Minimizing the time and cost of production of transgenic alfalfa libraries using the highly efficient completely sequenced vector pPZP200BAR

Cintia Jozefkowicz, Emilia Bottero, Cecilia Pascuan, Elba Pagano, Nicolás Daniel Ayub & Gabriela Soto

Plant Cell Reports

ISSN 0721-7714

Plant Cell Rep
DOI 10.1007/s00299-016-2026-7

Springer

Your article is protected by copyright and all rights are held exclusively by Springer-Verlag Berlin Heidelberg. This e-offprint is for personal use only and shall not be self-archived in electronic repositories. If you wish to self-archive your article, please use the accepted manuscript version for posting on your own website. You may further deposit the accepted manuscript version in any repository, provided it is only made publicly available 12 months after official publication or later and provided acknowledgement is given to the original source of publication and a link is inserted to the published article on Springer's website. The link must be accompanied by the following text: "The final publication is available at link.springer.com".

Springer

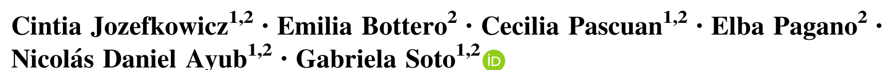
Author's personal copy

Plant Cell Rep
DOI 10.1007/s00299-016-2026-7

CrossMark

FOCUS ARTICLE

Minimizing the time and cost of production of transgenic alfalfa libraries using the highly efficient completely sequenced vector pPZP200BAR


Cintia Jozefkowicz[1,2] · Emilia Bottero[2] · Cecilia Pascuan[1,2] · Elba Pagano[2] · Nicolás Daniel Ayub[1,2] · Gabriela Soto[1,2]

Received: 2 July 2016 / Accepted: 9 July 2016
© Springer-Verlag Berlin Heidelberg 2016

Abstract Alfalfa is the most important forage legume worldwide. However, similar to other minor forage crops, it is usually harvested along with weeds, which decrease its nutrient quality and thus reduce its high value in the market. In addition, weeds reduce alfalfa yield by about 50 %. Although weeds are the limiting factor for alfalfa production, little progress has been made in the incorporation of herbicide-tolerant traits into commercial alfalfa. This is partially due to the high times and costs needed for the production of vast numbers of transgenic alfalfa events as an empirical approach to bypass the random transgenic silencing and for the identification of an event with optimal transgene expression. In this focus article, we report the complete sequence of pPZP200BAR and the extremely high efficiency of this binary vector in alfalfa transformation, opening the way for rapid and inexpensive production of transgenic events for alfalfa improvement public programs.

Keywords pPZP200BAR · Complete sequence · Binary vector · Alfalfa · Transformation

Communicated by N. Stewart.

Electronic supplementary material The online version of this article (doi:10.1007/s00299-016-2026-7) contains supplementary material, which is available to authorized users.

✉ Gabriela Soto
soto.gabrielacinthia@inta.gob.ar; gabysoto80@gmail.com

[1] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina

[2] Instituto de Genética Ewald A. Favret (CICVyA-INTA), De los Reseros S/N, Castelar C25 (1712), Buenos Aires, Argentina

Introduction and aims

The first binary vectors used antibiotic resistance cassettes (e.g., kanamycin and hygromycin) for efficient selection of transformed plant tissues. However, hypothetical lateral transfer of antibiotic resistance genes from transgenic plants to pathogenic bacteria have generated a strong public opinion against using antibiotic markers to produce transgenic crops (Tuteja et al. 2012). In this context, the bialaphos resistance (BAR) gene, originally cloned from *Streptomyces hygroscopicus* (Thompson et al. 1987), has been introduced into t-DNA of binary vectors as a non-antibiotic-selectable completely innocuous marker for both plant transformation and tolerance to the broad-spectrum non-systemic herbicide glufosinate (Liberty® Bayer Crop Science). Although binary vectors harboring the BAR gene have been used to produce transgenic plants, little is known of their molecular and functional characteristics, including detailed sequence and transformation efficiencies (Meek 2011). This limits the insertion of novel genes into t-DNA regions by endonucleases and the production and identification of events with potential biotechnological applications.

Due to the differential transcriptional and post-transcriptional transgene silencing in independent transgenic lines generated with the same binary vector, a vast number of events have to be screened to identify events showing high-level, constitutive and ubiquitous expression of the transgene (Schubert et al. 2004). This constraint is particularly hard in the case of alfalfa (*Medicago sativa*), the most important forage legume worldwide. For example, the identification of J101 and J163 alfalfa events with optimal glyphosate tolerance needed the production of thousands of transgenic events containing the same binary vector (PV-MSHT4) and the subsequent transgenic trait analysis of this

Published online: 22 July 2016

Author's personal copy

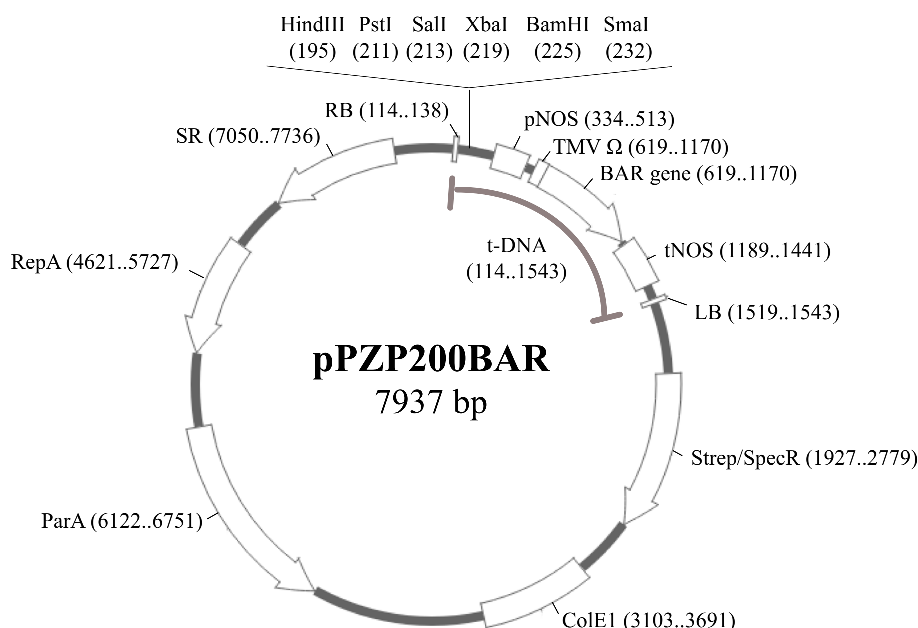


Fig. 1 Schematic representation of the pPZP200BAR binary vector. pPZP200BAR is 7937 bp in length and its GenBank accession number is KX227611. The right border (RB), a multiple cloning site (MCS) with unique restriction sites for *Hind*III, *Pst*I, *Sal*I, *Xba*I, *Bam*HI and *Sma*I, nopaline synthase promoter (pNOS), the 5′-untranslated region of RNA of tobacco mosaic virus (TMV Ω), the BAR gene, nopaline synthase terminator (tNOS), and the left border (LB) within the t-DNA region are shown. Gene orientation is indicated by *arrows*. *SR* serine recombinase, *RepA* replication protein A, *ParA* partitioning protein, *ColE1* origin of replication from *Escherichia coli*, *Strep/SpecR* cassette for tolerance to streptomycin and spectinomycin in *E. coli* and *Agrobacterium tumefaciens*

huge event library under field conditions (Rogan and Fitzpatrick 2004). In fact, currently, the only genetically modified alfalfa in the world market is Roundup Ready® alfalfa (alfalfa-RR), even though this important legume species has been transformed by *Agrobacterium* for over 25 years (D'Halluin et al. 1990). Unfortunately, the extensive use of glyphosate herbicides has generated a strong selection pressure for the emergence of glyphosate-tolerant weeds (Heap 2014), dramatically reducing the protein content and forage yield in alfalfa-RR, and thus showing the relevance for the rapid production of alternative herbicide-tolerant alfalfa cultivars to weed control (Rubiales 2014).

The production of vast numbers of transgenic events, as an empirical approach to bypass random transgene silencing, significantly increases the time and cost of development of efficient transgenic events, restricting the production and commercialization of transgenic alfalfa to international consortia. Moreover, it is expected that the common public studies do not provide solutions to this specific biotechnological problem because the expression levels of transgenes in alfalfa are usually sufficient for functional characterizations of novel genes (Garcia et al. 2014; Soto et al. 2011).

In this focus article, we report the complete sequence of the binary vector pPZP200BAR, which has been used for tobacco and potato transformations (Laguia-Becher et al. 2010; Rivero et al. 2012), and its extremely high efficiency in alfalfa transformation, opening the way for rapid and inexpensive production of transgenic events for alfalfa improvement public programs.

Materials and methods

To determine the complete sequence of the binary vector pPZP200BAR (CICVyA-INTA), a genomic walking analysis was performed starting with the primers bar1 (5′-TGCACCATCGTCAACCACT-3′) and bar2 (5′-ACAGCCACCACGCTCTTGAA-3′), designed against the coding sequence of the BAR gene. Sequences were aligned, assembled, and analyzed by using Bioedit Sequence Alignment Editor CAP (Hall 1999). All sequencing reactions were performed at Unidad de Genómica, Instituto de Biotecnología, CICVyA, INTA, Argentina. The complete sequence of pPZP200BAR has been deposited at GenBank under the accession number KX227611.

The alfalfa plants used in this study were the regenerative clone C2–3 (Garcia et al. 2014). Binary vectors harboring the BAR gene for glufosinate tolerance (pPZP200BAR, pGSFR761A, pGSFR780A, pBG-1 and pBL-1) were introduced into *Agrobacterium tumefaciens* LBA 4404 by electroporation (Shen and Forde 1989). Petioles of the alfalfa clone C2–3 were transformed with

Author's personal copy

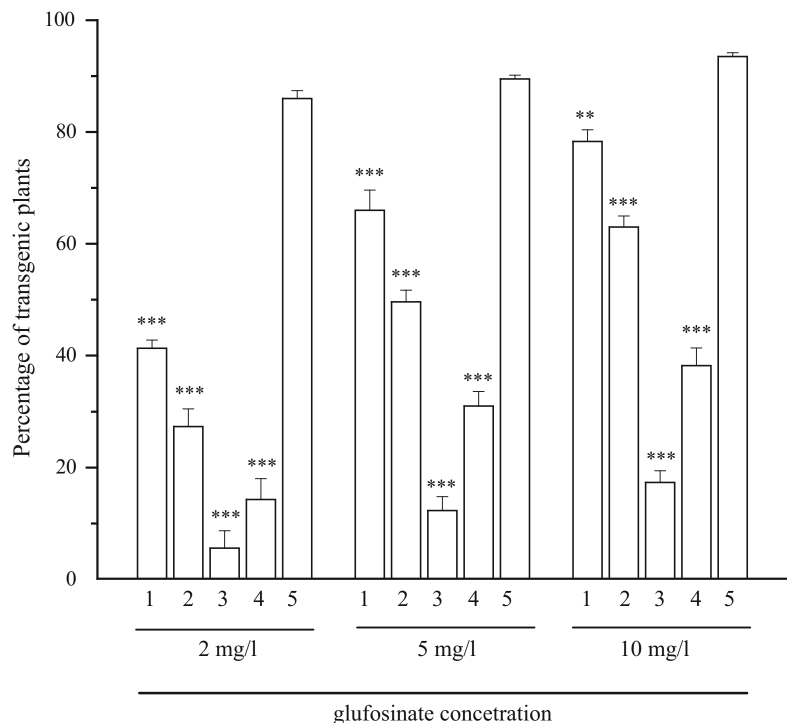


Fig. 2 Efficiency of pPZP200BAR in alfalfa transformation. Alfalfa transformation efficiency of different vectors harboring the BAR gene, including the binary vector pPZP200BAR, expressed as percentage of transgenic plants. Statistical analysis was carried out with ANOVA followed by Dunnett's contrast test comparing control binary vectors (1) pBG-1, (2) pBL-1, (3) pGSFR761A, (4) pGSFR780A with (5) pPZP200BAR (***$p < 0.001$, **$p < 0.01$). All values are mean ± SEM, $n = 3$ independent transgenic libraries, where each library contains 100 events

these binary vectors via *A. tumefaciens* and cultured in vitro, as we have previously described (Garcia et al. 2014), but placing kanamycin by 2–10 mg/l glufosinate in the selection/induction SHK medium. Alfalfa transgenic events were propagated by cuttings to increase the biomass available for molecular and phenotypic characterizations. Glufosinate-tolerant plants derived from different calluses were considered independent events. Expression of the transgenic trait (herbicide tolerance) in transgenic plants was corroborated by three applications 1.25 mg/l glufosinate (Liberty® Bayer Crop Science) as previously described (Montague et al. 2007). Genomic DNA of transgenic plants was isolated from leaf tissue with the DNeasy plant mini kit (Cat. # 69104, Qiagen). Transgenic plants were identified by PCR with the above described primers bar1 and bar2, under the following PCR conditions: 1 cycle at 94 °C for 5 min, 34 cycles of 94 °C for 1 min, 60 °C for 30 s and 72 °C for 1 min, and a final extension of 72 °C for 5 min. PCR reactions were performed with Taq (Cat. # 11615010, Invitrogen).

Findings

The complete sequence of the pPZP200BAR binary vector was determined (Fig. S1) and deposited at GenBank, with accession number KX227611. The vector pPZP200BAR is 7937 bp in length and has a GC content of 56.29 % (Fig. 1). The t-DNA region (1432 bp) contains the right border (RB), a multiple cloning site (MCS) with unique restriction sites for *Hin*dIII, *Pst*I, *Sal*I, *Xba*I, *Bam*HI and *Sma*I, which were confirmed by endonuclease analysis (Fig. S2), a complete cassette for tolerance to glufosinate *in planta*, and the left border (LB) (Fig. 1). The selectable marker cassette for plant transformation includes the nopaline synthase promoter (pNOS), the 5′-untranslated region of RNA of tobacco mosaic virus (TMV Ω), the BAR gene, and the nopaline synthase terminator (tNOS) (Fig. 1). pPZP200BAR also contains three ORFs that showed similarity to proteins associated with DNA transfer (ParA, RepA and SR) and a cassette for bacterial resistance to both streptomycin and spectinomycin (Strep/SpecR) (Fig. 1; Table S1). In contrast to the non-sequenced vectors pGSFR761A, pGSFR780A, pBG-1 and pBL-1 carrying the BAR gene and used for alfalfa transformation (Montague et al. 2007; White et al. 1990), pPZP200BAR has a small total size, a broad MCS and a small t-DNA region (Table S2), making it a binary vector easy to manipulate.

Previous to alfalfa transformation using pPZP200BAR, the natural tolerance of the regenerative alfalfa clone C2–3 to glufosinate was analyzed. A strong inhibition of regeneration was observed at concentrations of 2 mg/l of glufosinate and higher; therefore, 2, 5 and 10 mg/l were chosen as concentrations for in vitro selection in the C2–3 alfalfa clone. Similar results have been previously reported by other authors for the regenerable Regen-S (D'Halluin et al. 1990) and RSY27 (Montague et al. 2007) alfalfa genotypes, suggesting that 2 mg/l of glufosinate is a general limitation for alfalfa tissue regeneration. In addition, the alfalfa transformation efficiency of pPZP200BAR was

Author's personal copy

compared with that of pGSFR761A, pGSFR780A, pBG-1 and pBL-1. As previously reported in the Regen-S clone, pBG-1 showed higher transformation efficiencies than pBL-1 in the C2–3 genomic context (Fig. 2). In addition, pGSFR780A showed higher transformation efficiencies than pGSFR761A in the C2–3 clone, the same pattern as that previously observed in the RSY27 background (Fig. 2). Importantly, the use of pPZP200BAR produced significantly higher transformation efficiencies than the control vectors pGSFR761A, pGSFR780A, pBG-1 and pBL-1, at all the glufosinate concentrations tested (Fig. 2).

Conclusions and perspectives

In this short communication, we presented the complete sequence of pPZP200BAR, a small binary vector containing an innocuous marker for both plant transformation and tolerance to the herbicide glufosinate and a high-quality MCS, preventing the perceived risks of antibiotic resistance cassettes and allowing the introduction of additional genes into plants. Importantly, we here demonstrated the extremely high efficiency of this binary vector in alfalfa transformation, minimizing the time and cost of production of alfalfa event libraries, a critical constraint for the development of commercial transgenic alfalfa, which can only be financed by international consortia. Finally, we propose the use of pPZP200BAR for the rapid and inexpensive incorporation of different herbicide-tolerant traits into alfalfa in public breeding programs for long-term weed control.

Author contribution statement Conceived and designed the experiments: GS. Performed the experiments: CK, EB, CP, EP, NDA. Analyzed the data: CK, NDA, GS. Wrote the paper: GS.

Acknowledgments We are grateful to Dr. Esteban Hopp (IB-INTA) for his help in this work, especially during the initial phases. This work was supported by grants INTACD648/06, INTACD1016/12 and PICT Start-Up 2014-3759 to G.S.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

D'Halluin K, Botterman J, De Greef W (1990) Engineering of herbicide-resistant alfalfa and evaluation under field conditions. Crop Sci 30:866–871

Garcia AN, Ayub ND, Fox AR, Gomez MC, Dieguez MJ, Pagano EM, Berini CA, Muschietti JP, Soto G (2014) Alfalfa snakin-1 prevents fungal colonization and probably coevolved with rhizobia. BMC Plant Biol 14:248

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Heap I (2014) Global perspective of herbicide-resistant weeds. Pest Manag Sci 70:1306–1310

Laguia-Becher M, Martin V, Kraemer M, Corigliano M, Yacono ML, Goldman A, Clemente M (2010) Effect of codon optimization and subcellular targeting on Toxoplasma gondii antigen SAG1 expression in tobacco leaves to use in subcutaneous and oral immunization in mice. BMC Biotechnol 10:52

Meek S (2011) A review of the environmental safety of the PAT protein. Environ Biosafety Res 10:73–101

Montague A, Ziauddin A, Lee R, Ainley WM, Strommer J (2007) High-efficiency phosphinothricin-based selection for alfalfa transformation. Plant Cell Tissue Organ Cult 91:29–36

Rivero M, Furman N, Mencacci N, Picca P, Toum L, Lentz E, Bravo-Almonacid F, Mentaberry A (2012) Stacking of antimicrobial genes in potato transgenic plants confers increased resistance to bacterial and fungal pathogens. J Biotechnol 157:334–343

Rogan G, Fitzpatrick S (2004) Petition for determination of nonregulated status: roundup ready alfalfa (*Medicago sativa* L.) events J101 and J163. USDA Petition Number 04-110-101

Rubiales D (2014) Alfalfa: back to the future. J Int Legume Soc. ISSN: 2340-1559

Schubert D, Lechtenberg B, Forsbach A, Gils M, Bahadur S, Schmidt R (2004) Silencing in Arabidopsis T-DNA transformants: the predominant role of a gene-specific RNA sensing mechanism versus position effects. Plant Cell 16:2561–2572

Shen WJ, Forde BG (1989) Efficient transformation of *Agrobacterium* spp. by high voltage electroporation. Nucleic Acids Res 17:8385

Soto G, Stritzler M, Lisi C, Alleva K, Pagano ME, Ardila F, Mozzicafreddo M, Cuccioloni M, Angeletti M, Ayub ND (2011) Acetoacetyl-CoA thiolase regulates the mevalonate pathway during abiotic stress adaptation. J Exp Bot 62:5699–5711

Thompson CJ, Movva NR, Tizard R, Crameri R, Davies JE, Lauwereys M, Botterman J (1987) Characterization of the herbicide-resistance gene bar from *Streptomyces hygroscopicus*. EMBO J 6:2519–2523

Tuteja N, Verma S, Sahoo RK, Raveendar S, Reddy IN (2012) Recent advances in development of marker-free transgenic plants: regulation and biosafety concern. J Biosci 37:167–197

White J, Chang SY, Bibb MJ (1990) A cassette containing the bar gene of Streptomyces hygroscopicus: a selectable marker for plant transformation. Nucleic Acids Res 18:1062