non-transgenic leaves still survived on the fifth day, with 33% larval mortality observed overall (Table 1).

Little damage to leaf sections and very little skeletonisation (2.7 – 8%) were observed in transgenic leaves, whereas considerable amounts of tissue had been consumed in all replicates of the non-transgenic leaves which showed severe damage (over 30% skeletonisation) by the larvae (Table 2). About 57 – 66 cm^2 of the non-transgenic leaves showed skeletonisation, whereas only 3 cm^2 - 6cm^2 of the transgenic leaves were skeletonised (Figure 5). It was concluded that transgenic teak leaves expressing the *cry*1Ab gene were protected against damage by *Paliga damastesalis* larvae.

The larvae that fed on the two types of leaf also showed

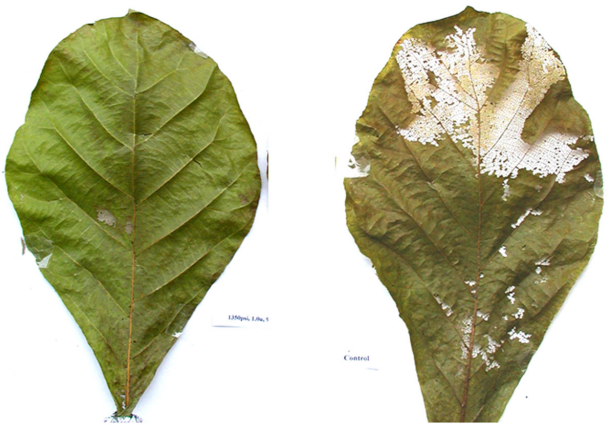

Figure 5. Insect bioassay. (a) Leaf of transgenic teak. **(b)** Leaf of non-transgenic teak. Differences in skeletonization areas seen after 5 days of exposure to *P. damastesalis* larvae which corresponds to the expression of *cry*1AB gene in the transgenic leaf.

Figure 6. Difference in size of *P. damastesalis* larvae after feeding with *cry*1Ab transformed leaves (a) and untransformed leaves (b).

Table 2. Skeletonisation area caused by *P. damastesalis* larvae on transgenic and non-transgenic teak leaves.

Source of the tested leaves for larvae feeding	Skeletonisation area on tested leaves (cm^2)	Percentage of skeletonisation area
Non-transgenic Teak	57 – 66	32 - 39
Transgenic Teak	3 – 6	2.7 - 8

differences in size. Those that fed on the non-transgenic leaves were bigger compared to larvae that fed on the transgenic leaves (Figure 6). The sizes of the larvae corresponded to the survival rate, so a higher survival rate was seen in larvae feeding upon the non-transgenic leaves compared to those feeding on transgenic leaves.

This insect bioassay study to determine the insecticidal activity of the *cry*1Ab gene showed differences between transgenic and non-transgenic teak leaves. According to Grace *et al.* (2005), the study on transgenic Monterey pine (*Pinus radiata*) expressing a *cry* toxin gene showed variable resistance to damage from painted apple moth larvae (*Tiea anartoides*), depending on the age (maturity) of needles. These studies emphasise the importance of transgene expression levels and tissue specificity.

CONCLUSION

Based on the bioassay, we were able to show that the *cry*1Ab gene had been successfully transferred through biolistic bombardment method and was expressing the toxin in the transgenic teak cells.

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