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IN PLANTA TRANSFORMATION OF BUCKWHEAT (*FAGOPYRUM ESCULENTUM* MOENCH.)

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Abstract - In order to develop a reliable and rapid transgenic system for functional study of specific buckwheat gene constructs, two different *in planta* transformation methods were analyzed: vacuum infiltration and infiltration by syringe. The results indicated that the vacuum infiltration method was much more efficient and can therefore be considered the method of choice for buckwheat transformation.

Key words: Buckwheat, GUS assay, syringe infiltration, vacuum infiltration

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INTRODUCTION

Agrobacterium-mediated *in vitro* transformation has been routinely used for the production of transgenic plants (H o r s c h et al., 1985), although it is usually time-consuming. Therefore, it is important to develop *in planta* transformation methods that do not require sterile conditions of tissue culture and enable rapid gene functional analysis.

Common buckwheat (*Fagopyrum esculentum* Moench) is a crop grown mainly in the northern hemisphere. The large amount of lysine and well-balanced content of other essential amino acids characteristic of buckwheat seed storage proteins make this plant highly recommendable for human consumption. Buckwheat is also unique among crops due to the presence of rutin, a flavonol glucoside serving as part of the defense system against oxidative stress. Aside from these traits, there is rising interest in this plant as an object of molecular biology and biotechnology research. Gene expression and functional promoter analysis of specific buckwheat genes could be an element in the process of understanding common mechanisms of gene regulation in plant cells.

Until now buckwheat has been transformed only by an *Agrobacterium*–mediated *in vitro* method (M i lj u š - D j u k i ć et al., 1992). This is the first paper reporting a rapid and effective method for *in planta* transformation of buckwheat. Two different *in planta* transformation methods were optimized: vacuum infiltration and infiltration by syringe.

MATERIAL AND METHODS

Plant material and vectors used

Buckwheat plants (*Fagopyrum esculentum* Moench) grown in a greenhouse were used for transformation experiments. The two vectors used for transformation – pCAMBIA2301 (Cambia, Australia) and pCAMBIA-PL – were electroporated into *Agrobacterium tumefaciens* strain EHA105. pCAMBIA2301 is a strong CaMV35S promoter upstream of the GUS reporter gene, while pCAMBIA-PL is a promoterless GUS reporter gene.

Transformation protocols

Vacuum infiltration

Vacuum infiltration was performed according to B e c h t o l d et al. (1993). An overnight culture of *A. tumefaciens* (10 ml) was inoculated in 1 L of LB medium supplemented with 150 μM acetosyringone (AS), 50 μg/ml kanamycin, and 10 μg/ml rifampicin. Bacterial cultures were grown overnight at 28°C to OD_{600} ranging from 0.8 to 2.1, centrifuged (5000 rpm/RT/15 min), and resuspended in infiltration medium [½ strength Murashige and Skoog macro and micro salts and vitamins, 5% sucrose, 2.6 μM MES, 44 nM 6-benzylaminopurine (BAP), 150 μM AS, 0.02% Silwett L-77, pH adjusted to 5.7]. Plants were removed from pots and immersed in 1

L of infiltration medium an hour before vacuum infiltration. Two different vacuum conditions were used for infiltration: pressure of 10^4 Pa was applied for 5 min and 10^2 Pa for 20 min, then released rapidly to increase the infiltration efficiency. After removal from the dessicator, plants were laid on their side for 15 min and wrapped in transparent plastic to maintain humidity. The next day they were uncovered and set upright in pots, but not watered for three days.

Infiltration by syringe

Infiltration by syringe was performed according to Y a n g et al. (2000). Briefly, *Agrobacterium* cultures were grown overnight at 28°C/150 rpm to OD_{600} ranging from 0.8 to 2.1. Cells were collected by centrifugation (5000 rpm/RT/15min) and resuspended in infiltration medium [10 mM $MgSO_4$, 10 mM MES (pH 5.5), 150 µM AS]. The bacterial suspension was infiltrated by applying pressure against the lower side of a young leaf with a needle-less syringe. After infiltration, plants were kept covered with transparent plastic bags for two days without watering.

Quantitative GUS assay

The level of transient GUS activity monitored by fluorescence GUS assay (J e f f e r s o n, 1987) was used as a measure of transformation efficiency. The protein concentration of sample extracts was determined using a BioRad Protein Assay Kit with BSA as the standard. GUS activity was expressed as pmol 4-MU per hour per mg of protein, where 4-MU was quantified with a Versa-Fluor fluorimeter (BioRad).

Statistical analyses

The results were statistically analyzed using the SPSS statistical program. Since the obtained values did not show normal distribution, the nonparametric Mann-Whitney test and median values were used for comparison of different groups of samples.

RESULTS AND DISCUSSION

According to numerous papers reporting successful *in planta* transformation of different plant species, the efficiency of transformation depended on several conditions, namely *Agrobacterium* culture density, vacuum conditions, and leaf maturity. It was shown that optimal values of these factors have to be determined for each plant species.

For optimization of the vacuum infiltration method for buckwheat, a total of 230 samples were analyzed. The influence of *A. tumefaciens* culture cell density, as well as pressure conditions and time elapsing between the transformation event and GUS measurement, were examined as factors that could be important for transient transformation efficiency.

Various plant species were successfully transformed using bacterial culture OD_{600} values varying from 0.8 to 2

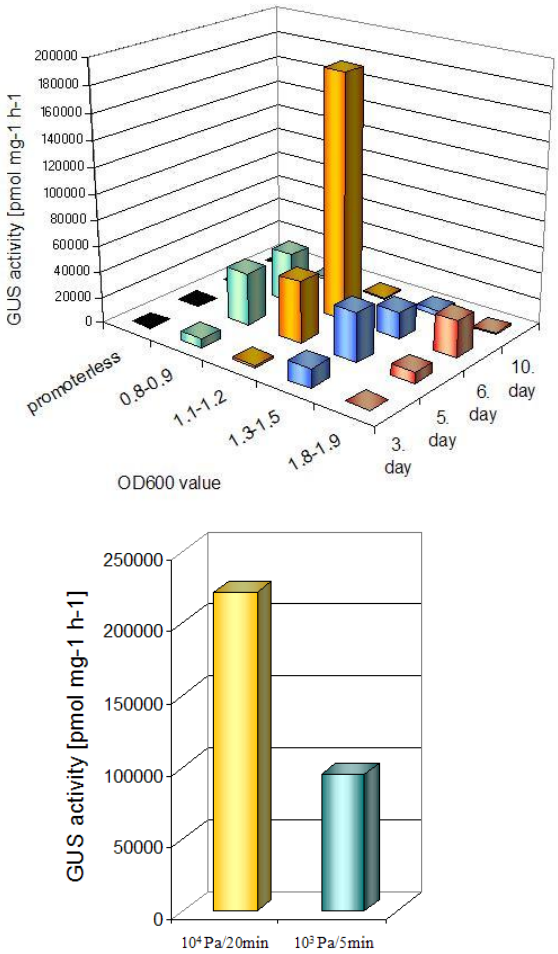

Fig. 1. Optimization of the vacuum infiltration method. Influence of bacterial culture OD_{600} and time elapsing between transformation event and GUS measurement on GUS activity. In all cases 10^4 Pa/20 min vacuum conditions were applied. Data are median values of GUS activities obtained by measuring 10-15 samples for each OD_{600}/day. Influence of vacuum conditions on GUS activity. EHA105/pCAMBIA2301 and bacterial culture OD_{600} 1.1 were used for both examined conditions. GUS activity was measured 6 days after transformation.

(Bechtold et al., 1993; Kapila et al., 1997; Amoah et al., 2000; Trieu et al., 2000; Bent and Clough, 1998). To determine the most suitable bacterial culture OD_{600} for buckwheat, we tested transformation efficiency using OD_{600} values within the following ranges: [0.8-0.9]; [1.1-1.2]; [1.3-1.5] and [1.9-2.1], under a pressure of 10^4 Pa/20 min. To find out when maximal GUS activity was reached, quantitative assays were performed 3, 5, 6, and 10 days after transformation for all tested OD_{600} values. Figure 1a shows the GUS values obtained for all examined conditions in plants transformed with EHA105/pCAMBIA2301 or with pCAMBIA-PL (negative control). On the third day after transformation, the detected GUS activity was low, but significantly higher than the GUS value measured in plants infiltrated with EHA105/pCAMBIA-PL, except for the case of OD_{600} [1.8-2]. Maximum GUS activities for all tested OD_{600} ranges were reached on the 5th and 6th days, but it was significantly decreased on the 10th day. The most suitable bacterial culture OD_{600} was [1.1-1.2] measured 6 days after transformation, since it resulted in distinctly higher GUS activity than under any other applied conditions.

As mentioned above, the efficiency of vacuum transformation also depends on the applied vacuum conditions, which have to be determined for each plant species. For example, tobacco was successfully transformed under conditions of $8x10^3$ Pa/20 min (Wang et al., 2002) and wheat under conditions of 10^5 Pa/60 min (Amoah et al., 2001), while for *Arabidopsis in planta* transformation different vacuum conditions in a range of from $1.6x10^3$ Pa/20 min to $5x10^4$ Pa/15 min were confirmed as suitable (Bechtold et al., 1993; Clough et al., 1998). To define optimal vacuum conditions for buckwheat *in planta* transformation, we tested two different pressures viz., 10^3 Pa and 10^4 Pa, and durations of 5 min to 25 min of vacuum treatment, applying the previously determined optimal OD_{600} 1.1. We found that 5 min was optimal for the vacuum pressure of 10^3 Pa, while 20 min was the most suitable for the vacuum pressure of 10^4 Pa (data not shown). Comparison of these two vacuum conditions showed that 10^4 Pa/20 min was the most suitable vacuum condition for buckwheat transformation, as the corresponding median value for GUS activity was 14 times higher than for leaves infiltrated under 10^3 Pa/5 min (Fig. 1b). It was also noticed that many leaves infiltrated under 10^3 Pa/5 min wilted, while leaves treated using 10^4 Pa/20 min showed faster recovery.

For optimization of the syringe infiltration method, the influence of bacterial culture OD_{600} was examined. GUS activity was measured 5 and 6 days after transformation of buckwheat, bearing in mind that maximal activity was reached within that period for vacuum infiltration. The results are summarized in Fig. 2a. For all examined OD_{600} ranges, it was shown that GUS activities were significantly higher than in plants infiltrated with the EHA105/pCAMBIA-PL strain. In contrast to vacuum infiltration, syringe transformation efficiency did not vary much among the different OD_{600} ranges higher than 1, as the corresponding values were not significantly different at $p<0.05$. Two groups of OD_{600} ranges were found to

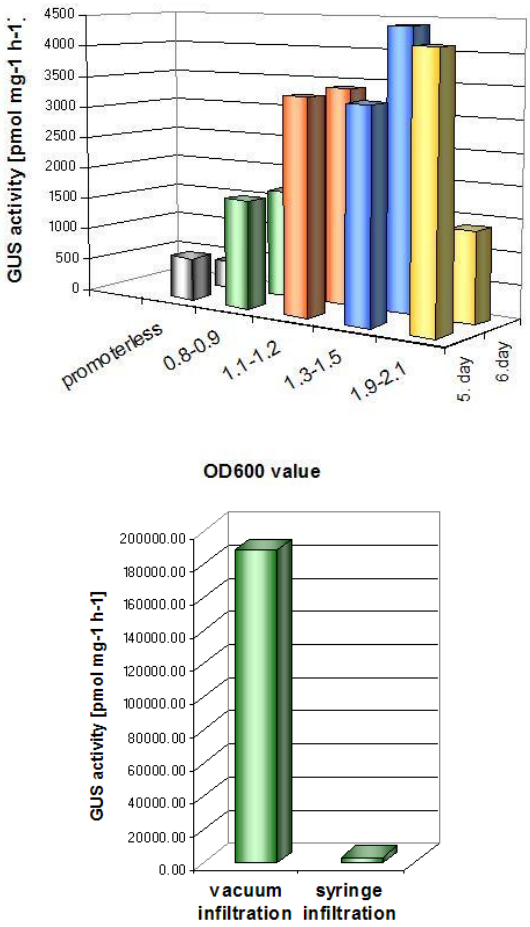

Fig. 2. Optimization of the syringe infiltration method. Influence of bacterial culture OD_{600} on transformation efficiency. Data for each OD_{600}/day represent median values of GUS activities (pmol MU mg^{-1} h^{-1}) obtained from 15 samples. Comparison of two different transformation methods under their optimal conditions (vacuum infiltration: OD_{600} 1.1 / 6th day; syringe infiltration OD_{600} 1-2 / 5th and 6th day).

have statistically significant differences in measured GUS activity. Buckwheat transformation was more efficient for the group within the OD_{600} range of from 1 to 2, since the corresponding median values were approximately twice as high as those obtained for OD_{600} 0.8 - 0.9.

The results obtained under the defined optimal conditions for both described transformation methods are compared in Fig. 2b. Vacuum infiltration appeared to be far more efficient, as the median value obtained for GUS activity was 57.3 times higher than that obtained with infiltration by syringe.

We conclude that vacuum infiltration can be considered the method of choice for transient buckwheat transformation. In further investigations, we plan to test the ability of the vacuum method to give stable transgenic T1 lines of buckwheat.

Abbreviations used

GUS - ß-glucuronidase; MES - 2[N-morpholino]ethane sulfonic acid; BAP - 6-benzylaminopurine; AS - acetosyringone; 4-MU - (4-methyl umbelliferol); BSA - bovine serum albumin.

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IN PLANTA ТРАНСФОРМАЦИЈА ХЕЉДЕ (*FAGOPYRUM ESCULENTUM* MOENCH.)

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За функционалну анализу потенцијалних промоторских фрагмената гена хељде неопходан је ефикасан метод трансформације. Имајући у виду да је микропропагација и регенерација хељде дуготрајан процес, оптимизована су две методе за *in planta* трансформацију: вакуум инфилтрација и инфилтрација помоћу шприца. Одређиване су оптималне вредности за: густину културе бактерија *Agrobacterium tumefaciens* (које носе вектор за трансформацију) и јачину вакуума. Ефикасност трансформације је праћена помоћу квантитативног GUS есеја. Резултати су показали да је вакуум инфилтрација ефикаснији метод трансформације хељде.