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Agrobacterium-mediated Transformation of Broad Bean *Vicia faba* L.

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Summary

Three broad bean cultivars, which varied in morphology and geographical origin, were inoculated with nine *Agrobacterium* strains to determine the best combination for potential use in transformation and to investigate the possibility of regenerating genetically transformed plants. The stems of seedlings were inoculated with *A. tumefaciens* wild type strains (A281 and B6S3), transconjugant strains (C58C1(pArA4abc) and C58C1(pArA4b)), the B6S3 rooty and shooty mutants (GV3101(pGV2255), GV3101(pGV2215) and GV3101(pGV2235)), and *A. rhizogenes* wild type strains (8196 and 15834). With all the tested strains only unorganized tumour tissue was obtained. Cultivars differed in their susceptibility to bacterium strains, and plant genotype *vs.* strain interaction was detected. The strain most virulent to cv. Lobab Lippoi was C58C1(pArA4b), while with the less susceptible cvs. Topolo and Ošlje the best results were obtained with strains A281 and B6S3, respectively. However, the size and phenotype of tumours depended on the bacterial strain exclusively, indicating that the difference in transformation efficiency of each bacterial strain might be the result of a different efficiency in one or more of the steps prior to expression of T-DNA genes within each particular cultivar. Established tumorous calli grown on hormone-free MS medium showed enhanced peroxidase activity. The *in vitro* transformation of cotyledon, leaf and internodal stem segments with Ri plasmids containing bacterium strains, was not successful.

Key words: *Vicia faba* L., *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, peroxidase

Introduction

Legumes are the third largest family of flowering plants and the most important source of plant proteins and energy. In developing countries, increased cultivation of legumes is the best hope for combating shortages in food supplies, especially vegetable proteins. However, some of the most important storage proteins in legume seeds are deficient in specific essential amino acids that must be made up in other ways. Increasing effort is being devoted to research directed towards the improvement of the broad bean (a traditional crop in Europe used for direct human consumption), due to its high protein content, its potential use as a high energy crop and its adaptability to temperate climates where it has been demonstrated to be high yielding *(1)*.

In recent years genetic transformation has been used to introduce new traits into commercially important plants thereby producing combinations of features which could not have been achieved by traditional breeding programs *(1,2)*. The most widely used method

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of plant transformation is based on the natural ability of *Agrobacterium* to insert T-DNA originating from Ti (tumour inducing) or Ri (root inducing) plasmids into the genome of many host plants *(3)*. The susceptibility of most legumes to *Agrobacterium* transformation and the difficulties of protoplast regeneration in many species make *Agrobacterium*-mediated transformation the obvious first choice. As far as we know, only four papers on genetic transformation of broad bean by *Agrobacterium* have been published *(4–7)*. Differences in response to infection with *A. rhizogenes in vivo* and *in vitro* have been detected among diverse cultivars, genetic stocks and landraces *(5,7)*. In addition to plant genotype, the response to *in vitro* infection with *A. rhizogenes* depended on the tissue type *(5)*. However, regeneration of transgenic plants of broad bean has not been successful yet.

A prerequisite for the practical use of *Agrobacterium*-mediated gene transfer to the broad bean is the identification of the most susceptible genotype or the most virulent *Agrobacterium* strain and the development of a protocol for the regeneration of fertile transgenic plants. In this paper, the susceptibility of three broad bean cultivars varying in morphology and geographical origin to infection with nine *Agrobacterium* strains was described. Besides the transformation with wild-type strains known to be virulent to a number of plant species, transformation with rooty and shooty mutants was demonstrated. Shooty mutants stimulate regeneration of transgenic shoots in several plant species (*8,9*). Therefore, such mutant strains might be helpful in the development of a regeneration protocol for the broad bean. Although the three broad bean cultivars were different in their susceptibility to transformation with each bacterial strain, the cells of all cultivars responded uniformly to expression of the same T-DNA.

Materials and Methods

Bacterial strains

Nine bacterium-strains of either *A. rhizogenes* or *A. tumefaciens* were used (Table 1): *A. rhizogenes* wild type strains 8196 and 15834 *(10)*, *A. tumefaciens* strain A281 containing plasmid pTiBo542 *(11,12)*, *A. tumefaciens* wild type strain B6S3 *(13,8)* and its T-DNA mutant strains GV3101(pGV2255), GV3101(pGV2215) and GV3101(pGV2235). Plasmid pGV2255 (a rooty type) is mutated in genes *4*, *6a* and *6b*, plasmid pGV2215 (a shooty type) is mutated in gene *2* and plasmid pGV2235 (a shooty type) is mutated in genes *5*, *7*, *2*, *1* and *6b* (for restriction map see ref. *8*). *A. tumefaciens* strains C58C1(pArA4b) and C58C1(pArA4abc) are transconjugant strains containing Ri plasmids of *A. rhizogenes* A4 *(10)*. Bacteria were grown either in liquid or on 1.0 % agar solidified medium with 0.8 % beef extract, 0.7 % yeast extract and 0.5 % sucrose (pH=7.2). For plant or explant inoculation, three-day-old bacterium cultures grown at room temperature were used.

Plant material and transformation

The three *Vicia faba* L. cultivars (cv. Lobab Lippoi – Hungary, cv. Topolo – Croatia, and cv. Ošlje – Bosnia and Herzegovina) were used in the experiments. Broad bean plants at the third trifoliate stage (2–3 weeks old) grown *in vivo* were wounded between nodes two and three with a sterile needle and inoculated with bacteria. The inoculum was smeared gently all over the wounds (1 cm long). About 300 plants of each cultivar were infected and 20 plants per cultivar were only injured (the control). Wound sites were examined over several weeks and six weeks after inoculation were scored for number of wounds producing tumours and size (diameter) of tumours.

For the *in vitro* assay, sterile seeds of cv. Lobab Lippoi (the most susceptible cultivar *in vivo*) were germinated in glass tubes. After ten days, excised cotyledons, leaves and internodal stem segments were injured with a sterile needle and:

a) Co-cultivated for 30 or 50 min in the bacterium suspension (strains C58C1(pArA4b), C58C1(pArA4abc), 15834 or 8196) on a horizontal shaker. Explants were blotted dry on sterile filter paper and placed on 1 % agar solidified Murashige and Skoog medium *(14)* without hormones (MS0) in Petri dishes. After 48 or 96 h the explants were washed in liquid MS0 medium supplemented with carbenicillin (1 g/L) and transferred to solidified MS0 medium with carbenicillin (1 g/L).

b) Wounded explants were inoculated with the same strains of bacteria from the solid media by needle. After infection, the excised explants were placed with the cut uninfected bases on agar-solidified MS0 medium (with 1 g/L carbenicillin).

The frequency and development of tumours were studied during a six-week period. The *in vivo* experiment was repeated three times (all together 30–40 seedlings of each cultivar were inoculated with each bacterial strain) and the *in vitro* experiment was repeated twice.

Establisment of tumour tissue culture

Primary tumours developed *in vivo* were used for *in vitro* culture 6 weeks after inoculation. Removed tumours were surface sterilised by immersion in 70 % ethanol for 2 min, in 1.5 % sodium hypochlorite for 20 min, followed by 5x5 min rinses in sterile distilled water. The outer cortex of the tumours was cut off and the remaining tissue was cut into 2–3 mm cubes and placed onto a solidified MS0 medium with carbenicillin (0.5 g/L). Explants were subcultured to fresh medium every four weeks. Cultures were incubated at 24 °C under fluorescent tube light (16 h photoperiod).

Peroxidase activity

Peroxidase activity was studied in established tumour tissue cultures achieved by strains B6S3, GV3101(pGV2255) and A281 on plants of cv. Lobab Lippoi and cv. Ošlje. Internodal stem segments of both cultivars were used as a control. The total guaiacol peroxidase activity was determined spectrophotometrically at 470 nm. The test solution was prepared according to Siegel and Galston *(15)*. Enzyme activity is expressed as A_{470}/g fresh weight/min, or as specific activity in relation to protein content, determined according to Bradford *(16)*.

Results

Tumour formation on seedlings

In the experiments carried out *in vivo*, tumours induced by strains B6S3 and A281 (Ti plasmid carrying strains) began to appear at about two weeks after inoculation on plants of all three cultivars. *A. tumefaciens* strains C58C1(pArA4b) and C58C1(pArA4abc) carrying Ri plasmids and the *A. tumefaciens* rooty mutant GV3101(pGV2255) also induced tumour development on the three cultivars. Tumours induced by those strains became visible 3–4 weeks after inoculation. At the same time small, 1-mm-sized outgrowths appeared on a few stems of all the cultivars at the site infected with *A. tumefaciens* shooty mutant GV3101(pGV2215). Although those structures did not grow any further they might be considered as a result of transformation as no outgrowths were seen on stem slits of the control plants. Stem slits of all cultivars inoculated with strains 8196, 15834 and shooty mutant GV3101(pGV2235) healed as in noninfected plants. Thus *in vivo* neither root nor shoot formation were obtained on any cultivar from inoculation with all tested strains.

The percentage of inoculated plants that developed visible tumours and the size of tumours (diameter) were evaluated six weeks after inoculation to determine the efficiency of transformation. The response of three broad bean cultivars to infection with virulent strains of bacteria was different. The most susceptible cultivar was Lobab Lippoi (44 % of plants with tumours), followed by cv. Ošlje (28 %) and cv. Topolo (24 %). Significant differences in the transformation ability of the same bacterial strain with respect to each particular cultivar have been detected (Fig. 1). Cultivar Lobab Lippoi was the most susceptible to transformation with *A. tumefaciens* C58C1 carrying Ri plasmid pArA4b (92.5 % of plants responded to infection); the highest transformation frequency on cultivar Topolo was achieved with strain A281 (76.5 %) and cv. Ošlje was the most susceptible to strain B6S3 (62.9 %). However, the size of tumours did not correlate with the frequency of tumour induction, neither did it depend on the genotype of the plant. Each

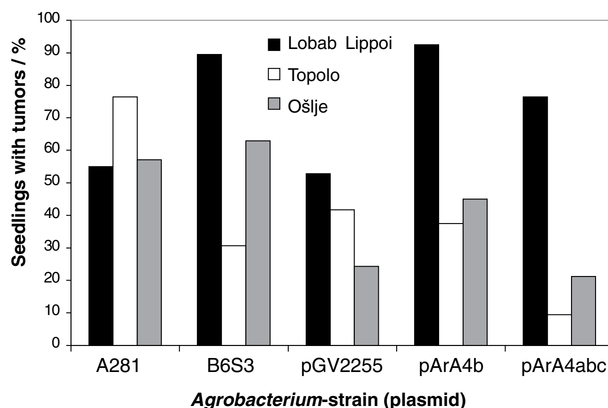

Fig. 1. The frequency (%) of seedlings of three *Vicia faba* L. cultivars forming tumours on a stem inoculated with different *Agrobacterium*-strains, 6 weeks after inoculation; inoculation was performed three times on c. 10 seedlings per cultivar with each strain

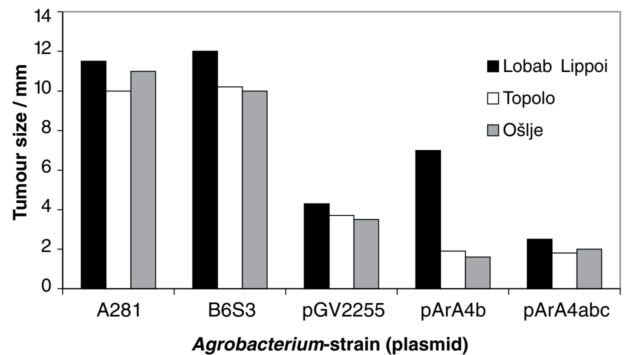

Fig. 2. Average diameter (mm) of tumours induced by different *Agrobacterium*-strains on seedling stems of three broad bean cultivars, 6 weeks after inoculation; three to 37 tumours were measured for each strain/cultivar combination

bacterial strain, except C58C1(pArA4b), induced tumours of similar size on all three cultivars (Fig. 2).

Establishment of tumour callus cultures

Axenic tumour cultures growing on solidified MS0 medium were established from tumours induced *in vivo* with strains B6S3, A281 and GV3101(pGV2255) on all the cultivars as well as from tumours induced with C58C1-(pArA4b) on cvs. Lobab Lippoi and Ošlje. Phenotypic differences among established tumour callus cultures were observed depending on the bacterial strain. The long-term B6S3 cultures were dark green and very compact. The A281 calli were light green and less compact, and the GV3101(pGV2255) calli were soft and yellow green. The callus lines (friable and yellow-brownish) established from the C58C1(pArA4b) tumours of cv. Lobab Lippoi rarely produced hairy roots but attempts to culture separated hairy roots on MS0 medium have not been successful. The phenotypic characteristics of an individual callus clone remained stable during subculturing.

Estimating the total peroxidase activity in long term tumour cultures (induced by B6S3, GV3101(pGV2255) and A281 on plants of cv. Lobab Lippoi and cv. Ošlje), higher activity was detected in all tumour calli in comparison with the control. The difference in specific peroxidase activity between tumorous and normal tissue was even more pronounced (Table 2).

Table 1. *Agrobacterium*-strains used to inoculate three broad bean cultivars

Strain	Plasmid	Comments	Source/Ref.
A. tumefaciens			
A281	pTiBo542	wild-type	(*11,12*)
B6S3	pTiB6S3	wild-type	(*8,13*)
GV3101	pGV2215	shooty-type	C. Koncz, J. Schell*
GV3101	pGV2235	shooty-type	C. Koncz, J. Schell*
GV3101	pGV2255	rooty-type	C. Koncz, J. Schell*
C58C1	pRiA4b	transconjugant	(*10*)
C58C1	pRiA4a,b,c	transconjugant	(*10*)
A. rhizogenes			
8196	pRi8196	wild-type	(*10*)
15834	pRi15834	wild-type	(*10*)

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Table 2. Protein content and peroxidase activity in transformed and normal (internodal segments) broad bean tissue

Cultivar	Tumour type	Protein fraction mg/g FW (a)	Peroxidase activity ΔA_{470}/g FW/min (b)	Specific activity (b/a)
Lobab Lippoi	B6S3	3.03	1826.86	602.92
	pGV2255	4.27	2028.81	430.11
	A281	2.66	1787.29	671.91
	Control	6.88	314.29	45.72
Ošlje	B6S3	3.76	1081.84	287.80
	pGV2255	5.95	3052.06	512.78
	A281	9.62	5593.02	581.70
	Control	6.44	321.18	49.91

FW – Fresh weight

Tumour induction in different explants

Explants (cotyledons, leaves and internodal stem segments) of the most susceptible cultivar Lobab Lippoi were infected *in vitro* with Ri plasmid containing strains C58C1(pArA4b), C58C1(pArA4abc), 15834 and 8196, but even after 6 weeks of postinoculation there were no morphological changes in comparison with noninfected explants. Thus the *in vitro* transformation failed.

Discussion

Three broad bean cultivars were tested for their response to nine *Agrobacterium*-strains *in vivo*. Significant differences in susceptibility to each strain were noticed among the cultivars. Two groups of authors who used various strains of *A. rhizogenes* for transformation of different genetic stocks and landraces of broad bean reported analogous results *(5,7).* Although neither of the two *A. rhizogenes* strains (8196 and 15834) used in this study were virulent on any of the three broad bean cultivars, Siefkes-Boer *et al.* *(7)* induced hairy roots *in vivo* on seven and nine out of ten genotypes of *Vicia faba* with strains 8196 and 15834, respectively. Schiemann and Eisenreich *(4)* also obtained hairy roots *in vivo* on cv. Fribo with strain 15834, but *in vitro* experiments with epicotyl explants failed. However, Saalbach *et al.* *(6)* succeeded in the transformation of *Vicia faba* epicotyl explants *in vitro* with strain 15834 after 0.2 M acetosyringone was added to the medium during co-cultivation. As the *in vitro* transformation was unsuccessful, even with bacterium strains that were virulent *in vivo*, the addition of acetosyringone might be also required for *in vitro* transformation of the cultivars used in this study.

This study is the first to demonstrate the transformation of *Vicia faba* with wild *A. tumefaciens* strains. Strains A281 and B6S3 are supervirulent to a number of plant species including some legumes *(8,11,13,17–19).* Although they were more virulent in comparison with most of the other strains used in these experiments, the highest frequency of tumour formation has been obtained with transconjugant *A. tumefaciens* strain C58C1 carrying Ri plasmid pArA4b on cv. Lobab Lippoi. Strain C58C1(pArA4abc), which in addition to plasmid pArA4b carries two more plasmids pArA4a and pArA4c *(10)*, was less efficient than C58C1(pArA4b). It is known that the smallest plasmid pArA4a contains genes whose products allow bacteria to utilise mannopine, mannopinic acid and agropinic acid, while plasmid pArA4c is a cointegrate of pArA4a and pArA4b *(10).* Although the strain with the two plasmids would appear to have a selective advantage over a strain with only one since it would be able to use all of the opines produced by transformed plant tissue *(10)*, the strain carrying only the plasmid pArA4b was more efficient in transformation of all three cultivars. To our knowledge there are no published data dealing with the comparison of the transformation efficiency of the strains C58C1(pArA4b) and C58C1(pArA4abc) on other plant species. Therefore, it is difficult to make any general conclusion concerning the different efficiencies of those two strains. In the strain containing both pArA4a and pArA4b plasmids the cointegrated form (pArA4c) is the predominant plasmid in the bacterial cell *(20)*. Therefore, one could imagine that some *cis* elements involved in the regulation of the expression of genes responsible for different steps of the transformation process might not be equally efficient in cointegrated and dissociated plasmids. In the present study, *A. tumefaciens* strain C58C1 carrying plasmid pArA4b was the most virulent strain and a promising tool for foreign gene delivery into *Vicia faba* cv. Lobab Lippoi, while on the less susceptible cvs. Topolo and Ošlje best results were obtained with supervirulent strains A281 and B6S3, respectively.

The induction of tumour formation by wild *A. tumefaciens* strains depends largely on the expression of two auxin biosynthetic genes *iaaM* (also called gene *1*) and *iaaH* (gene *2*) and of a cytokinin biosynthetic gene *ipt* (gene *4*). Expression of those genes leads to the over-accumulation of both auxin and cytokinin inside the tumour tissue *(8).* Strains mutated at either of the genes *1* and *2* (shooty mutants) can induce shoot formation while those mutated at gene *4* (rooty mutants) can cause root proliferation *(8,9).* Transformation of *Vicia faba* with shooty or rooty strains has not been reported previously. In the experiments described in this paper two shooty mutants (pGV2215 and pGV2235) and one rooty mutant (pGV2255) of the plasmid pTiB6S3 were used. Although in contrast to GV3101(pGV2235), strain GV3101(pGV2215) induced visible outgrowths on a few plants of all tested cultivars, neither of the two mutants appeared to be useful for plant regeneration under these experimental con-

ditions. Since the results obtained with plasmid pTiB6S3 have shown that T-DNA has been transferred and expressed quite efficiently into the genome of all three cultivars, the absence of clear morphological symptoms of transformation after infection with both shooty mutants has led to the conclusion that broad bean cells transformed with either GV3101(pGV2215) or GV3101(pGV2235) have not been stimulated to divide by the expression of mutated T-DNAs. On the other hand, rooty mutant GV3101(pGV2255) has been able to induce tumours in all three cultivars, but no roots were obtained either *in vivo* or *in vitro* (from established tumour cultures), which is in contrast to the results obtained with analogous mutants in some other plant species *(8,9).* This result indicates that either overproduction of auxin, due to expression of mutated T-DNA from pGV2255, does not stimulate root proliferation in transformed broad bean tissue or that the overproduction of auxin in transformed broad bean cells is efficiently downregulated.

Plant cells transformed with wild-type agrobacteria proliferate autonomously in *in vitro* culture in the absence of the phytohormones (auxins and cytokinins) that are otherwise needed for the growth of normal plant cells. Another feature of transformed cells is an increase of total peroxidase activity *(21,22).* Therefore, enhanced peroxidase activity might be a useful biochemical transformation marker. Higher peroxidase activity, in comparison with the control, was detected in all tested tumour cultures in this study.

Each bacterial strain has shown different efficiency in the transformation of different broad bean cultivars. However, the size and phenotype of tumour tissue depended on the strain rather than on the plant genotype (except in the C58C1(pArA4b)/cv. Lobab Lippoi combination). This indicates that the three broad bean cultivars do not respond differently to the change in the balance of growth regulators in the cells transformed with the same T-DNA. As tumour formation is the final result of a complex process in which a large number of discrete steps are involved, the different transformation efficiency of each bacterial strain in this study might be the result of different efficiency in one or more steps prior to the expression of T-DNA genes within each particular cultivar. However, tumours induced with strain C58C1(pArA4b) on cv. Lobab Lippoi were approximately four times larger than in other cultivars, while there were no differences in the size among tumours induced with strain C58C1(pArA4abc). As both of those strains transfer the same T-DNA to the plant cell they are expected to induce tumours of a similar size. This observation indicates that there might be very specific metabolic differences among the cultivars that must be taken in consideration. Therefore more attention has to be paid to the *Agrobacterium*-strains used for optimizing the transformation of each cultivar.

Although a cultivar very susceptible to transformation with *Agrobacterium in vivo* was identified, the regeneration of transgenic broad bean plants remains the main problem for the practical use of *Agrobacterium*-mediated gene delivery in the broad bean.

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Transformacija boba *Vicia faba* L. s pomoću bakterija roda *Agrobacterium*

Sažetak

Radi utvrđivanja najpogodnije kombinacije za transformaciju i mogućnosti regeneracije genetički promijenjenih biljaka, tri sorte boba različite morfologije i geografskog podrijetla inficirane su s devet sojeva bakterije roda *Agrobacterium*. Stabljike klijanaca inokulirane su *in vivo* divljim sojevima (A281 i B6S3) bakterije *A. tumefaciens*, transkonjugantnim sojevima (C58C1(pArA4abc) i C58C1(pArA4b)), korijen-inducirajućim (GV3101(pGV2255)) i izdanak-inducirajućim (GV3101(pGV2215) i GV3101(pGV2235)) mutantima soja B6S3, te divljim sojevima (8196 i 15834) bakterije *A. rhizogenes*. Virulentni sojevi potaknuli su jedino rast neorganiziranog tumorskog tkiva na svim sortama. Utvrđena je različita osjetljivost sorata na pojedine bakterijske sojeve. Najbolji rezultat postignut je sa sojem C58C1(pArA4b) na sorti Lobab Lippoi, cv. Topolo bio je najosjetljiviji na soj A281, a cv. Ošlje na soj B6S3. Unatoč tome, veličina i fenotip tumora ovisili su jedino o bakterijskom soju. To opažanje upućuje na zaključak da su razlike u učestalosti transformacije pojedinih kultivara istim bakterijskim sojem posljedica različite učinkovitosti procesa koji prethode ekspresiji T-DNA. U pokusima *in vitro* na eksplantatima stabljike, listova i kotiledona, primjenom korijen-inducirajućih bakterija, nije postignuta transformacija. U kulturama tumorskoga tkiva ustaljenim na podlozi MS bez hormona utvrđena je pojačana aktivnost peroksidaza.