Phaseolus vulgaris ***L. regeneration systems and their application for*** **Agrobacterium*-mediated genetic transformation***

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REPORT

ABSTRACT

Procedures for regeneration and genetic transformation of *Phaseolus vulgaris* L. were established. Pre-culture of explants in liquid medium with TDZ, which has not been used for common bean regeneration before, was a critical step for multiple bud induction. The optimization of several parameters involved in DNA-transfer combined with efficient direct organogenesis allowed to regenerate plants from tissue transformed with *Agrobacterium tumefaciens*. Somatic embryo formation on cotyledons from immature seeds of *P. vulgaris* was achieved. For this species, it was the first time that somatic embryos reaching the cotyledonary stage were obtained by these procedures, and a protocol was established for the in vitro regeneration of *P. vulgaris* via indirect organogenesis. The use of cotyledonary nodes with one or two cotyledons attached, which have not been used as initial explants for common bean regeneration before, was an important factor for the successful formation of morphogenetic green nodular compact calli. This last was successfully used as target explants to obtain transformed plants, based on the indirect organogenesis regeneration pathway. The protocol established for the CIAP7247F variety was shown reproducible in other four commercial cultivars. These results provide for the first time ever a protocol for the genetic transformation of common bean via *A. tumefaciens*, which can be used for cultivar breeding. This research granted the 2015 Award of the Cuban National Academy of Sciences.

Keywords: common bean, green nodular calli, indirect organogenesis, somatic embryos

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RESUMEN

Sistemas de regeneración de *Phaseolus vulgaris* **L. y su aplicación en la transformación genética vía** *Agrobacterium tumefaciens***.** Se establecieron procedimientos para la regeneración y transformación genética de *Paseolus vulgaris* L. El precultivo de los explantes en medio líquido con TDZ, no descrito para esta especie en la literatura científica consultada, fue un paso determinante para la inducción de yemas múltiples. La optimización de varios parámetros que intervienen en la transferencia de ADN en combinación con la organogénesis directa, permitió por vez primera regenerar plantas de *P. vulgaris* a partir de tejido transformado con *Agrobacterium tumefaciens*. Se logró la formación de embriones somáticos en cotiledones de semillas inmaduras de *P. vulgaris*. Por vez primera para esta especie, se obtuvieron embriones somáticos que alcanzaron la etapa cotiledonal. Además, se estableció un protocolo para la regeneración in vitro de *P. vulgaris* vía organogénesis indirecta. El uso del nudo cotiledonal con uno o dos cotiledones como explante inicial para la regeneración de frijol común, fue un factor determinante para la formación eficiente de callos morfogenéticos. El protocolo establecido para la variedad CIAP7247F fue reproducido en cuatro variedades comerciales. Sobre la base de la regeneración vía organogénesis indirecta, se llevó a cabo la transformación mediada por *A. tumefaciens* en frijol común. El uso de callos nodulares verdes, que no se habían utilizado antes como explante para la transformación de frijol, jugó un papel importante para obtener plantas transgénicas. Estos resultados permitieron disponer por vez primera de un protocolo de transformación genética vía *A. tumefaciens*, el cual puede ser empleado para la obtención de variedades de frijol mejoradas. Este trabajo mereció el Premio Anual de la Academia de Ciencias de Cuba para el año 2015.

Palabras clave: frijol común, callos verdes nodulares, organogénesis indirecta, embriones somáticos

Introduction

Major improvements in agronomic traits of cultivated common bean (*Phaseolus vulgaris* L.) have been achieved throughout years of conventional breeding. However, such improvements have been limited and restricted to the traits available in the bean genetic pool, in spite of breeding contributing to the improvement of desirable agronomic traits of this major edible crop. As a consequence, many problems of this crop had not been overcome by existing breeding techniques.

Moreover, the conventional improvement of common bean has been hampered in many aspects, and due to genetic difference among bean species, common bean can only be crossed with a bunch of wild or cultivated species. Traditional breeding methods are also hampered by the low inheritance of some important characteristics (total yield and yield components), as well as sexual barriers and embryo abortion in interspecific hybrids [1]. Therefore, genetic

transformation of common bean is of surmount importance for increased crop adaptation, production yields and profitability, and it would allow breeders to introduce novel traits which could contribute to improve crop performance, quality and tolerance to detrimental abiotic and biotic stress factors [2].

Unfortunately, really routine and easily applicable protocols for common bean transformation are still a promise, despite the numerous regeneration protocols already developed [3]. In fact, this crop is still regarded as recalcitrant to transformation, its limited regeneration capacity as the main bottleneck. Furthermore, any efficient system for gene transformation has to surpass key technical constraints, including a high regeneration capacity, the efficient delivery of transgenes to a large number of cells from the target explants, and also, effective selectable markers have to be considered [4].

Our long-term aim to cope with the abovementioned limitations and needs was to establish an efficient, reproducible, genotype independent procedure, to routinely obtain stable transgenic plants of *P. vulgaris* using *Agrobacterium tumefaciens* as gene transfer vector. The main requirements to be addressed in order to reach this goal were: i) the development of an efficient regeneration system, capable to support the regeneration of transformed bean cells; and ii) the optimization of *Agrobacterium*-mediated transformation techniques to allow an efficient DNA-transfer to the bean cells. For this purpose, different regeneration systems were investigated in *P. vulgaris*, the successful ones been applied to develop a reproducible *Agrobacterium*-based transformation method. This research granted the 2015 Award of the Cuban National Academy of Sciences.

Somatic embryogenesis

Common bean, like most legumes, is recalcitrant to in vitro culture, and the establishment of a regeneration procedure via somatic embryogenesis in this species has not been possible yet. Therefore, determining the effect of factors that influence somatic embryo formation constitutes an essential step to develop a regeneration protocol via somatic embryogenesis. To achieve somatic embryo formation in *P. vulgaris* cv. CIAP7247F, cotyledons of immature seeds were employed as initial plant material. Five 2,4-D concentrations (10, 20, 30, 40 and 50 mg/L) and cotyledon orientation on culture medium were studied. The highest number of explants that formed somatic embryos and the highest number of somatic embryos formed per explant were obtained at 40 and 50 mg/L 2,4-D.

Somatic embryos were formed on the abaxial side from the cotyledons, the explants positioned with the adaxial side into contact with the culture medium [5]. Nevertheless, the embryos obtained by this method did not regenerate into plants, further requiring new insights in the process to obtain completely developed somatic embryos. The analysis of this situation allowed us to identify immature cotyledons as an optimum tissue which would support the generation of somatic embryos in common bean. In this line of research, other factors could be involved in such processes, including the influence of the nitrogen source and the availability of plant growth regulators in the culture medium, among other changes in the culture conditions.

Direct organogenesis

Common bean direct regeneration has been investigated by several research groups before [6-10]. None of the published approaches had been successfully used for common bean genetic transformation. The main disadvantage found in those procedures was that the regeneration from a pre-existing meristematic tissue [3]. Therefore, a reproducible procedure was developed for the regeneration of fertile plants by organogenesis from cultures of the economically relevant *P. vulgaris* L. cultivars: CIAP7247F, BAT93, BAT304, BAT482 and ICA Pijao using epicotyl sections as initial explant. Unlike the previously published regeneration protocols, this system is based on the production of multiple buds from non-meristematic tissue. Pre-culture of explants in liquid medium with TDZ, which had not been used for common bean regeneration before, was a critical step for multiple bud induction [11]. Optimization of culture media and parameters involved with the explant quality supported the standardization of several culture phases including shoot formation and plantlet acclimatization, which ultimately yielded fertile plants.

Agrobacterium-mediated transformation using direct organogenesis

The optimization of several parameters involved in *Agrobacterium* DNA-transfer and the combination of the optimized method with direct organogenesis from epicotyl sections successfully led to the generation of transgenic plants [11]. Noteworthy, the recent attempts to transform common bean with *Agrobacterium* [3, 12] failed, and transformed tissue could not be recovered mainly due to poor regeneration. The full regeneration of beans from tissue transformed via *A. tumefaciens*, either chimeric or stable transgenic plants, had not been demonstrated. Using the genetic transformation procedure [11], geneticin-resistant plantlets were obtained which survived in soil and yielded seeds. Nevertheless, only chimeric plants or escapes were recovered.

Several factors can affect the efficiency of a transformation system, among which the regeneration way and selection procedure play an important role. Regarding our results, the resulting escapes and chimeric plants were attributed in descending relevance to the multicellular origin of the multiple buds, the use of a selection marker (*npt*II) which may not have been appropriate, and to a probably wrong selection strategy. Alternative selection markers such as the bialaphos-resistance (*bar*) or imidazolinone-resistance (ahas) genes could provide a more strict selection as needed in this transformation system. Also the earlier application of the selection with more selection cycles, possibly based on repetitive multiplication of the primary multiple buds on selective medium, could reduce the regeneration of escapes and chimeric plants. Despite these improvements, that could increase the effectivity of the transformation procedure established [11], it was considered that an optimum solution could be a regeneration system based on indirect organogenesis,

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through a callus phase as that previously described for *P. acutifolious* [13].

Indirect organogenesis

A protocol for the successful *in vitro* regeneration of *P. vulgaris* L. cv. CIAP7247F via indirect organogenesis was established [14]. The use of CN-1 and CN-2 explants, which have not been used as initial explants for common bean regeneration before, was determinant for the formation of morphogenetic green nodular compact calli. As far as we know, just three studies on indirect regeneration of *P. vulgaris* have been published yet; the first two protocols reported were genotype dependent [15, 16] and the third one [7] showed low efficiency. The regeneration frequency that we obtained was high as compared to the results reported previously [15, 16]. The established protocol [14] was efficiently applied to other four commercial *P. vulgaris* varieties: BAT93, BAT304, BAT482 and ICA Pijao. This denoted the extensible value of this procedure, which may therefore be useful to regenerate other common bean genotypes.

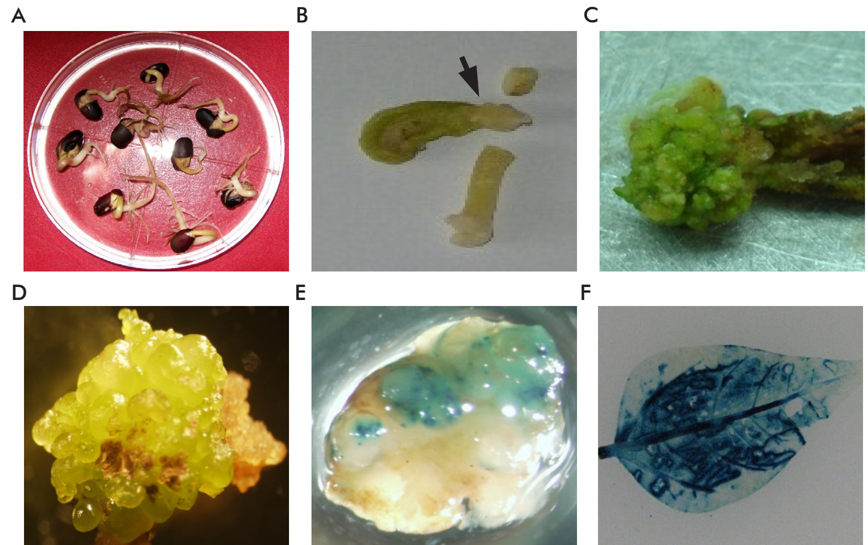

Figure. Transformation of common bean cultivar CIAP7247F using green nodular calli as explants. A) Germinating seeds 3-days old; B) Explant preparation from seedlings (arrow points to the cotyledonary node with one cotyledon); C) Primary green nodular callus; D) green nodular callus seven days after second subculture; E) GUS staining in transformed green nodular calli; F) GUS staining in leave of a geneticin-resistant plant.

Agrobacterium-mediated transformation using indirect organogenesis

Derived from above mentioned results, a transformation system was implemented, integrating *Agrobacterium*-mediated DNA transfer techniques with efficient regeneration via indirect organogenesis, and using the *bar* gene as selectable marker [17] (Figure). Up to our knowledge, all the systems for common bean transformation, regardless of the DNA delivery method, use direct organogenesis to regenerate the transformed cells. Therefore, the work scheme proposed in our strategy [17] was the very first transformation protocol developed for common bean in which organogenic calli are used as target explant. In line with the aim of this study, the system supported the obtainment of putative transgenic lines. In fact, the T_1 progeny of five of these lines showed Mendelian inheritance of the foreign genes, as confirmed by PCR analysis [17]. These results validated the effectiveness of the regeneration protocol of transformed beans cells via indirect organogenesis for the five commercial varieties assayed [14].

Relevance of the study

It is concluded that, based on the results obtained with the two transformation systems established, *Agrobacterium*-mediated transformation using indirect regeneration is the most promising strategy. They constitute important tools for bean breeding programs. Moreover, the regeneration protocols reported could be applied for genetic transformation using *A. tumefaciens* in combination with particle bombardment DNA delivery methods, for commercial genotypes. These procedures may also be used to regenerate other important bean cultivars, further modifying them by either genetic transformation or induced mutations, and to regenerate bred genotypes with resistance to biotic and abiotic stress. Additionally, it could be utilized to breed cultivars with specific traits cording to breeding purposes, such as: improved yield, taste quality, adaptability to mechanical harvesting and processing. Future research projects would have to address the use of the varieties tested in this study for its application in the field, particularly to introduce resistance to the Bean Golden Mosaic Virus, increase salinity and drought tolerance, and enhance the contents of valuable amino acids such as methionine and threonine, all of them relevant aspects to improve plant development and productivity.

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