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Factors influencing the efficiency of *Agrobacterium tumefaciens*-mediated transformation and regeneration in Brussels sprouts

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Abstract

An efficient method for *Agrobacterium tumefaciens*-mediated genetic transformation of Brussels sprouts was developed. We tested two strains, LBA4404 and EHA105 both with the binary vector p35SGUSINT containing the *NPT11* gene for kanamycin resistance. Explants were derived from *in vitro*-grown seedlings of two commercial cultivars, Winter Pick and Troika. Transformation and regeneration efficiency were based on transient *GUS* expression and the ability of the explants to grow on media supplemented with kanamycin. Factors influencing *GUS* expression and shoot regeneration included the plant genotype, the *A. tumefaciens* strain, and the explant type. Winter Pick had significantly ($P \leq 0.05$) higher *GUS* expression, up to 44.3% in leaf sections compared to 25.6% in Troika, and significantly ($P \leq 0.05$) higher shoot regeneration. *Agrobacterium* strain LBA4404 was easier to control in culture and it achieved significantly ($P \leq 0.05$) higher infection rate, 46.1 %, than EHA105 (22.0%), ultimately achieving higher shoot regeneration. The length and temperature of co-cultivation did not significantly affect *GUS* expression, but co-cultivation for 2 d at either 21°C or 24°C resulted in more and healthier shoots as opposed to the vitreous shoots obtained from 3 d and 5 d co-cultivation periods. Compared to hypocotyl and petiole explants, leaf sections were the best, with highest *GUS* expression (44.8%) and moderate shoot regeneration of 45.8% on MS medium with 2.0 mg L^{-1} BAP, 5.0 m L^{-1} $AgNO_3$ and 50 mg L^{-1} kanamycin. This optimised protocol can be used to introduce the *thaumatin* gene for sweetness to mask the bitter taste in Brussels sprouts.

Keywords: genetic transformation; glucosinolates; *GUS* gene; thaumatin; tissue culture.
Abbreviations: $AgNO_3$_Silver nitrate; BAP_6-Benzylaminopurine; BM_Basal medium; CIM_Callus induction medium; CM_Co-cultivation medium; 2,4-D_2,4-Dichlorophenoxyacetic acid; GM_Germination medium; MS_Murashige and Skoog (1962); NAA_1-Naphthaleneacetic acid; RM_Rooting medium; SRM_Shoot regeneration medium; SRSM_Shoot regeneration selection medium.

Introduction

Brussels sprouts belong to the *gemmifera* group of *Brassica oleracea* of the family Cruciferae, and is thought to have derived from the wild cabbage (Singh et al., 2004; Sanchez-Yelamo, 2014). The axillary buds or sprouts contain high levels of glucosinolates, vitamins: B_1, B_2, B_6, B_9, C and K as well as minerals particularly iron, manganese, phosphorous and potassium (Singh et al., 2004). The B_9 vitamin, folic acid in particular, is converted in the body to co-enzymes that are essential for the normal functions of various enzymes required for normal growth, development and reproduction in humans (Sommers, 2000). Indeed, folic acid reduces the incidence of babies being born with brain and spinal cord abnormalities (neural tube defects) and it is therefore highly recommended during pregnancy (Shellack et al., 2015).
Brussels sprouts contain higher levels of glucosinolates than other *Brassica* crops (Table 1). Glucosinolates are nitrogen and sulphur containing compounds that are derived from glucose and an amino acid. They contain an R-side chain that varies between and within species resulting in over 120 different forms (Cartea and Velasco, 2008), of which over 90 occur in the Cruciferae (Lee et al., 2013) and about 20 are detected in the *Brassica* crops (Ishida et al., 2014). The consumption of *Brassica* crops such as Brussels sprouts and broccoli has been shown to enhance the breakdown of suspected carcinogenic heterocyclic aromatic amines in the human body (Smith et al., 2005; Razis and Noor, 2013). Hence, glucosinolates have been associated with reduced risk of cancers of the colonrectum, lung, prostrate and breast (Herr and Buchler, 2010; Ishida et al., 2014). Phenethyl isothiocyanate is particularly an effective inhibitor of lung tumour induction by the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (Mi et al., 2007). Despite these health benefits, glucosinolates are associated with the characteristic taste of *Brassica* spp with the bitterness being attributed to 2-propenyl glucosinolates (Shonhof et al., 2004). Glucosinolates normally remain intact, but as a natural plant defence mechanism following pest attack, food processing or chewing, they are hydrolysed by the enzyme, *myrosinase* (β-*thioglucoside glucohydrolase*), releasing glucose and breakdown products, including isothiocyanates (Agrawal and Kurashige, 2003). Isothiocyanates contribute significantly to inducing the

pungent and acrid flavours while the 2-propenyl derivative of isothiocynates is associated with the garlic-like off-flavours in *Brassicas* (Williams and Pun, 2011). The characteristic bitter taste and unpleasant odour have been correlated with a low preference score by consumers (Drewnowski and Gomez-Carneros, 2000; Schonhof et al., 2004).

Previous efforts to produce milder tasting Brussels sprouts cultivars have included reduction of soil sulphur levels (Yusuf, 1997) and conventional breeding (Oram and Kirk, 1993). However, reducing the amount of glucosinolates in bud tissue may reduce the health benefits of Brussels sprouts. It has been proposed that masking the bitter taste and off-flavour in Brussels sprouts by use of sweeteners or flavour enhancers such as thaumatin, without changing the level of glucosinolates may improve the taste and still retain the health benefits of glucosinolates. Thaumatin is among a group of sweet proteins that have been isolated from plants, others being monelin, pentadin, mabinlin, miraculin, brazzein and curculin (Pawar et al., 2013a). Thaumatin is isolated from the fruits of the West African plant, *Thaumatococus danielli* and it is about 3000 times sweeter than sucrose on a weight basis (Szwacka et al., 2012). It is approved as a flavour enhancer and intensive sweetener in many countries (Yebra-Biurrun, 2005).

The transfer and expression of the gene encoding for thaumatin into Brussels sprouts could enhance flavour and sweetness and therefore mask the bitter taste thereby encouraging its consumption. The cDNA encoding *thaumatin II* is available and so is a binary plasmid vector where the CaMV 35S is fused upstream of this cDNA. Indeed, several transgenic plant species expressing the thaumatin sweet phenotype have been produced (Szwacka et al., 2002; Firsov et al., 2012). Previously, attempts to transform Brussels sprouts with *Agrobacterium rhizogenes* resulted in plants with abnormal phenotypes (Hosoki and Kigo, 1994) while (Gu, 1998) reported limited success with *A. tumefaciens*. Here, we tested various factors that could influence transformation efficiency and regeneration of transformed shoots and present an optimised protocol for *A. tumefaciens*-mediated transformation that can now be used to transfer the thaumatin gene into Brussels sprouts.

Results

Effect of cefotaxime concentration on removal of A. tumefaciens from explants and shoot regeneration frequency

For explants that were inoculated with strain LBA4404, cefotaxime at 150 mg L^{-1} was effective at eliminating the bacteria from both cultivars (Table 2). When strain EHA105 was used, cefotaxime at 500 or 650 mg L^{-1} was only effective on cv. Winter Pick but not cv. Troika. The effect of cefotaxime concentration was not significant on shoot regeneration, however, increasing the concentration from 150 mg L^{-1} to 250 mg L^{-1} doubled shoot regeneration from 3.4 to 7.8 % for explants of cv. Winter Pick while it reduced by more 50% from 17.2 to 7.7 % from explants of cv. Troika Collectively, strain EHA105 had higher shoot regeneration but it was difficult to control its overgrowth in culture with the tested cefotaxime concentrations (Table 2).

Effect of co-cultivation temperature and duration on GUS expression, contamination, shoot and root regeneration

Total *GUS* expression at both 21°C and 24°C was highest (7.6%) when explants were co-cultivated for 2 d compared to 6.1% and 4.8% resulting from co-cultivation for 3 d and 5 d, respectively (Table 3). Further, *GUS* expression occurred at both temperature regimes in explants co-cultivated for 2 d but was inconsistent in explants co-cultivated for 3 d and 5 d. The interaction between the duration and temperature of co-cultivation with strain LBA4404 was however not significant on *GUS* expression. Contamination of explants generally increased as co-cultivation temperature was increased from 21°C to 24°C and significantly ($P \leq 0.05$) increased as the duration was increased from 2 d to 5 d (Table 4). Similarly, death of cultures generally increased as co-cultivation temperature was increased from 21°C to 24°C and significantly ($P \leq 0.01$) increased as the duration was increased from 2 d to 5 d. Shoots appeared in 2^{nd} – 3^{rd} wk after transfer of explants to SRSM. The interaction between duration and temperature during co-cultivation on green shoot regeneration frequency was significant at $P \leq 0.05$. Increasing co-cultivation duration from 2 d to 5 d increased green shoot regeneration on SRSM at 21°C but reduced it at 24°C (Fig. 1). After 6 wk on SRSM green and pale green shoots were excised and cultured on RM with 150 mg L^{-1} cefotaxime. Callus formation was observed from the 2^{nd} wk on SRSM and roots appeared in the 2^{nd} – 3^{rd} wk. New shoots were observed in the 3^{rd} wk. After 4 wk, they were excised from the initial callus and sub-cultured onto similar medium. Highest total percentage of rooted green shoots on selection medium was observed in explants co-cultivated for 2 (59) d compared to 53 and 38 in 3 d and 5 d co-cultivation, respectively (Table 5). Additionally, shoots produced by explants co-cultivated for 3 d and 5 d were mostly stunted and vitreous.

Effect of explant type on GUS expression and shoot regeneration

GUS expression was observed on callus on the cut ends of hypocotyls but not on the epidermis along their entire length. For the leaf sections, blue spots were observed on both the cut edges and also interspersed within the leaf tissue either on one side or on both the abaxial and adaxial surface. *GUS* expression in hypocotyls and leaf sections of cv. Winter Pick are shown in Fig. 2. Leaf sections had significantly higher *GUS* expression (44.8%; 4.7 blue spots per explant) than hypocotyls explants (6.6%; 0.9 blue spots per explants) and petioles explants (0.0%). On the contrary, highest shoot regeneration was obtained from petiole sections (80.0%) followed by hypocotyls sections (65.8) and then leaf section (45.8%; Fig. 3).

Factors affecting GUS expression and shoot regeneration from leaf explants of Brussels sprouts.

The effect of cultivar on *GUS* expression was not significant (Winter Pick, 46.0% and Troika 44%), when explants were inoculated with strain LBA4404, but after inoculation with strain EHA105, *GUS* expression was 40.0% in Winter Pick compared to 5.0% in Troika. Collectively in both cultivars, strain LBA4404 gave significantly higher *GUS* expression (46.1%; LSD = 19.7; $P \leq 0.05$) than EHA105 (22.0%). The effect of preconditioning alone was not significant on *GUS* expression in explants inoculated either immediately after sectioning, 35.6%, or after 2 d preconditioning, 32.5%, but most explants from Troika inoculated immediately with strain EHA105 died within the 1^{st} wk. Shoot regeneration was significantly higher in Winter Pick (73.0%; LSD = 17.4; $P \leq 0.05$) than in Troika (54.7%) and shoots regenerated from Winter Pick were generally more vigorous than those from Troika (Fig. 4).

Table 1. Major glucosinolates in some *Brassica* vegetables mg/100g fresh weight.

Name	R Side chain	Brussels sprouts	Cabbage	Cauliflower
Sinigrin	2-propenyl	44.5	26.3	14.2
Gluconapin	3-butenyl	25.2	1.8	0.7
Progoitrin	2-hydroxy-3-butenyl	47.8	3.8	2.3
Glucoiberin	3-methylsulphinylpropyl	35.3	45.0	17.3
Glucobrassicin	3-indolymethyl	62.4	29.5	22.7
Neoglucobrassicin	1-methoxy-3-indolymethyl	11.0	2.5	4.8
Total		226.2	108.9	62.0

Adapted from Gu (1998); Ishida et al. (2014).

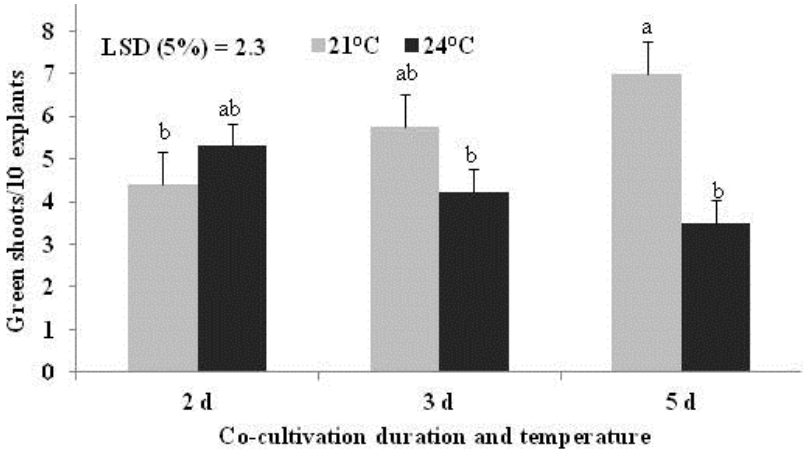

Fig 1. Effect of co-cultivation duration and temperature on the number of green shoots that developed on hypocotyl explants of Brussels sprouts cv. Troika inoculated with *A. tumefaciens* strain LBA4404. Explants were cultured on SRSM for 6 wk after co-cultivation (values are means of 7 replicates with 10 hypocotyls each with standard errors). Bars followed by the same letter are not significantly different at the 5% level using Duncan's new multiple range test.

Table 2. Effect of cefotaxime concentration on contamination and shoot regeneration from hypocotyls explants of Brussels sprouts cvs. Troika and Winter Pick. Explants were inoculated with *A. tumefaciens* strains LBA4404 and EHA105 and cultured on SRSM for 4 wk after co-cultivation (values are means of 2 replicates).

Cefotaxime (mg L-1)	Cultivar of Brussels sprouts	Strain of *A. tumefaciens*	Contamination by *A. tumefaciens* (%)	Explants with shoots (%)
150	Troika	LBA4404	0.0	17.2
150	Winter Pick	LBA4404	0.0	3.4
250	Troika	LBA4404	0.0	7.7
250	Winter Pick	LBA4404	0.0	7.8
500	Troika	EHA105	20.0	30.0
500	Winter Pick	EHA105	5.0	14.1
650	Troika	EHA105	45.0	22.0
650	Winter Pick	EHA105	0.0	25.0

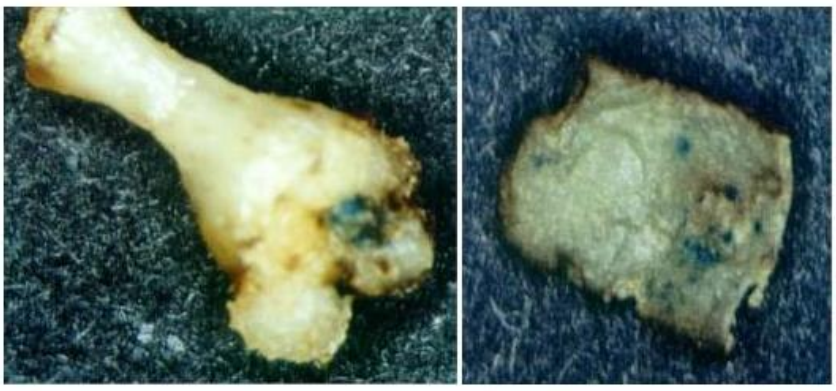

Fig 2. *GUS* expression in explants of Brussels sprouts cv. Winter Pick after inoculation with *A. tumefaciens* strain LBA4404 (a) hypocotyl explants were tested 18 d after co-cultivation (b) leaf section were tested 7 d after co-cultivation.

Table 3. Effect of co-cultivation duration and temperature on *GUS* expression in hypocotyl explants of Brussels sprouts cv.Troika inoculated with *A. tumefaciens* strain LBA4404 (values are the percentage of explants expressing *GUS* and are means of 3 replicates).

Duration of co-cultivation (d)	Temperature of co-cultivation (°C)	*GUS* expression Days after co-cultivation			
		7 (%)	21 (%)	Total per temperature (%)	Total per duration (%)
2	21	2.8	0.0	2.8	
	24	0.0	4.8	4.8	7.6
3	21	3.3	2.8	6.1	
	24	0.0	0.0	0.0	6.1
5	21	0.0	0.0	0.0	
	24	0.0	4.8	4.8	4.8
Total		6.1	12.4		

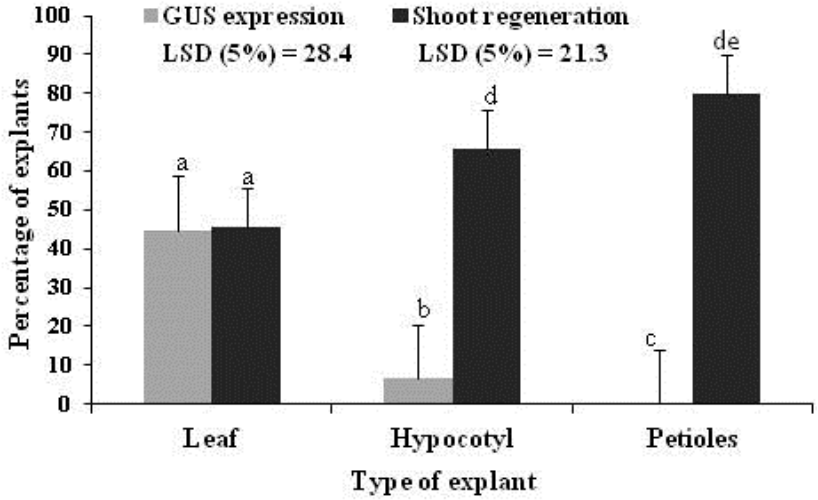

Fig 3. Effect of explants type on *GUS* expression and shoot regeneration from explants of Brussels sprouts cvs Winter Pick and Troika after inoculation with *A. tumefaciens* strain LBA4404. Explants were cultured on SRSM for 4 wk after co-cultivation (values are means of 2 cultivars with 2 replicates of each explant type with standard errors). Bars followed by the same letter are not significantly different at the 5% level using Duncan's new multiple range test.

Table 4. Effect of co-cultivation duration and temperature on contamination and death of hypocotyl explants of Brussels sprouts cv. Troika after inoculation with *A. tumefaciens* strain LBA4404. Explants were cultured on SRSM for 6 wk (values are the means of 7 replicates with 10 hypocotyls each).

Co-cultivation duration (d)	Co-cultivation temperature (°C)	No. of contaminated explants	No. of dead explants*
2	21	1.23 ± 0.45a	1.43 ± 0.90a
	24	0.71 ± 0.70a	0.86 ± 0.35a
3	21	1.00 ± 0.92a	3.71 ± 1.83bc
	24	1.14 ± 0.83a	4.14 ± 2.10bc
5	21	1.43 ± 1.40ab	3.29 ± 1.58b
	24	2.57 ± 1.40b	5.57 ± 1.68c

Means followed by the same letter in a column are not significantly different at $P \leq 0.05$ using Duncan's new multiple range test (*significant at $P \leq 0.01$).

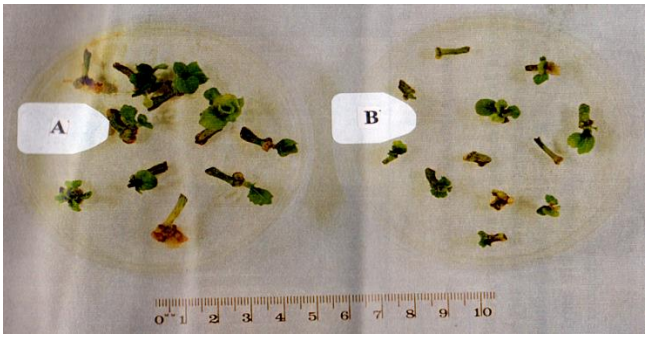

Fig 4. Direct shoot regeneration from petioles of Brussels sprouts after inoculation with *A. tumefaciens* strain LBA4404. Explants were cultured on SRSM for 3 wk after co-cultivation (a) cv. Winter Pick and (b) cv. Troika.

Further, explants inoculated with strain LBA4404 produced significantly more shoots (45.2 L%; LSD = 17.1; P ≤ 0.05%) than those inoculated with EHA105 (2.0%) as most explants inoculated with strain EHA105 were lost due to bacterial overgrowth. Increasing the level of BAP from 2.0 to 5.0 mg L^{-1} and addition of 1.0 mg L^{-1} IBA did not significantly affect *GUS* expression. However, the number of shoots regenerated per explant was significantly increased when leaf sections were inoculated without preconditioning (Fig. 5).

Discussion

Concentration of 150 mg L^{-1} cefotaxime was effective in eliminating the bacteria from both cultivars when explants were inoculated with strain LBA4404. However, strain EHA105 required much higher concentrations to effectively eliminate the bacteria from explants of Winter Pick and neither 500 nor 650 mg L^{-1} was effective for cv. Troika. In the event of wounding, different plant genotypes may produce different levels of the phenolic compound *asetosyringone* that is responsible for plant pathogen interactions (Baker et al., 2005), hence attracting more agrobacterium and subsequently complicating the effectiveness of antibiotic on a particular *Agrobacterium* strain. Additionally, EHA105 is a hyper-virulent strain (Kumar and Sopory, 2008), and some recent studies (Pawar et al., 2013b) found that its overgrowth could only be effectively controlled if cefotaxime was used in combination with carbenicillin. Similar to other studies cefotaxime had both bacteriostatic and morphogenic effects on the two cultivars. For example, Teixera da Silva and Fukai (2001) observed enhanced shoot regeneration in presence of cefotaxime in tobacco transformed with *Agrobacteium* strain LBA4404. Cefotaxime also promoted growth and morphogenenesis from maize callus (Danilova and Dolgikh, 2003). In contrast to other *Brassica* crops where co-cultivated for 3 d was more favourable (Khan et al., 2009), co-cultivation of Brussels sprouts explants for 2 d at either 21°C or 24°C was found to be better as it maintained high *GUS* expression and regeneration of healthy non-vitreous shoots. It has been demonstrated that *vir*D2 is mostly responsible for T-DNA transfer from *Agrobacterium* spp (Gelvin, 2003) and that the optimum temperature for production of the two *vir*D2 proteins products of 56 kDa and 43 kDa are 20 – 25 and 15 – 20°C, respectively (Alt-Moerbe et al., 1998). This indicates that temperatures ranging 15 – 25°C are suitable for T-DNA transfer and this covers the range 21 – 24°C used in the present study. Leaf sections achieved significantly higher *GUS* expression compared to hypocotyl and petiole explants in our study It has been demonstrated that explants that are capable of wound induced cell division usually ensure competence for transformation due to increased levels of secreted endogenous *acetosyringone* (Bulley and James, 2004; Yong et al., 2006) and eventually shoot regeneration. This suggests that leaf sections either contain more transformation-competent cells or they secreted higher levels of *asetosyringone*, very likely due to increased wounded surface area, thereby attracting more *Agrobacterium* and hence more transformation events. Gnasekaran et al. (2014) also found that wounding of protocorm-like bodies (PLBs) of orchid by a scapel rather than a needle increased transformation efficiency. However, despite having highest *GUS* expression, leaf sections had lower, shoot regeneration compared to the other explants, posing the need to balance between high transformation frequencies with high shoot regeneration. This difficulty of striking the balance between transformation competence and totipotence of plant cells has been reported (Delporte et al., 2012). Nonetheless, green shoots regeneration from leaf explants on kanamycin supplemented media was appreciably high and that could still be multiplied further in tissue culture. When leaf explants were used, cv. Winter Pick significantly achieved higher shoot regeneration with more vigorous shoots than Troika. This is in agreement with other studies where cultivar-dependent regeneration frequencies have been observed in *Brassica* crops (Gu, 1998; Zhang et al., 2000). Thomzik (1996) indicated that only certain cells or cell types are transformation and regeneration-competent or are capable of being made so, and it again appears that such cells may be more widely distributed in the leaves of cultivar Winter Pick compared to Troika. Although the hyper-virulence of strain EHA105 has benefited genetic transformation of other *Brassica* crops such as rapeseed (Radchuk et al., 2000) and oilseed mustard (Dutta et al., 2008) it neither improved transformation efficiency nor shoot regeneration in Brussels sprouts as it was difficult to control its overgrowth in the culture leading to death of cultures. Hence, LBA4404 was more suitable for transformation as it consistently achieved higher *GUS* expression and shoot regeneration from leaf explants and less contamination. Increasing the level of BAP from 2.0 to 5.0 mg L^{-1} and addition of 1 mg L^{-1} IBA did not affect *GUS* expression in our study despite auxins being implicated in enhancing expression of *ß-glucuronidase* by He et al. (2003). However, the number of shoots per explants when inoculated without preconditioning was significantly increased. These were explants sub-cultured from shoot regeneration non selective medium and therefore had overcome the effect of non-preconditioning. Hence, increased hormone levels would greatly benefit further multiplication of transgenic shoots *in-vitro*.

Materials and methods

Plant materials

Two commercial cultivars of Brussels sprouts, Winter Pick (Arthur Yates & Co. Ltd., Milperra, NSW, Australia) and Troika (Mr. Fothersgill's Seeds, Pty. Ltd, NSW Australia), were tested. Winter Pick is an early maturing hybrid that produces firm green heads that retain colour for a long time after maturity. Troika is high yielding with good disease resistance and is ideal for freezing. Seeds were surface sterilised in 70% ethanol for 2 min and rinsed twice in sterile MQ water. They were then washed for 30 min on an orbital shaker in 25% Milton bleach (Proctor and Gamble, NSW, Australia), with 1 – 2 drop of Tween 20, and then thoroughly rinsed 5 times with sterile MQ water. Seeds were then aseptically germinated for 7 d in 90 x 14 mm Petri dishes half-filled with GM (Table 6). Thereafter, they were germinated at 21 ± 1°C in continuous darkness for the first 2 d and the rest 5 d at growth room conditions (16 hours light/8hour darkness, 40 µmols m^{-2} s^{-1}) at 24 ± 1°C.

Agrobacterium strains

Two strains of *Agrobacterium tumefaciens*, LBA4404 (Hoekima et al., 1983) and EHA105 (Hood et al., 1993) were tested. These strains carry disarmed binary vectors containing a P35S*GUS* intron (Supplementary Fig. 1). The plasmids include the *GUS* reporter gene encoding for ß-*glucuronidase* that breaks down a number of ß-glucuronides to compounds

Table 5. Effect of co-cultivation duration and temperature on rooting of shoots excised from hypocotyl explants of Brussels sprouts cv. Troika inoculated with *A. tumefaciens* strain LBA4404. Shoot were excised from original explants after 6 wk on SRSM and rooted on RM for 4 wk and sub-cultured on similar medium for a further 4 wk.

Co-cultivation duration (d)	Co-cultivation temperature (°C)	No. of originally green shoots	Green shoots after 8 wk on RM	Green shoots with roots after 8 wk on RM	Rooting frequency per duration (%)[a]
2	21	50	13	8	
	24	35	21	12	59
3	21	36	9	2	
	24	16	10	8	53
5	21	61	51	22	
	24	19	12	4	38

[a]Rooting frequency = Total number of green shoots with roots after 8 wk x 100.
Total number of green shoots after 8 wk.

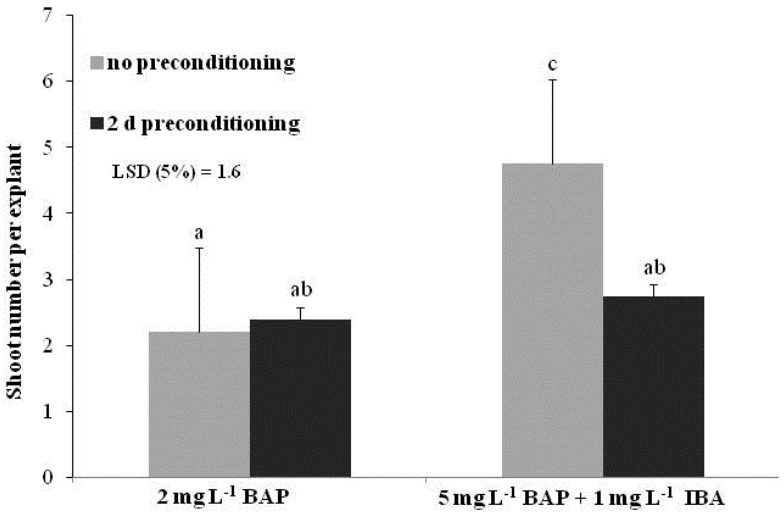

Fig 5. The interaction between plant growth regulators and preconditioning on the number of shoots per leaf explant of Brussels sprouts cv. Winter Pick after inoculation with *A. tumefaciens* strain LBA4404. Explants were culture on SRSM for 3 wk after co-cultivation (values are means of 2 replicates of each treatment with standard errors). Bars followed by the same letter in a series are not significantly different at the 5% level using Duncan's new multiple range test).

Table 6. Composition of medium used in *Agrobacterium tumefaciens*-mediated transformation and regeneration of Brussels sprouts.

Medium*	Composition
BM	MS + 150, 250, 500 or 650 mg L^{-1} cefotaxime
GM	MS (½ macrosalts) + 30 g L^{-1} sucrose + 4 g L^{-1} PhytaGel
CM	MS supplemented with 0.1 mg L^{-1} 2,4-D and 2.0 mg L^{-1} BAP
CIM	MS + 0.1 mg L^{-1} 2,4-D and 0.5 mg L^{-1} BAP + 7 g L^{-1} PhytaGel)
SRM	MS + 2.0 mg L^{-1} BAP, 5.0 mg L^{-1} $AgNO_3$ + 7 gL^{-1} PhytaGel
SRSM	MS + 2.0 mg L^{-1} BAP, 5.0 mg L^{-1} $AgNO_3$ + 50 mg L^{-1} kanamycin + 7 g L^{-1} PhytaGel
RM	MS + 0.1 mg L^{-1} NAA, 50 mg L^{-1} kanamycin +150 mg L^{-1} cefotaxime + 7 g L^{-1} PhytaGel

*All media were supplemented with 30 g L^{-1} sucrose, autoclaved at steam pressure of 103.4 kPa at 121°C for 20 min and stored at 4°C.

that turn blue after oxidation, hence enabling putatively transformed tissues to be selected early in the transformation process. They also contain the *NPTII* gene that encodes for *neomycin phosphotransferase* for kanamycin resistance. Overnight *Agrobacterium* suspensions were prepared from a single colony from actively growing cultures grown on yeast extract broth (YEB; Difco laboratories, Detroit, USA) supplemented with 10 mg L^{-1} kanamycin (Sigma, St Louis Mo., USA) and maintained at -80°C. A log phase *Agrobacterium* inoculum of OD_{600} = 0.300 was prepared according to Gu (1998) and used for infection.

Transformation procedure

Explants were excised from 7 d old seedlings and pre-cultured on CIM (Table 6) in 90 x 14 mm Petri dishes and maintained in continuous darkness at 21 ± 1°C for 2 d. In 55 x 14 mm Petri dishes, between 10 – 15 explants were inoculated with 15 ml of *A. tumefaciens* inoculum and placed on an orbital shaker for 10 min. Explants were blotted on sterile filter paper to remove excess bacteria and then placed on sterile filter paper saturated with liquid CM in 90 x 14 mm Petri dishes. Co-cultivation was carried out in continuous darkness for 2 d 21 ± 1°C. Thereafter explants were decontaminated with appropriate concentration of cefotaxime (Hoechst, NSW, Australia), blotted on sterile filter paper and placed horizontally on SRM with appropriate concentration of cefotaxime in 90 x 14 mm Petri dishes for 7 d. Selection of putatively transformed shoots was done on SRSM and sub-culturing on similar medium every 10 – 14 d. All aseptic procedures were carried out in a laminar flow hood (Southern Cross Science Pty. Ltd).

Histochemical GUS assay

GUS gene expression in inoculated explants and in regenerated shoots was carried out as described by Jefferson (1987). Randomly selected explants were tested after 7 d on SRM and a further 14 days on SRSM (Table 6).

Evaluation of cefotaxime concentration on decontamination and shoot regeneration

Pre-cultured hypocotyl explants (0.5 – 0.7 cm) of both cultivars were inoculated with either *A. tumefaciens* strain LBA4404 or EHA105 in two separate experiments and co-cultivated for 2 d. Explants were then decontaminated in BM containing 500 or 650 mg L^{-1} cefotaxime and cultured on SRM supplemented with either 150 or 250 mg L^{-1} for those inoculated with LBA4404 or 500 or 650 mg L^{-1} cefotaxime for explants inoculated with strain EHA105. After 7 d under growth room conditions, explants were assessed for contamination, appearance of shoot and/or roots and then transferred to SRSM with sub-culturing on to similar medium every 10 d. Contamination by the *Agrobacterium* and shoot regeneration were assessed for 4 wk.

Evaluation of co-cultivation duration and temperature on transformation efficiency, contamination, shoot and root regeneration

Pre-cultured hypocotyl explants were inoculated with *A. tumefaciens* strain LBA4404 for 10 min and after blotting off excess *Agrobacterium* they were cultured in CM in total darkness for 2, 3 or 5 d at either 21 ± 1°C or 24 ± 1°C. Thereafter, explants were washed with BM with 250 mg L^{-1} cefotaxime and cultured on SRM and maintained at growth room conditions at 24 ± 1°C. After 7 d, explants were transferred to SRSM with 250 mg L^{-1} cefotaxime, and sub-cultured on to similar medium every 10 – 14 d. Some explants were randomly selected after 7 and 21 d and tested for transient *GUS* expression. Shoots were excised at 6 wk and cultured on to RM and assessed for eight weeks.

Evaluation of explants type on GUS gene expression and shoot regeneration

Three explants types: hypocotyls (0.5 – 0.7 cm), leaf petioles (0.5 – 1.0 cm) and leaf sections (0.4 x 1.0 cm) from both cultivars were tested. Explants were inoculated with *A. tumefaciens* strain LBA4404 and after decontamination with 150 mg L^{-1} cefotaxime, they were cultured on SRM at 24 ± 1°C. After 7 d some explants of each type were randomly selected and assessed for *GUS* expression while the remaining explants were cultured on SRSM. They were assessed after 6 wk for *GUS* expression, contamination, explants survival, callus formation, shoot and root regeneration.

Evaluation of factors affecting GUS expression and shoot regeneration frequency from leaf explants

The effects of plant genotype, *Agrobacterium* strains, pre-conditioning of explants, and plant growth regulators were assessed. Leaf sections from both cultivars: Winter Pick and Troika were inoculated with either LBA4404 or EHA105 either immediately after sectioning or after a 2 d preconditioning on CIM. After a 2 d co-cultivation with the *Agrobacterium* explants were washed 150 mg L^{-1} (for LBA4404) or 500 mg L^{-1} cefotaxime (for EHA105). Explants were cultured on SRM for 7 d and then transferred to SRSM supplemented with either 2.0 mg L^{-1} BAP or 5.0 mg L^{-1} BAP and 1.0 mg L^{-1} IBA. After 7 d, explants were transferred to similar selection medium. In all experiments, *GUS* expression was assessed after 7 d and after 21 d.

Experimental design and data analysis

Treatments were replicated 5 – 15 times with 10 – 28 explants depending on availability of explants for individual experiments. All experiments were laid out in a completely randomised design. Analysis of variance (ANOVA) was conducted using GENSTAT 5 (Release 4.1) with significance of difference levels of $P \leq 0.05$. Means were compared using the least significant difference (LSD) or Duncan's new multiple range test (DMRT; $P \leq 0.05$).

Conclusion

Strain LBA4404 was better for transformation of Brussels sprouts as it consistently achieved higher transient *GUS* expression and shoot regeneration. It was also easier to remove from explants than strain EHA105 requiring only 150 mg L^{-1} cefotaxime as opposed to 650 mg L^{-1} needed for EHA105. Cultivar Winter Pick consistently gave higher *GUS* expression and regenerated more shoots that were also more vigorous compared to Troika. A 2 d co-cultivation of explants with the *Agrobacterium* at either 21°C or 24°C was found to be optimum for both *GUS* expression and shoot regeneration. Leaf explants proved better than the hypocotyls and petiole explants with substantial transformation efficiency and almost all explants that expressed *GUS* also regenerated green shoots with or without pre-conditioning. The optimised protocol for transient *GUS* expression and shoot regeneration in Brussels sprouts is shown in Supplementary Fig. 2 and it can immediately be used for transformation with the *thaumatin* gene.

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