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Optimization conditions for *Agrobacterium*-mediated transformation of *Brassica rapa* with a bacterial isochorismate synthase gene

(Kaedah optimum transformasi tanaman *Brassica rapa* dengan gen bakteria isokorismik sintase melalui pengantara Agrobakterium)

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Keywords: *Brassica rapa*, *Agrobacterium tumefaciens*, isochorismate synthase, genetic transformation, GFP

Abstract

The aim of the study was to transform *Brassica rapa* plants with a bacterial isochorismate synthase (*entC*) gene in order to obtain transgenic plants with elevated levels of salicylic acid (SA) to increase pathogen resistance. Transgenic plants of *B. rapa* ssp. *oleifera* carrying the *entC* gene which encodes for *Escherichia coli* isochorismate synthase were developed via *Agrobacterium tumefaciens*-mediated transformation. This method involved the use of hypocotyl explants co-cultivated with disarmed strain LBA 4404 harbouring the binary vector pCAMBIA 1301 carrying the gene for *entC*, hygromycin phosphotranferase and sGFP in the T-DNA region and driven by CaMV (Cauliflower Mosaic Virus) 35S promoter. Prior to this, several important parameters including type of agrobacterial strains, concentration of acetosyringone, preconditioning days, pH and light effect during co-cultivation were evaluated for their influence on the transformation efficiency and subsequently optimized. The presence of the transgene in the genome of the greenhouse-grown transgenic plants was verified by PCR (Polymerase Chain Reaction) whereas the expression of the *entC* gene in mRNA (messenger ribonucleic acid) was revealed by RT-PCR (reverse transcription-PCR). The result indicated that the exogenous gene was successfully integrated into the genome and expressed in transgenic plants of *B. rapa* ssp. *oleifera*.

Introduction

Brassica rapa or turnip rape (Brasssicaceae) is one of the major important crops throughout the world due to its nutritional quality of vegetables, seed oil and to some extent feedstock. Vegetable oil from *Brassica* such as *B. napus* (canola), *B. juncea* (Indian mustard) and *B. rapa* (turnip) is one of the important sources of seed oil production after soybean and cotton seed (Rakow 2004). Therefore, in recent years there is a growing interest to improve the performance of these species such as to increase pathogen resistance or improve nutritional quality either by conventional breeding or genetic engineering technologies. The latter, including *Agrobacterium*-mediated transformation

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potentially has the added benefit that it can overcome some of the barriers of conventional breeding.

The most common method employed for *Brassica* transformation is using *Agrobacterium*, due to simplicity of handling the bacteria and straightforwardness of transformation protocols (Pua and Lim 2004). Although genetic transformation for most of the major *Brassica* crops has been achieved especially for *B. napus*, the successful transformation of *B. rapa* is relatively difficult due to low regeneration from tissue culture, inefficient transgene transformation as well as genotype dependence. Indeed this species is considered as one of the recalcitrant members in the genus of Brassicaceae for shoot regeneration *in vitro* (Jain et al. 1988) and the reports on successful transformation of this species are very few.

The success of transformation by *Agrobacterium* as a gene delivery system relies on the susceptibility to the bacteria and an efficient shoot regeneration system. This depends on many factors, such as preculturing and co-cultivation condition, phytohormone treatments, bacterial strains, methods of infection and the cultivar of *B. rapa* used. Rafat et al. (2010) reported the successful transformation of cabbage (*Brassica oleracea* ssp. *capitata*) with heat shock protein gene by optimizing several important parameters such as pre-culture medium, acetosyringone application, bacterial application and inoculation time.

Salicylic acid (SA) is a signal compound that plays an important role in mediating plant defences against pathogen attack. It is required by both local and systemic acquired resistance (LAR and SAR respectively), which induce the expression of plant pathogenesis-related genes as well as accumulation of defensive compounds (Wildermuth et al. 2001). In plants, SA is thought to be derived from the phenylpropanoid pathway via phenylalanine, trans-cinnamic acid (CA) and benzoic acid (BA) (Lee et al. 1995), whereas in microorganisms, SA synthesis proceeds via isochorismate synthase (ICS) (Verberne et al. 1999). Evidence for the involvement of the isochorismate pathway in plants is still evolving.

Wildermuth et al. (2001) showed that an *Arabidopsis* mutant in which ICS is mutated has lower SA levels and is more susceptible to microbial infections. Extensive work has been carried out in previous years to manipulate the SA regulation in tobacco and *Arabidopsis*. Verberne et al. (2000) transformed tobacco plants with two bacterial genes encoding the enzymes that convert chorismate to SA in a two-step process. High accumulation of SA was observed in plants expressing the bacterial gene encoding the ICS from *E. coli* and isochorismate pyruvate lyase (IPL) from *Pseudomonas fluorescence* when these enzymes were targeted to the chloroplast.

Research by Mauch et al. (2001) demonstrated that when two bacterial genes *pchA*, encoding ICS and *pchB*, encoding IPL from *P. aeruginosa* were introduced and expressed in *Arabidopsis*, the accumulation of free and conjugated SA increased more than 20-fold compared to the wild type. However, the plants showed a strong dwarfed phenotype and produced very little seeds.

The aim of this study was to transform *B. rapa* plants with a bacterial isochorismate synthase (*entC*) gene via *A. tumefaciens*-mediated transformation in order to obtain transgenic plants with elevated levels of SA to increase pathogenic resistance. Only the ICS was chosen as IPL overexpression might result in channeling isochorismate away from essential metabolic pathways, resulting in severe phenotypic effects as observed in *Arabidopsis* (Mauch et al. 2001).

Materials and methods

Plant material and growth conditions

Brassica rapa ssp. *oleifera* seeds, obtained from Boreal Plant Ltd, Jokionen, Finland, were surface-sterilized with 70% ethanol

for 5 min followed by double sterilization with 15% and 10% sodium hypoclorite solution for 30 and 15 min respectively. The sterilized seeds were germinated on half strength MS (Murashige and Skoog 1962) medium without any plant growth regulators. The seeds were germinated at 24 °C under a 16/8-h (light/dark) photoperiod (Philip Coolwhite 110 W fluorescence bulb, 50–100 μmol m^{-2} s^{-1}). After 4 days, the *in vitro* hypocotyls and cotyledonary-petioles were aseptically harvested for regeneration and transformation experiments.

Culture media

The media used for plant regeneration and transformation were according to Wahlroos et al. (2003) with a few modifications. For co-cultivation, the medium was supplemented with 100–200 μM of acetosyringone (AS) and for regeneration/ selection of the putative transformants, 20 mg/litre hygromycin was added to the medium. All media were adjusted to pH 5.7 with NaOH and autoclaved at 121 °C for 20 min.

Construction of plant expression vector

Previously, the *entC* sequence from *E. coli* (Ozenberger et al. 1989) was fused to the chloroplast targeting sequence (*ss*) from the small subunit of ribulose biphosphate carboxylase and inserted between Cauliflower Mosaic Virus (CaMV) 35S promoter and Potato proteinase inhibitor I terminator (PIt) and cloned into a transformation vector as reported by Verberne et al. (2000). For this present study, pIC-20H containing $_{ss}$*entC* gene was digested with Xba1 and cloned into the transformation vector *pCAMBIA* 1302 containing hygromycin resistance marker and sGFP (S65T) reporter genes as Xba1 fragment. The binary vector was named *pCAMBIA::ssentC* (*Figure 1*).

Parameter optimization

Several important parameters which have been reported to influence the efficiency of *Agrobacterium*-mediated transformation were optimized. Among them were the different strains of *Agrobacterium* used (LBA 4404, LBA 1118, LBA 1119), duration of co-cultivation (24, 48, 72 and 96 h) as well as the effect of light and pH (5.2, 5.6) during co-cultivation. Other parameters such as different concentration of acetosyringone, preconditioning days and the dilution factor of *Agrobacterium* used for transformation were also evaluated.

Genetic transformation experiments

The binary vector *pCAMBIA::ssentC* was introduced into *A. tumefaciens* strains LBA 4404, LBA 1118 and LBA 1119 by electroporation (Bio-Rad Gene Pulser II, Hercules, California, USA) and the plasmid-harbouring *Agrobacterium* culture was then used for plant transformation experiments. A single colony of the bacteria was inoculated in 5 ml liquid LB medium (5 g/litre NaCl, 5 g/litre yeast extract, 10 g/litre tryptone,

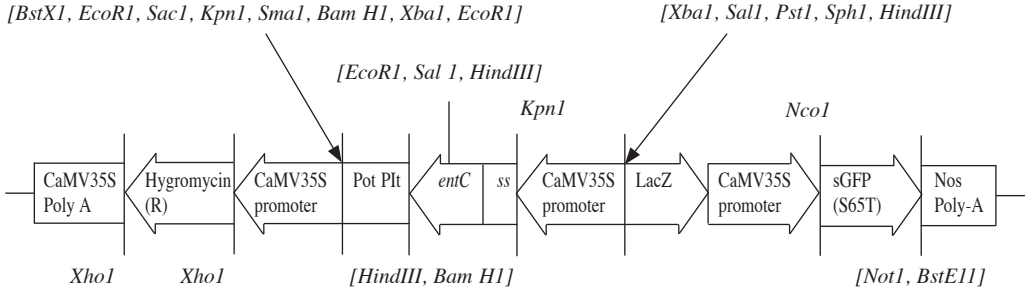

Figure 1. Schematic representation of the pCAMBIA binary vector containing $_{ss}$entC gene (isochorismate synthase)

pH 7.0) supplemented with the appropriate antibiotics: for LBA 4404 and LBA 1119–50 µg/ml kanamycin (kan) and rifampicin (rif), for LBA1118–50 µg/ml kan and rif and 100 µg/ml spectinomycin (spec). The culture was incubated at 28 °C in a rotary shaker at 200 rpm. After 2 days, 1 ml of culture was transferred into 50 ml fresh LB medium containing the appropriate antibiotics. The culture was further incubated at the above mentioned conditions overnight.

The *Agrobacterium* culture (OD_{600}= 1.0–1.2) was then centrifuged at 5,000 rpm for 15 min and the pellet obtained was resuspended and diluted in liquid MS medium. Two hundred microliters of this suspension was plated uniformly onto the appropriate co-cultivation media. Aseptically harvested hypocotyls and cotyledonary-petioles were excised and placed onto this medium. After 3–4 days of co-cultivation in 16/8-h (light/dark) photoperiod or in total darkness, the infected explants were washed twice with half strength MS liquid medium supplemented with 250 mg/litre cefotaxime and carbenicillin (Duchefa Biochemie, Netherlands) followed by several times with sterile distilled water to remove excess *Agrobacterium* suspension. The explants were then blotted dry on sterile filter paper and placed onto the regeneration/selection medium containing 200 mg/litre carbenicillin and cefotaxime to inhibit *Agrobacterium* growth and 20 mg/litre hygromycin (Duchefa Biochemie, Netherlands) for selection of transformed cells.

The culture was incubated at 24 ± 1 °C with a 16/8-h (light/dark) photoperiod at low light intensity (25–50 µmol m^{-2} s^{-1}). Micropore (3M) paper tape was used to seal the plates throughout the experiments. Hygromycin-resistant shoots obtained were subcultured several times onto fresh regeneration media containing reduced levels of cefotaxime and carbenicillin. Plantlets with well-developed shoots were transferred into hormone-free full strength MS medium containing hygromycin for root development. The rooted plantlets were then transferred into half strength MS medium supplemented with 0.50 mg/litre NAA for root elongation. Plantlets with well-developed roots were acclimatized and grown under greenhouse conditions with a 16/8-h (light/dark) photoperiod at 300–500 µmol m^{-2} s^{-1}).

Molecular confirmation of transgenic plants

PCR screening Genomic DNA was isolated from fresh leaves of greenhouse grown putative transformed and non-transformed control plants following the CTAB procedure (Lichtenstein and Draper 1985). Polymerase chain reactions (PCR) were performed to detect the presence of the *entC* gene in the genome of the putative transformants by using the following primer (specifically for *entC* gene) set: 5'–GCA ACA CTT GCG CCC AAT CGC-3' (forward) and 5'–CCG TTA CCT TCG CTG TCA CAC-3' (reverse). The total volume of the PCR mixture was 50 µl containing 1 µl template DNA, 2 µl of each primer (1 µM), 1 µl Phusion dNTPs mix (10 mM), 10 µl 5x Phusion HF Buffer and 0.5 µl of Phusion DNA polymerase (100 U (2U/µl), (Finnzymes, Espoo, Finland). The PCR conditions were 98 °C for 30 s as initial denaturation, followed by 33 cycles at 98 °C for 30 s, 65 °C for 30 s, and 72 °C for 45 s. Final extension was carried out at 72 °C for 10 min. Amplified DNA was analysed on a 1% (w/v) Tris-acetate-EDTA (TAE) agarose gel. The expected PCR product was 980 bp.

Semi quantitative one step RT-PCR screening Total RNA was isolated from the fresh young leaves of putative transformed and control plants using the RNeasy Plant Mini Kit (Qiagen Benelux B.V, Venloo, Netherlands). Semi-quantitative RT-PCR was performed using the Qiagen One Step RT-PCR Kit to detect the presence of *entC* mRNA in total RNA preparation. The reaction mixtures were incubated in a thermocycler at 50 °C (30 min) for cDNA

synthesis. The PCR reaction conditions and the oligonucleotide primers used were the same as for the PCR analysis. To eliminate false positive RT-PCR products due to contamination of genomic DNA, control reactions were performed following the Qiagen One Step RT-PCR Kit manufacturer's instructions.

Results and discussion

Evaluation of factors affecting the transformation efficiency

Several parameters known to influence the transformation efficiency of *B. rapa* were evaluated to determine the optimal conditions for transformation (*Table 1*). All the parameters were optimized on the basis of transient gene fluorescence protein (GFP) expression of the explants.

Agrobacterial strains The susceptibility of many crop species to *Agrobacterium* infection is one of the prerequisites to obtain transgenic plants, which depends among others on the bacterial strains used. Therefore in this study, three strains: LBA 4404, LBA 1118 and LBA 1119 were used to identify the strain with the highest rate of transient expression based on GFP fluorescence in the explant tissue. Strain LBA 4404 was found to be the most effective, followed by LBA 1118 and LBA 1119 for transgene expression after 10 days of co-cultivation (*Table 1*).

The transient GFP expression was 2-fold higher with LBA 4404 than with LBA 1118. Hence for further transformation experiments, strain LBA 4404 was used. The effectiveness of strain LBA 4404 in comparison to other strains of *Agrobacterium* for *B. rapa* transformation was reported by Kuvshinov et al. (1999). LBA 4404 was successfully used for transformation of other *Brassica* species such as Chinese cabbage (Jun et al. 1995), cauliflower (Bhalla and Smith 1998) and broccoli (Chen et al. 2001).

Table 1. Effect of different parameters on transient GFP expression of cotyledonary-petioles of *Brassica rapa* ssp. *oleifera* after 10 days of co-cultivation. Each experiment contained 200 explants

Different parameters	*Brassica rapa* ssp. *oleifera* GFP positive	
	n	Infection frequency (%)
Agrobacterium tumefaciens strains		
LBA 4404	70	35
LBA 1118	30	15
LBA 1119	22	11
pH of co-cultivation medium		
5.2	28	14
5.6	68	34
Co-cultivation period (hour)		
24	22	11
48	25	13
72	70	35
96	82	41
Co-cultivation condition		
Dark	27	14
16/8 light/dark	51	26
Concentration of AS in co-cultivation medium (μM)		
0	48	24
100	43	21
200	52	26
Dilution factor of *Agrobacterium* culture		
1:1	26	13
1:10	35	18
1:20	63	32
Preconditioning (days)		
0	43	22
1	45	23
3	41	21
5	39	20

n = no. of GFP positive explants

Concentration of acetosyringone (AS) The positive role of AS has been demonstrated on genetic transformation of many plants including recalcitrant species (Godwin et al. 1991; James et al. 1993). In this present study, two different concentrations of AS, i.e. 100 and 200 μM were evaluated to determine its effect on

the transformation efficiency. Shimoda et al. (1990) reported that AS is a phenolic compound that is able to induce the virulence (*vir)* gene in *Agrobacterium*-mediated transformation. Research by Zhang et al. (2000) on transformation of Chinese cabbage revealed that addition of AS to the co-cultivation medium enhanced the infection frequency by 3-fold compared to transformation without AS. A similar result was reported by Chakrabarty et al. (2002) on cauliflower transformation. However, in this study, no improvement in GFP expression was observed by adding AS, suggesting that it is not essential for *Agrobacterium*-mediated transformation of *B. rapa* ssp. *oleifera*.

Duration of co-cultivation The most effective duration for co-cultivation was determined by co-cultivating the explants for an increasing length of time (24, 48, 72 and 96 h). Evaluation on this parameter showed that the infection frequency increased by increasing the co-cultivation duration. However, increasing the co-cultivation period to 96 h resulted in severe necrosis of the explants and the bacterial growth could not be controlled with 250 mg/litre cefotaxime and carbenicillin. From literature, most of the transformation methods used 2–3 days of co-cultivation since longer co-cultivation periods resulted in necrosis or death of the explants. Previous research by Park et al. (2005) on *B. napus* and Tsukazaki et al. (2002) on cabbage transformation demonstrated that co-cultivation up to 3 days was effective to yield high transformation rates. Thus, a 72 h co-cultivation period was chosen for these subsequent experiments.

pH of co-cultivation media To examine the effect of pH of the co-cultivation media on the transformation efficiency, two different pH regimes were tested. The explants cultured on a medium with pH 5.6 showed a higher percentage of GFP expression (34%) compared to the explants cultured on a medium with pH 5.2. Several researchers have reported that *vir* induction leading to transformation is effective when the pH of the co-cultivation medium is lower than that commonly used in tissue culture medium (pH 5.6) (Stachel et al. 1985; Vernade et al. 1988). Takasaki et al. (1997) and Zhang et al. (2000) observed higher transient GUS expressions of *Brassica* spp. when the co-cultivation medium was at pH 5.2 when compared with pH 5.8. However, the effect of the pH in this experiment was not observed.

Light effect during co-cultivation Assessment on the effect of light was performed by comparing co-cultivation under total darkness and under a day/light regime. Co-cultivation under a photoperiod of 16/8 h (light/dark) resulted in a higher infection frequency, i.e. 26% compared to co-cultivation under total darkness. The influence of light on the transformation efficiency has been given less attention by researchers. However recently, several reports showed the positive effect of light/dark regime on transformation efficiency (Clercq et al. 2002; Wang and To 2004). Research by Zambre et al. (2003) showed that the transient GUS expression of *Arabidopsis* and *Phaseolus acutifolius* was highly and positively correlated with the co-cultivation under light. It increased under continuous light when compared with a 16 h light/dark condition and was strongly suppressed under total darkness.

Dilution factor of *Agrobacterium culture* To determine the effect of bacterial density on transformation efficiency, the *Agrobacterium* cultures grown to OD_{600}=1.0–1.2 were diluted with MS liquid medium at several dilution factors, (1:1, 1:10 and 1:20). Highest infection frequency was obtained with the dilution of 1:20, followed by 1:10 and 1:1. These results were in accordance with Chakrabarty et al. (2002) who reported similar results during transformation of cauliflower. They also

observed that necrosis of the explants were reduced by increasing the dilution factor. In the present study, necrosis of the explants was not observed even when the dilution factor 1:1 was used.

Preconditioning Explants of *B. rapa* ssp. *oleifera* were preconditioned for 0, 1, 3 and 5 days in the same medium as for co-cultivation prior to *Agrobacterium* infection, to investigate the effect of preconditioning on infection frequency. It was observed that preconditioning had no apparent effect on the infection frequency although it was prolonged to 5 days. The effectiveness of preconditioning to increase the transformation efficiency of *Brassica* sp. had previously been mentioned (Cardoza and Stewart 2003). Sangwan et al. (1992) suggested that preconditioning of explants with phytohormones prior to transformation would activate the cell division upon wounding resulting in competent cells for transformation. This is in line with Zambryski (1988) who suggested that the molecules that activate the *vir* genes in *Agrobacterium* during transformation were only present in metabolically active dividing cells.

However, the results of this study suggest that the preconditioning of explants did not have any effect on the transformation of ssp. *oleifera* and confirmed the earlier observation by Wahlroos et al. (2003) on transformation of this variety. Preconditioning is regarded as a way to avoid the hypersensitive response that leads to the necrosis of the explant upon infection with *Agrobacterium* (Babic et al. 1998). Probably, the necrosis of the explants was avoided by lowering the dilution factor of *Agrobacterium* culture during co-cultivation as mentioned previously. Other factors that might be beneficial to increase efficiency were examined, such as addition of glucose and acetosyringone in the *Agrobacterium* culture instead of in the co-cultivation medium and by using SAAT (sonication assisted *Agrobacterium* transformation), but none of these factors had a positive effect on the transformation efficiency. The use of SAAT for crops transformation had been reported previously by a few researchers (Santarem et al. 1998; Beranova et al. 2008) but recently, Li and coworkers (Li et al. 2009) reported for the first time a successful seed transformation of *B. napus* using this method.

Transformation and selection of putative transformants

GFP fluorescence sectors at the cut end of hypocotyls and cotyledonary-petioles were detected as early as 3–4 days after co-cultivation and the percentage of GFP expression over explants were calculated after 2 weeks in the selection medium. Green fluorescence in the calli was detected after the calli had just emerged from the cut edges of explants after 1–2 months in the regeneration/selection medium (*Plate 1a*). However, it was observed that the green fluorescent sectors were not homogenously distributed throughout the callus. When the green fluorescent sectors were isolated and transferred into the regeneration/selection medium, they resulted in browning and subsequent death of the callus. Despite its higher regeneration frequency from cotyledonary-petioles (data not shown), transformation experiments using strain LBA 4404 produced GFP positive green callus only from hypocotyls.

In this study, the finding that the GFP positive callus of ssp. *oleifera* was generated from hypocotyls is in contrast with Wahlroos et al. (2003) and Malyshenko et al. (2003), who obtained transgenic plants of the same variety from cotyledonary-petioles. However, this result is consistent with Mukhopadhyay et al. (1992) who obtained the transgenic plants from hypocotyls of *B. rapa* cv. Pusa Kalyani. Explants such as hypocotyl and cotyledonary-petioles are the most common source for *Brassica* transformation because of its high regeneration capacity. However, its response to *Agrobacterium* infection is

dependent on the variety and cultivar used (Christey and Braun 2004). In *Brassica* species, higher regeneration frequencies and lower transformation rates have been observed from cotyledons (Mukhopadhyay et al. 1992; Park et al. 2005) compared to hypocotyls, which had lower regeneration capacities but higher transformation rate.

The green calli/shoots were transferred onto appropriate regeneration/selection medium containing 20 mg/litre hygromycin after 3–4 weeks for shoot generation/ elongation. It was observed that the GFP positive green calli of ssp. *oleifera* were able to produce shoots. This is in contrast with the previous experiment with *B. rapa* cv. Raapstelen (data not shown) which showed that calli of cv. Raapstelen failed to generate any shoot from all the transformation experiments. The tiny shoots of cv. Raapstelen that regenerated directly from cotyledonary-petioles without an intervening callus phase also failed to develop further, although the GFP fluorescence was detected in the explants 10 days post inoculation.

Similar observations were reported by Mukhopadhyay et al. (1992) on transformation of *B. campestris,* which showed that the green emerging buds that generated from the cotyledonary-petioles were not able to survive in selection medium. Similar results were reported by Takasaki et al. (1997), who also failed to obtain transgenic plants from cotyledons of *B. rapa*. However, transgenic *Brassica* spp. obtained by indirect regeneration via the callus phase have been reported by several researchers (Wahlroos et al. 2003; Park et al. 2005).

In this case, the tiny shoots of ssp. *oleifera* that were able to elongate further in selection medium were also obtained via the callus phase. Research by Babic et al. (1998) on *B. carinata* transformation showed that only explant cells that underwent dedifferentiation into callus were able to produce transgenic shoots. This is in agreement with Sangwan et al. (1992) on transformation of *Arabidopsis,* who reported that only dedifferentiated mesophyll cells were competent for transformation. The authors suggested that such dedifferentiation (that occurs upon phytohormone treatment) alters the cell wall to become more susceptible to take up macromolecules such as T-DNA into the cytoplasm.

The developing calli of ssp. *oleifera* that were maintained on regeneration/ selection medium were regularly screened for GFP expression. It was observed that the intensity of the green fluorescence became less apparent over time and was no longer detectable in the newly developed shoots. Green putative transgenic shoots developed from callus after 2–3 months in the regeneration/selection media containing 2 mg/litre $AgNO_3$ (*Plate 1b*). Shoots were then separated from the callus and further maintained on regeneration/selection medium (*Plate 1c*). Well-developed shoots were transferred onto hormone-free full strength MS medium for root induction (Wahlroos et al. 2003) with the hygromycin still present (*Plate 1d*). However, a problem in obtaining roots in this medium was encountered. Therefore, the rooting medium was supplemented with 0.5 mg/litre NAA, which resulted in rooting of 66% of these shoots. Plantlets with roots that had developed after 2–3 months in this medium were then moved into hormone-free half strength MS medium containing 0.5 mg/litre NAA for root elongation. Finally plantlets with well-developed roots were acclimatized (*Plate 1e*) and planted in soil.

Out of 20 putative transgenic plantlets that survived in selection medium, only 13 were successfully established in soil and grown in the greenhouse until maturity. The total time to obtain the greenhouse grown plant was 8–9 months. The regenerated plants were morphologically identical to wild type and control non-transformed plants. Only three of the transgenic plants set seeds and produced flowers after 15–18 months old (*Plate 1f*). However, it was found that during the later stages of regeneration media, GFP expression in the mature leaf of

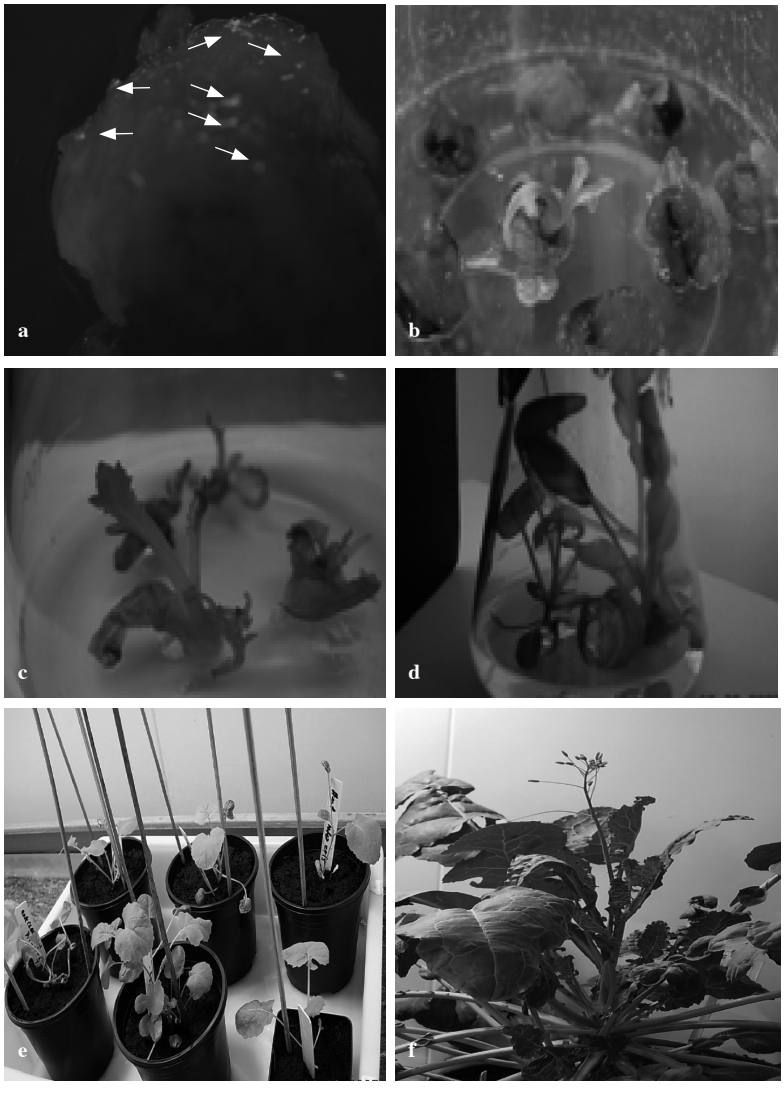

Plate 1. Transformation and regeneration of hygromycin-resistant shoots of ***Brassica rapa*** ssp. ***oleifera.*** *(a) Green fluorescent exhibited in 2-month-old callus (Arrows indicate the green fluorescent cell), (b) Putative transformed shoot formed on callus, (c) Transgenic shoots on regeneration/selection medium, (d) Transgenic plantlets in rooting/selection medium, (e) Transgenic plantlet under acclimatization, and (f) Transgenic plant produced flower*

the transgenic plants were no longer visible. Instability of the GFP gene as a marker for the presence of the transgene has been reported previously (Halfhill et al. 2003; Zhou et al. 2005). Baranski et al. (2006) also observed the declining intensity of fluorescence on adventitious roots of carrots after *A. rhizogens* transformation.

Similarly, Halfhill et al. (2001) reported changes of the intensity of GFP fluorescence throughout the life cycle of transgenic *B. napus*. High intensity was observed in the young leaves compared to the mature leaves. The authors suggested that the increasing chlorophyll concentration in the mature leaves might interfere with

GFP fluorescence detection. Blumenthal et al. (1999) reported the different levels of GFP expressed from gene driven by the 35S CaMV promoter in various tissues of tobacco plants and similar results were reported by Harpe and Stewart (2000). Halfhill et al. (2003) concluded that the reduction in fluorescence intensity was closely related with the decrease of the soluble protein during the leaf aging process.

Molecular analysis of putative transgenic plants by PCR and RT-PCR

Expression of the *entC* gene was initially studied by Northern Blot analysis in all 13 hygromycin resistant-T_0 plants grown in the greenhouse. However, the results yielded no detectable amount of *entC* in any of the transgenic plants. PCR analysis was then performed to detect the presence of the transgenes in the genome. This analysis verified the presence of *entC* gene in the genome DNA in seven of the putative transgenic plants. The PCR amplification produced a fragment of approximately 1.0 kb, which was the expected size (980 bp) of *entC* gene as shown in *Plate 2a*. No PCR product was observed in control non-transformed plants.

To determine whether the absence of a positive signal in the Northern Blot analysis was due to the low expression level of the gene, expression of *entC* gene was verified by semi-quantitative one-step RT-PCR using the same gene specific primers as in the PCR of the genomic DNA. RT-PCR was performed by isolating RNA taken from all the PCR-positive transgenic plants. RT-PCR products revealed in all cases the presence of the expected 980 fragment and its absence in non-transformed control (*Plate 2b*) confirming the expression of *entC* gene in mRNA of the transgenic tissue.

Dean et al. (2002) suggested that although Northern Blot analysis is a common and efficient tool to quantify gene expression levels, RT-PCR is much more sensitive to detect lower levels of gene expression and requires smaller quantities of RNA. Low transgene expression has been reported by previous researchers (Goring et al. 1991; Maghuly et al. 2006) and it

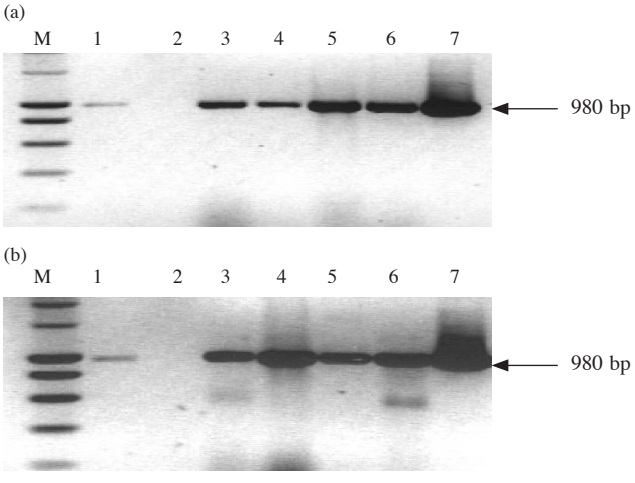

Plate 2. Molecular analysis of putatively transformed ***Brassica rapa*** *plants. (a) PCR and (b) RT-PCR analysis using the entC gene specific primers for detection of the transgene from T_0-generated greenhouse grown of* ***Brassica rapa*** *ssp.* ***oleifera****. Lanes M Lambda DNA marker, 1-positive control entC gene, 2-non transform control, 3-6- transgenic lines 2, 4, 5, 6, and lane 7- positive control entC gene (at increased concentration than lane 1)*

was suggested to be associated with a high copy number and subsequent gene silencing (Flavell 1994; Vaucheret et al. 1998).

In this study, the transformation efficiency (based on the number of hygromycin resistant shoots per number of explants) achieved was 3.6% (data not shown). However, only seven out of the 13 hygromycin resistant plantlets were transgenics carrying the *entC* gene. We concluded that 20 mg/litre hygromycin is not sufficient to prevent the 'escape' of some of the hygromycin-sensitive regenerants during selection.

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References

Babic, V., Datta, R.S., Scoles, G.J. and Keller, W.A. (1998). Development of an efficient *Agrobacterium*-mediated transformation system for *Brassica carinata*. *Plant Cell Rep*. 17: 183–188

Baranski, R., Klocke, E. and Schumann, G. (2006). Green fluorescent protein as a selection marker for *Agrobacterium rhizogenes* mediated carrot transformation. *Plant Cell Rep*. 265: 190–197

Bhalla, P.L. and Smith, N. (1998). *Agrobacterium*-mediated transformation of cauliflower *Brassica oleracea* var. *botrytis*. *Mol*. *Breed*. 4: 531–541

Beranova, M., Rakousky, S., Vavrova, Z. and Skalicky, T. (2008). Sonication assisted *Agrobacterium*-mediated transformation enhances the transformation efficiency in flax (*Linum usitatissimum* L). *Plant Cell, Tiss*. *Org*. *Cul*. 94: 253–259

Blumenthal, A., Kuznetzova, L., Edelbaum, O., Raskin, V., Levy, M. and Sela, I. (1999). Measurement of green fluorescence proteins in plant: quantification, correlation to expression, rapid screening and differential gene expression. *Plant Sci*. 142: 93–99

Cardoza, V. and Stewart, C.N. (2003). Increased *Agrobacterium*-mediated transformation and rooting efficiencies in canola (*Brassica napus* L) from hypocotyls segment explants. *Plant Cell Rep*. 21: 599–604

Chakrabarty, R., Viswakarma, N., Bhat, S.R., Kirti, P.B., Singh, B.D. and Chopra, V.L. (2002). *Agrobacterium*-mediated transformation of cauliflower: optimization of protocol and development of Bt-transgenic cauliflower. *J*. *Biosci*. 27: 495–502

Chen, L.F.O., Hwang, J.Y., Chang, Y.Y., Sun, C.W. and Yang, S.F. (2001). Transformation of broccoli (*Brassica oleracea* var. *italica*) with isopentenyltransferase gene via *Agrobacterium tumefaciens* for post harvest yellowing retardation. *Mol*. *Breed*. 7: 243–257

Christey, M.C. and Braun, R. (2004). Production of transgenic vegetable *Brassica*. In: *Biotechnology in agriculture and forestry*, Vol. 54. *Brassica* (Pua, E.C. and Douglas, C. J., eds.), p. 113–126. Heidelberg: Springer-Verlag

Clercq, J.D., Zambre, M. and Van Montagu, M. (2002). An optimized *Agrobacterium*-mediated transformation procedure for *Phaseolus acutifolius* A. Gray. *Plant Cell Rep*. 21: 333–340

Dean, J.D., Goodwin, P.H. and Hsiang, T. (2002). Comparison of relative RT-PCR and Northern Blot analyses to measure expression of β-1,3-glucanase in *Nicotiana benthamiana* infected with *Colltotrichum destructivum*. *Plant Mol*. *Biol*. *Rep* 20: 347–356

Flavell, R.B. (1994). Inactivation of gene expression in plants as a consequence of a specific sequence duplication. *Proc*. *Natl*. *Acad*. *Sci*. *USA*. 91: 3490–3496

Godwin, I., Todd, G., Ford-Lloyd, B. and Newbury, H.J. (1991). The effect of acetosyringone and pH on *Agrobacterium*-mediated transformation vary according to plant species. *Plant Cell Rep*. 9: 671–675

Goring, D.R., Thompson, L. and Rothstein, S.J. (1991). Transformation of a partial nopaline synthase gene into tobacco suppresses the expression of a resident wild-type gene. *Proc .Natl*. *Acad*. *Sci*. *USA* 88: 1770–1774

Halfhill, M.D., Millwood, R.J., Rufty, T.W., Weissinger, A.K. and Stewart, C.N. (2001). Spatial and temporal patterns of green fluorescent protein (GFP) fluorescence during leaf canopy development in transgenic oilseed rape, *Brassica napus* L. *Plant Cell Rep*. 22: 338–343

Halfhill, M.D., Richards, H.A., Mabon, S.A. and Stewart Jr, C.N. (2003). Expression of GFP and Bt transgenes in *Brassica napus* and hybridization with *Brassica rapa*. *Theor. Appl*. *Genet* 103: 362–368

Harpe, B.K., and Stewart Jr, C.N. (2000). Patterns of green fluorescent protein expression in transgenic plants. *Plant Mol. Biol. Rep*. 18: 141a–141i

Jain, R.K., Chowdhury, J.B., Sharma, D.R. and Friedt, W. (1988). Genotype and media effects on plant regeneration from cotyledon explant cultures of some *Brassica* species. *Plant Cell Tis. Org. Cult*. 14: 197–206

James, D.J., Uratsu, S., Cheng, J., Negri, P., Viss, P. and Dandekar, A.M. (1993). Acetosyringone and osmoprotectants like betaine or praline synergistically enhance *Agrobacterium*-mediated transformation of apple. *Plant Cell Rep*. 13: 28–31

Jun, S.I., Kwon, S.Y. and Paek, K.Y. (1995). *Agrobacterium*-mediated transformation and regeneration of fertile transgenic plants of Chinese cabbage (*Brassica campestris* ssp. pekinensis cv.'spring flavor'). *Plant Cell Rep* 14: 620–625

Kuvshinov, V., Koivu, K., Kanerva, A. and Pehu, E. (1999). *Agrobacterium tumefaciens*-mediated transformation of greenhouse-grown *Brassica rapa* ssp. *oleifera*. *Plant Cell Rep*. 18: 773–777

Lee, H.I., Leon, J. and Raskin, I. (1995). Biosynthesis and metabolism of salicylic acid. *Proc. Natl. Acad. Sci. USA*. 92: 4076–4079

Li, S., Zhao, D., Wu, Y. J. and Tian, X.E. (2009). A simplified seed transformation method for obtaining transgenic *Brassica napus* plants. *Agricultural Sciences in China* 8: 658–663

Lichtenstein, C. and Draper, J. (1985). Genetic engineering of plants. In: *DNA cloning*: (Glover, D.M., ed.), p. 67–119. Oxford: IRL Press

Maghuly, F., Leopold, S., Machado, A.D.C., Fernandez, E.B., Khan, M.A., Gambino, G., Gribaudo, I., Schartl, A. and Laimer, M. (2006). Molecular characterization of grapevine plants transformed with GFLP resistance gene. *Plant Cell Rep*. 25: 546–553

Malyshenko, S.I., Tyul'kina, L.G., Zvereva, S.D. and Raldugina, G.N. (2003). Transgneic *Brassica campestris* plant expressing the GFP gene. *Russ. J. Plant Physiol*. 50: 276

Mauch, F., Mauch-Mani, B., Gaille, C., Kull, B., Haas, D. and Reimmann, C. (2001). Manipulation of salicylate content in *Arabidopsis thaliana* by the expression of an engineered bacterial salicylate synthase. *Plant J*. 25: 67–77

Mukhopadhyay, A., Arumugam, N., Nandakumar, P.B.A., Pradhan, A.K., Gupta, V. and Pental, D. (1992). *Agrobacterium*-mediated genetic transformation of oilseed *Brassica campestris*: transformation frequency is strongly influenced by the mode of shoot regeneration. *Plant Cell Rep*. 11: 506–513

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*. 15: 473–479

Ozenberger, B.A., Brickman, T.K. and McInrosh, M.A. (1989). Nucleotide sequence of *Escherichia coli* isochorismate synthase gene *entC* and evolutionary relationship of isochorismate synthase and other chorismate-utilizing enzymes. *J. Bacteriol*. 171: 775–783

Park, B.J., Liu, Z., Kanno, A. and Kameya, T. (2005). Genetic improvement of Chinese cabbage for salt and drought tolerance by constitutive expression of a *B. napus* LEA gene. *Plant Sci*. 169: 553–558

Pua, E.C. and Lim, K.L. (2004). Transgenic Oilseed *Brassica*. In: *Biotechnology in Agriculture and Forestry* Vol. 54. *Brassica* (Pua, E.C. and Douglas, C.J, eds.), p. 238–260. Heidelberg: Springer-Verlag

Rafat, A., Aziz, M.A., Rashid, A.A., Abdullah, S.N.A., Kamaladini, H., Sirchi, M.H.T. and Javadi, M.B. (2010). Optimization of *Agrobacterium tumefaciens*-mediated transformation and shoot regeneration after co-cultivation of cabbage (*Brassica oleracea* subsp. *capitata*) cv. KY Cross with AtHSP101 gene. *Scientia Horticulturae* 124: 1–8

Rakow, G. (2004). Species origin and economic important of *Brassica*. In: *Biotechnology in Agriculture and Forestry* Vol. 54. *Brassica* (Pua, E.C. and Douglas, C.J., eds.), p. 238–260. Heidelberg: Springer-Verlag

Sangwan, R.S., Bourgeois, Y., Brown, S., Vasseur, G. and Sangwan-Norreel, B. (1992). Characterization of competent cells and early events of *Agrobacterium*-mediated genetic transformation in *Arabidopsis thaliana*. *Planta* 188: 439–456

Santarem, E.R., Trick, H.N., Essig, J.S. and Finer, J.J. (1998). Sonication-assisted Agrobacterium-mediated transformation of soybean immature cotyledons; optimization of transient expression. *Plant Cell Rep* 17: 752–759

Shimoda, N., Toyoda-Yamamoto, A., Nagamine, J., Usami, S., Katayama, M., Sakagani, Y. and Machida, Y. (1990). Control of expression of *Agrobacterium vir* genes by synergistic action of phenolic signal molecules and monosaccharides. *Proc. Natl. Acad. Sci. USA*. 87: 6684–6688

Stachel, S.E., Messens, E., Van Montagu, M. and Zamryski, P. (1985). Identification of

signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. *Nature* 318: 624–629

Takasaki, K., Hatakeyama, K., Ojima, K., Watanabe, M., Toriyama, K. and Hinata, K. (1997). Factors influencing *Agrobacterium*-mediated transformation of *Brassica rapa*. *Breed. Sci*. 47: 127–134

Tsukazaki, H., Kiginuki, Y., Aida, R. and Suzuki, K. (2002). *Agrobacterium*-mediated transformation of a doubled haploid line of cabbage. *Plant Cell Rep*. 21: 257–262

Vaucheret, H., Beclin, C., Elmayan, T., Feuerbach, F., Godon, C., Morel, J.B., Mourrain, P., Palauqui, J.C. and Vernhettes, S. (1998). Transgene-induced gene silencing in plants. *The Plant J*. 16: 651–659

Verberne, M.C., Budi Muljono, R.A. and Verpoorte, R. (1999). Salicylic acid biosynthesis. In: *Biochemistry and molecular biology of plant hormone* (Hooykaas, P.P.J., Hall, M.A. and Libbenga, K.R., eds.), p. 295–314. Amsterdam: Elsevier Science B.V.

Verberne, M.C., Verpoorte, R., Bol, J.F. and Linthorst, H.J.M. (2000). Overproduction of salicylic acid in plants by bacterial transgene enhances pathogen resistance. *Nature Biotechnol*. 18: 779–782

Vernade, D., Herrera-Estrella, A., Wang, K. and Van Montagu, M. (1988). Glycine betaine allows enhanced induction of the *Agrobacterium tumefaciens vir* genes by acetosyringone at low pH. *J. Bacteriol*. 170: 5822–5829

Wang, H.S., and To, K.Y. (2004). *Agrobacterium*-mediated transformation in the high-value medicinal plant *Echinacea purpurea*. *Plant Sci*. 166:1087–1096

Wahlroos, T., Susi, P., Tylkina, L., Malyshenko, S. and Zvereva, S. (2003). *Agrobacterium*-mediated transformation and stable expression of the green fluorescence protein in *Brassica rapa*. *Plant Physiol. Biochem*. 41: 773–778

Wildermuth, M.C., Dewdney, J., Wu, G. and Ausubel, F.M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defense. *Nature* 414: 562–565

Zambre, M., Terryn, N., Clercq, J.D., Buck, S.D., Dillen, W., Van Montagu, M., Van De Straeten, D. and Angenon, G. (2003). Light strongly promotes gene transfer from *Agrobacterium tumefaciens* to plant cells. *Planta* 216: 580–586

Zambryski, P. (1988). Basic process underlying *Agrobacterium*-mediated DNA transfer to plant cells. *Annu. Rev. Genet*. 22: 421–477

Zhang, F.L., Takahata, Y., Watanabe, M. and Xu, J.B. (2000). *Agrobacterium*-mediated transformation of cotyledonary explants of Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*). *Plant Cell Rep*. 19: 569–575

Zhou, X., Carranco, R., Vitha, S. and Hall, C.T. (2005). The dark side of green fluorescent protein. *New Phytl*. 168: 313–322

Abstrak

Tujuan kajian ini adalah untuk transformasi tanaman *Brassica rapa* dengan gen bakteria isokorismik sintase untuk menghasilkan pokok transgenik yang tinggi kandungan asid salisilik dan seterusnya rintang terhadap serangan perosak. Pokok transgenik *B. rapa* ssp. *oleifera* yang membawa gen *entC* daripada bakteria *Escherichia coli* yang mengekod enzim asid korismik sintase telah dibangunkan dengan menggunakan *Agrobacterium tumefaciens* sebagai pengantara transformasi. Kaedah ini menggunakan hipokotil sebagai eksplan yang telah diinfeksi dengan strain *Agrobacterium* LBA 4404 yang mengandungi vektor transformasi *pCAMBIA* 1301. T-DNAnya telah diselitkan dengan jujukan gen bagi *entC*, enzim 'hygromycin phospotransferase', dan 'green fluorescence protein' (sGFP) yang kesemuanya bawah pengawalan promoter 'Cauliflower Mosaic Virus' (CaMV) 35S. Beberapa parameter penting seperti strain agrobakteria yang digunakan, kepekatan bahan asetosiringone, kesan jangka masa, pH, dan cahaya semasa ko-kultivasi telah dioptimumkan untuk mendapatkan transformasi yang lebih efisien. Kehadiran gen *entC* ke dalam genom tanaman *B. rapa* yang ditanam di dalam rumah hijau telah dikenal pasti menggunakan 'Polymerase Chain Reaction' (PCR) sementara ekspresi gen tersebut di dalam 'messenger ribonucleic acid' (mRNA) telah dikenal pasti menggunakan 'reverse transcription-PCR (RT-PCR). Keputusan menunjukkan bahawa gen *entC* daripada *E. coli* telah berjaya diintegrasi dan seterusnya diekspres dalam tanaman *B. rapa* ssp. *oleifera*.

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