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ORIGINAL ARTICLE

Maximizing the expression of transgenic traits into elite alfalfa germplasm using a supertransgene configuration in heterozygous conditions

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Abstract

Key message **A novel process for the production of transgenic alfalfa varieties.**

Abstract Numerous species of legumes, including alfalfa, are critical factors for agroecosystems due to their ability to grow without nitrogen fertilizers derived from non-renewable fossil fuels, their contribution of organic nitrogen to the soil, and their increased nutritional value. Alfalfa is the main source of vegetable proteins in meat and milk production systems worldwide. Despite the economic and ecological importance of this autotetraploid and allogamous forage crop, little progress has been made in the incorporation of transgenic traits into commercial alfalfa. This is mainly due to the unusually strong transgene silencing and complex reproductive behavior of alfalfa, which limit the production of events with high transgene expression and the introgression of selected events within heterogeneous synthetic populations, respectively. In this report, we describe a novel procedure, called supertransgene process, where a glufosinate-tolerant alfalfa variety was developed using a single event containing the BAR transgene associated with an inversion. This approach can be used to maximize the expression of transgenic traits into elite alfalfa germplasm and to reduce the cost of production of transgenic alfalfa cultivars, contributing to the public improvement of this legume forage and other polyploid and outcrossing crop species.

Introduction

Alfalfa (*Medicago sativa* L.), also known as the "Queen of Forages", is a major forage worldwide. This legume crop is particularly rich in proteins and vitamins, providing highly nutritious hay and pasture for animal and dairy productions (Lei et al. 2017). Alfalfa is also an integral factor of crop rotation, adding fixed nitrogen to the soil and improving the soil structure for future crops (Lopes et al. 2015). However, similarly to that observed in other forage crops, weeds are often harvested along with alfalfa, reducing its potential protein content and feed digestibility. In addition, weed infestations normally result in a dramatic decrease in alfalfa yield, showing the need to generate novel technologies to control weeds in alfalfa (Rubiales 2014). Over the past 40 years, herbicides have improved crop yield and enabled the development of modern agriculture. However, the extensive use of herbicides has generated a strong selection pressure for the emergence of herbicide-tolerant weeds, threatening the sustainability of current agriculture (Green 2014). This selection pressure is particularly strong in systemic herbicides such as glyphosate, the most important and most widely used herbicide in world agriculture (Duke and Powles 2008). Currently, it is well known that the success of an individual herbicide is only temporary, and that long-term weed control requires the continuous production of novel herbicide-tolerant cultivars. To this end, it is necessary to

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have rapid and inexpensive processes to incorporate different herbicide-tolerant traits into important crops, including cultivated alfalfa.

Cultivated alfalfa is tetraploid ($2n = 4x = 32$) and exhibits high levels of self-incompatibility and inbreeding depression (Busbice et al. 1972). Due to this particular reproductive behavior, an alfalfa cultivar normally comprises a large number of genetically and phenotypically heterozygous parent plants (Rumbaugh et al. 1988). Therefore, alfalfa is recalcitrant to introgression of favorable alleles or transgenic traits into elite heterogeneous populations. For instance, the identification of J101 and J163 events with suitable glyphosate tolerance required the production of vast numbers of transgenic alfalfa events to bypass the transgenic silencing, hindering the development of the glyphosate-tolerant alfalfa cultivar (alfalfa-RR). This cultivar was developed using the dihomogenic process (McCaslin et al. 2002; Rogan and Fitzpatrick 2004), where heterogeneous parental plants carry two independent transgenic events (Fig. 1). Although this pioneer process offers an elegant solution for the polyploid and outcrossing nature of alfalfa, it also has two main economic limitations that restrict its application to alfalfa improvement public programs. First, the dihomogenic process requires the production of two independent events that combine high-level, constitutive and ubiquitous expression of the transgene showing Mendelian inheritance (Fig. 1). Due to the transgene silencing in plants (Schubert et al. 2004), it is necessary to screen hundreds or thousands of events to identify alfalfa events showing optimal transgene expression (Rogan and Fitzpatrick 2004). Thus, the production of two optimal events for the development of a transgenic alfalfa cultivar is very expensive. In addition, inversions are a general feature of t-DNA-transformed plants (Takano et al. 1997), and genes associated with inversions act as a "supergene" due to the suppression of recombination between differently oriented chromosomal segments (Hoffmann et al. 2004). Thus, transgenes ligated to inversions have non-Mendelian inheritance (Zhu et al. 2010) and should be discarded in the dihomogenic process, even if they have an optimal pattern of transgene expression. Second, the production of a single transgenic alfalfa cultivar via the dihomogenic process needs the introgression of each of the two independent events individually (Beazley et al. 2012; Liang and Skinner 2004), doubling the regular cost of development of a commercial transgenic crop where only one transgenic trait is introduced (Fig. 1).

Previously, we reported the *Agrobacterium*-mediated transformation of the alfalfa regenerative clone C23 (Garcia et al. 2014) and the high efficiency of the pPZP200BAR binary vector for rapid and low-cost production of large transgenic event libraries (Jozefkowicz et al. 2016). Here, we present an alternative process for the production of transgenic alfalfa varieties using a single transgenic event containing the BAR transgene for tolerance to the non-systemic herbicide glufosinate, where the BAR transgene is ligated to an inversion. This procedure, named super-transgene process, combines the high transgene expression of events associated with inversions and the ability of RT-qPCR analysis to identify individual plants with two copies of the BAR transgene per genome to maximize the expression of transgenic traits into elite germplasm (Fig. 1).

Materials and methods

Plant material

The alfalfa plants used were the regenerative clone C23 (Garcia et al. 2014), the three independent transgenic libraries of alfalfa transformed with the binary vector pPZP-200BAR (Jozefkowicz et al. 2016), the alfalfa breeding population SIMA 1728 (EEA Manfredi, INTA, Argentina), and the alfalfa cultivars Super Sonic (Seed Genetics, Australia), Bacana II (Forage Genetics, USA), and CW 1010 (Cal West, USA).

Genome walking assays and nucleotide sequence analyses

Genomic DNA of transgenic plants was isolated from leaf tissue with the DNeasy plant mini kit (Cat. #69104, Qiagen). Transgenic plants were confirmed by PCR with the primers bar1 and bar2 (Online Resource 1), designed against the coding sequence of the BAR transgene. The PCR conditions comprised: 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). High-throughput screening of t-DNA/plant DNA junctions of alfalfa transgenic events was performed using the Genome Walker kit (Cat. #638904, Clontech, Mountain View, CA, USA), following the manufacturer's instructions. Four Genome Walker libraries were constructed using the restriction enzymes EcoRV, PvuII, StuI and SmaI. The nucleotide sequences of the nested primers complementary to the t-DNA right- and left-border sequences were GSP1-RB and GSP2-RB, and GSP1-LB and GSP2-LB, respectively (Online Resource 1). The amplified fragments were cloned into the pCR2.1-TOPO vector (Invitrogen), and subsequently transformed into competent *Escherichia coli* DH5α. The selected clones were sequenced using the universal primers M13 and T7. Genome analyses of the wild-type alfalfa clone C23 were performed by TAIL-PCR according to (Soto et al. 2011). The right- and left-border junction nucleotide sequences

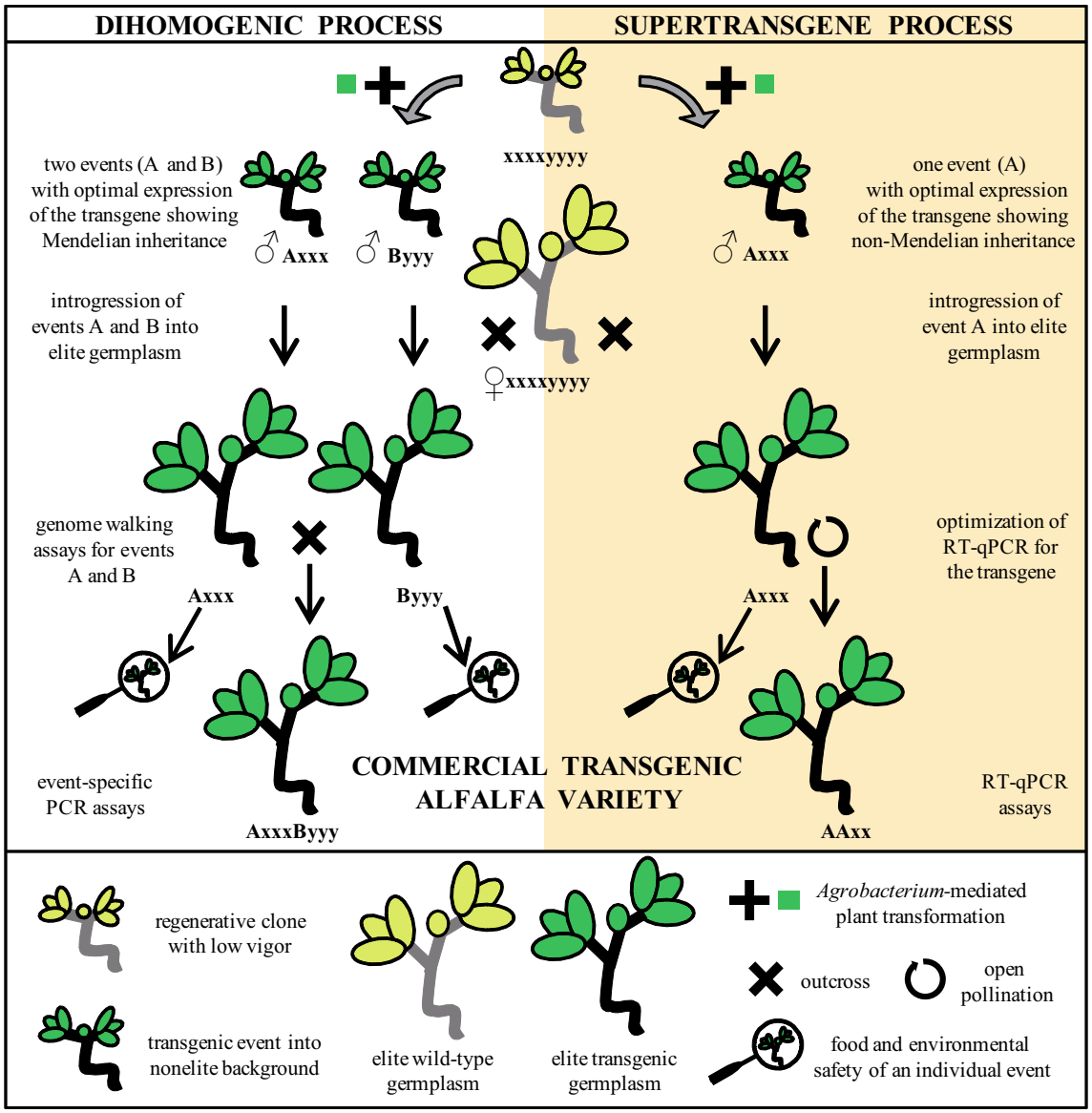

Fig. 1 Schematic representation of the dihomogenic and supertransgene processes for the production of a commercial alfalfa variety containing only one transgenic trait. The dihomogenic and supertransgene approaches contain the same three critical steps: the transformation of a high regenerative alfalfa clone with a binary vector via *Agrobacterium tumefaciens*, the introgression of the transgene into elite alfalfa germplasm, and the deregulation of the transgene in the market. However, the dihomogenic approach requires the production and, introgression of two events, whereas the supertransgene process requires only one event. The latter thus significantly reduces the cost of producing a commercial transgenic alfalfa variety. Axxx and Byyyy are transgenic plants where the dominant transgenes "A" or "B" are randomly integrated into chromosomes. The genetic positions in the non-transgenic members of the chromosome sets are represented by "x" and "y". "A" and "B" are the same transgene derived from the same binary vector

of alfalfa transgenic events were used as query to search against *Medicago truncatula* genome databases, including Phytozome (http://www.phytozome.net) and NCBI (http://www.ncbi.nlm.nih.gov), using BLASTN (nucleotide identity cutoff ≤ 75%). Sequences were aligned, assembled, and analyzed using Bioedit Sequence Alignment Editor CAP (Hall 1999). Sequencing reactions were performed at Unidad de Genómica, CICVyA, INTA, Argentina.

Quantitative RT-PCR (RT-qPCR) assays

Total RNA was extracted from leaves using an RNeasy Plant Mini Kit (QIAGEN Cat 74903, Germany), and used as a template to generate first-strand cDNA synthesis using Reserve Transcriptase M-MuLV (Roche Cat 11062603001, Germany), random primers and dNTPs (Invitrogen, Life Technologies, USA). Then, 4 µg total RNA was used for each reaction. For RT-qPCR analyses, PCR amplification

was performed with 5 µl of RT (1:5 diluted) per reaction, using 1 U iQ SYBR green Supermix (BIO-RAD) and 0.2 mM primers, with the iCycler iQ system (BIO-RAD). Primers for the real-time qPCR were bar3 and bar4 (Online Resource 1) according to (Grohmann et al. 2009). The qPCR conditions comprised: 1 cycle at 95 °C for 5 min, 35 cycles of 95 °C for 16 s, 60 °C for 40 s, and 72 °C for 30 s. At each cycle, accumulation of PCR products was detected. The amplification fragment (60 bp) was sequenced and found to be identical (100% nucleotide identity) to the BAR transgene. The expression level of the BAR transgene was normalized using aspartate aminotransferase as a housekeeping gene using primers p12FW and p13RV (Online Resource 1) according to (Garcia et al. 2014). The efficiency of primer binding was determined by linear regression by plotting the cycle threshold value versus the log of the cDNA dilution (Soto et al. 2010). RT-qPCR experiments were performed three times with independent RNA samples (biological replicates). For each biological replicate, the qPCR reactions were carried out in duplicate.

Southern blot and event-specific PCR assays

For Southern hybridizations and event-specific PCR analysis, genomic DNA of the PL1 event was isolated from leaf tissues using the DNeasy Plant Maxi kit (Cat. # 68161, Qiagen), following the manufacturer's instructions. DNA was digested with the HindIII restriction enzyme, which cleaves the pPZP200 bar t-DNA only once. After that, 25 µg of DNA from each sample was digested overnight and blotted after separation on 1% (w/v) agarose gel, using the TAE buffer. The DNA fragments in the gels were transferred to a positively charged Nylon membrane (Cat. # 11209272001, Roche). Nylon membranes were cross-linked, and then used for hybridization with a DIG-labeled probe. Prehybridization and hybridization were carried out according to the manufacturer's instructions. The hybridization probe BAR-digoxigenin-labeled DNA was generated by PCR using the PCR DIG probe synthesis kit (Cat. # 11573152910, Roche), using the primers bar1 and bar2 (Online Resource 1). PCR amplification was performed under standard conditions (25 µl volume using 0.8 µM of each primer, 1X PCR buffer, 0.2 mM each dNTP, 2 mM $MgCl_2$ and 20 ng of template) and using the same cycling program described above for the identification of transgenic plants. PL1-event-specific PCR was carried out using the primers es-up and es-low (Online Resource 1), designed against pPZP200 bar t-DNA and the upstream inverted region, amplifying a 650-bp fragment. The PCR conditions comprised: 1 cycle at 94 °C for 5 min, 34 cycles of 94 °C for 1 min, 55 °C for 30 s and 72 °C for 1 min, and a final extension of 72 °C for 5 min. The PCR reactions were performed with Taq (Cat.# 11615010, Invitrogen).

Western blot assays

For western blot assays, tissues were homogenized in liquid nitrogen and buffer was added (50 µM Tris–Cl pH: 8, 100 µM NaCl, 1 µM EDTA, 1 µM DTT, 0.1% Triton X-100, protease inhibitor). After centrifugation at 13,000*g* for 15 min at 4 °C, the supernatants were collected and protein concentration was determined by the Bradford protein assay (Bradford 1976). Then, 3 × sodium dodecyl sulfate (SDS) sample buffer (2% SDS, 10% (v/v) glycerol, 62.5 mmol/l Tris–HCl, pH 6.8, 0.2% bromophenol blue, 10 mmol/l 2-mercaptoethanol) was added to the supernatants. Proteins were separated by SDS-PAGE on 12% polyacrylamide gels, transferred to nitrocellulose membranes and blocked with Tris-buffered saline (TBS) containing 0.2% triton, 1% of glycine and 2% non-fat dry milk overnight at 4 °C. Precision Plus Protein™ Dual Color Standards (Bio-Rad, CA 94547, USA) were used as molecular weight markers. Membranes were incubated with the rabbit anti-mouse PAT antibody (1:5000, P0249, Sigma-Aldrich, St. Louis, MO, USA) for 2 h, and then incubated for 1 h with HRP-conjugated goat anti-rabbit immunoglobulin (Ig)G as secondary antibody (1:2500, Santa Cruz Biotechnology, Dallas, Texas, USA). Membranes were analyzed by chemiluminescence (ECL, Thermo Scientific, Meridian, Rockford, USA), and the densitometric analysis was performed using the Gel-Pro Analyzer System (Media Cybernetics, USA).

Analysis of glufosinate tolerance and production of the alfalfa-BAR transgenic variety

For the analysis of inheritance and expression of the transgenic trait of individual events from the three transgenic alfalfa libraries used (Jozefkowicz et al. 2016), pPZP200BAR-transformed alfalfa plants were crossed manually with CW 1010 wild-type alfalfa using transgenic parental plants as pollen donors. Progenies of individual events were analyzed by applying 1–1000 mg/l glufosinate at the third trifoliate stage under hydroponic conditions (Setten et al. 2013). For the production of the alfalfa-BAR transgenic variety, transgenic plants were crossed manually with four elite wild-type alfalfa germplasms (SIMA 1728, Super Sonic, Bacana II and CW 1010) using transgenic parental plants as the pollen donors. In each generation, 353–758 glufosinate-tolerant individual plants were crossed with 100 individual wild-type elite plants. Glufosinate-tolerant individual plants were selected by applying 100 mg/l glufosinate at the third trifoliate stage under soil conditions (Jozefkowicz et al. 2016).

Characterization of alfalfa-BAR under field conditions

For cultivar performance assays, 45-day-old plants of alfalfa-BAR were harvested periodically (every 20–30 days) to prevent their flowering and evaluate their productivity for 6 months. Plant regrowth was evaluated in July (i.e., winter in Argentina), and plant survival was analyzed after 1 year of planting. In these experiments, the cuts were performed at 8 cm from the soil and the weeds were mechanically removed. SIMA 1728, Super Sonic, Bacana II and CW 1010 were used as elite germplasm controls. To analyze the expression of the transgenic trait into alfalfa-BAR, 3-month-old plants of alfalfa-BAR were exposed to two applications of glufosinate using 0, 2 or 3.5 l/ha of glufosinate containing 1% $SO_4(NH_4)_2$ surfactant. Three months after herbicide treatments, the vegetal material of plants was harvested at 8 cm from the soil to evaluate plant productivity. A mix of wild-type plants (WT) from SIMA 1728, Super Sonic, Bacana II and CW 1010 was used as glufosinate-sensitive controls. To measure plant productivity, vegetal material was dried for 5 days at 90 °C. Field assays using alfalfa-BAR were performed under strict normative to GMOs (CONABIA Exp. S05:3543/15). Germplasm quality under field conditions was evaluated in one localization (INTA-Castelar, Buenos Aires) using three replicate plots ($n = 3$) with 40 plants per plot. Significant differences between alfalfa varieties were calculated using ANOVA followed by Dunnett's multiple comparison tests. Tolerance experiments under field conditions were repeated in two different locations (INTA-Castelar, Buenos Aires, Argentina, and INTA-Manfredi, Córdoba, Argentina), with similar results. Significant differences between treatments were calculated using ANOVA followed by Tukey's test.

Results

Identification and initial characterization of superevents

We identified transgenic events associated with inversions, named here superevents, by taking advantage of the low, but stable incidence of inversions in plant transgenic libraries and the particular features of t-DNA integrations related to inversions including t-DNA right-border truncations and suppression of homologous recombination between inverted and normal chromosomes. Specifically, we screened three libraries of alfalfa transformed with pPZP200BAR by *Agrobacterium tumefaciens* (Fig. 2a) for superevents using genome walking assays (Fig. 2b) and transgene inheritance analyses (Fig. 2c). Most of the events (74.77%) had a complete t-DNA right border (cRB events) (Online Resource 2). Events displaying t-DNA truncations at the right border (tRB events) represented an average of 25.33% of all the events present in the transgenic libraries (Online Resource 3), while 21.03% of this subset of tRB events showed a 1:3

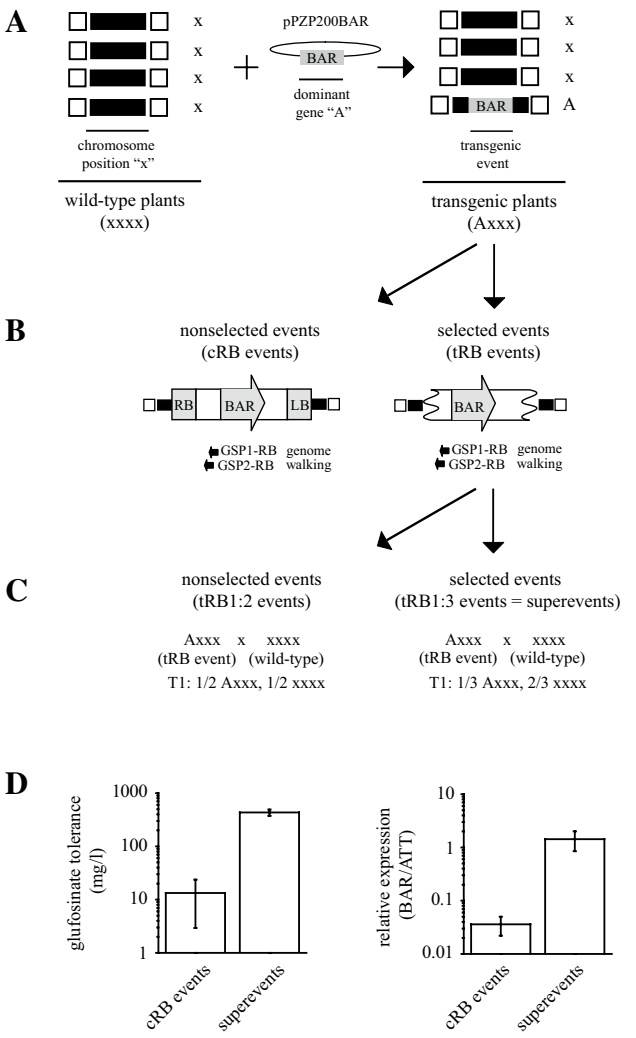

Fig. 2 Steps for the identification of transgenic events associated with inversions (superevents) and their phenotypic and molecular characterization. **a** Schematic representation of alfalfa transformation with the pPZP200BAR binary vector, where the dominant transgene "A" (BAR gene) is randomly integrated into a chromosome. The genetic position in the non-transgenic members of the chromosome set is represented by "x". **b** Scheme showing the genomic walking assays using the GSP1-RB and GSP2-RB primers against pPZP200BAR t-DNA for the rapid discrimination between cRB events (left) and tRB events (right) containing complete or truncated versions of t-DNA RB sequences, respectively. **c** Schematic representation of the segregation of the transgenic trait (glufosinate tolerance) for tRB1:2 (left) and tRB1:3 events (right) showing Mendelian and non-Mendelian inheritance patterns, respectively. In tRB1:3 events, named superevents, the incorporation of the BAR transgene (allele "A") is ligated to an inversion. **d** Compares the glufosinate tolerance (left) and the expression of the BAR transgene (right) between the most abundant events within transgenic libraries (cRB events) and superevents. Values are mean ± SEM, $n = 3$ independent transgenic events

non-Mendelian inheritance of the BAR transgene (Online Resource 3), which fits to a dominant transgene associated with an inversion in simplex condition in a tetraploid species such as alfalfa. These results suggest a low, but significant incidence of superevents (5.32%) in alfalfa transgenic libraries. Interestingly, the glufosinate tolerance of superevents was more than one order higher than that of cRB events, and the large difference of expression of the transgenic trait between inverted and common transgenic events can be attributed to the higher BAR transgene expression of superevents than cRB events (Fig. 2d). These results suggest that events associated with inversions can bypass, at least partially, the transgenic silencing, a normal phenomenon observed in transgenic plants.

Integral molecular characterization of superevent PL1

Since the introgression of a transgenic event into elite germplasm and its commercial deregulation are long-term and expensive procedures, the event selection normally comprises not only the transgene expression analyses, but also an integral molecular characterization of the event including the genomic locus modifications, the genetic stability of the insert, the heritability of the transgene and its codifying protein, and a rapid assay for an unequivocal identification of the specific event. Thus, we decided to perform an extensive molecular study of an individual superevent (named PL1) prior to its introgression into elite germplasm. Genomic analysis of superevent PL1 confirmed that the t-DNA insertion (between the R-III and R-IV regions) is associated with an inversion (at the R-II region) (Fig. 3a). The analysis of putative open reading frames in this genomic region (from R-I to R-IV) showed that superevent PL1 is localized into an intergenic context, suggesting no endogenous gene disruptions. The Southern blot assay showed that superevent PL1 has both a single copy of the t-DNA insertion and the integrity of the heritability of the BAR supertransgene in T0 and T1 transgenic plants (Fig. 3b). Furthermore, the western blot analysis showed that the BAR protein from T0 and T1 transgenic plants containing superevent PL1 has similar immunoreactivity and equivalent electrophoretic mobility, demonstrating the integrity of the heritability of the BAR protein from supervent PL1 (Fig. 3c). In addition, event-specific PCR assays based on genome walking data were able to distinguish superevent PL1 from other event (named PL2) constructed with the same binary vector (Fig. 3d), suggesting that this type of event can be easily identified. Therefore, molecular characterization of superevent PL1 suggests that superevents can display all molecular features to undergo both introgression and deregulation processes like a conventional event.

Development of a transgenic alfalfa variety from superevent PL1

To avoid inbreeding depression, the introgression of superevent PL1 into elite alfalfa germplasm was done using five different wild-type cultivars (SIMA 1728, Super Sonic, Milonga II, Bacana II and CW 1010) through five backcrossing generations (Fig. 4a). All these wild-type alfalfa varieties have the same winter hardiness rating (fall dormancy group 9/10), which is appropriate for the template regions of the Pampean (Buenos Aires) and Central Valley regions of Buenos Aires (Argentina) and California (USA), respectively. Due to the dramatic reduction in plant vigor observed in inbred alfalfa plants, we used additional measures to maximize the increase in heterozygosity during the introgression process, including the selection of vigorous and phenotypically heterogeneous plants in each backcrossing generation as parental plants and the selection of glufosinate-tolerant alfalfa plants as pollen donors and conventional alfalfa plants as the females (Fig. 4a). During the introgression process, we observed a 26.4–27.9% inheritance ratio for the glufosinate-tolerance trait in backcrossing generations (T1–T5), considerably less that the expected 33.3% (Fig. 4a), which is congruent with the low but stable frequency of alfalfa selfing during non-emasculated hand crosses. After the introgression process, and to increase the transgene copy number, T5 plants were randomly intercrossed by hand, producing a T6 generation where the observed (54.9%) and expected (55.5%) inheritance ratios for the transgenic trait were similar (Fig. 4a). T6 individual plants with two copies of the BAR transgene at a single locus were selected as parent plants from the synthetic variety of transgenic alfalfa (syn) using RT-qPCR assays (Fig. 4b). The random intercrossing of syn plants produces high amounts of the transgenic trait into its progeny (93.8%), which is expected for a population with balanced copies of inverted and normal chromosomes (93.7%) (Fig. 4a). Interestingly, the original regenerative alfalfa clone (C23) and its derivative transformed alfalfa clone PL1 showed a non-elite low-vigor phenotype characterized by uniformly small leaves and short shoot growth, while backcrossing generations led to more vigorous plant growth (Fig. 4c). These results suggest the success in the incorporation of a transgenic trait into an elite alfalfa germplasm (alfalfa-BAR).

The high expression of the transgenic trait and the elite performance of alfalfa-BAR

Normally, a commercial transgenic variety has to express the transgenic trait at all growth stages. In the specific case of weed–alfalfa competition, the expression of the herbicide-tolerance trait at different growth stages can be particularly important for an integral control of weeds in this perennial

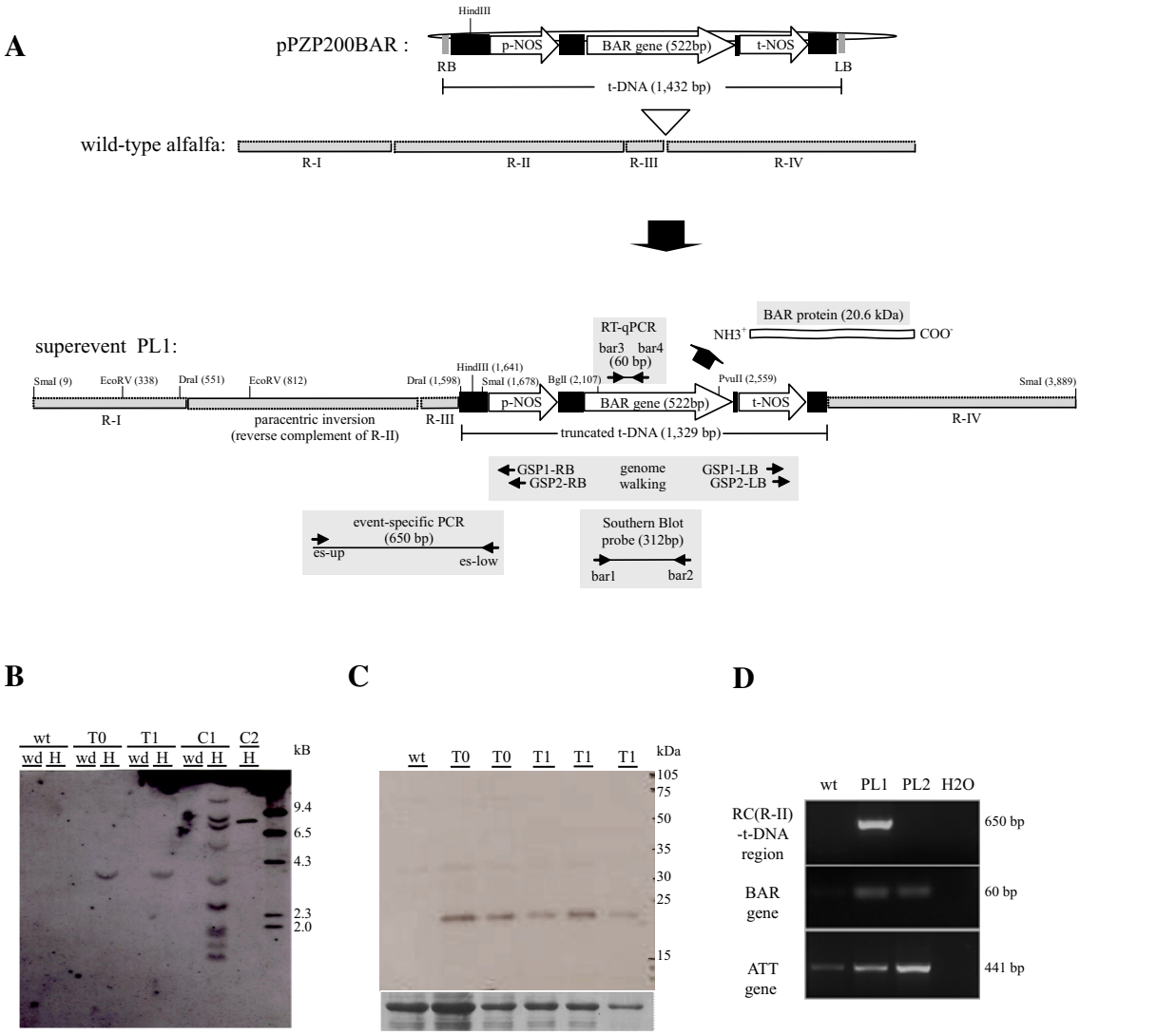

Fig. 3 Molecular characterization of superevent PL1. **a** Schematic representation of the binary vector pPZP200BAR, the clone C23 wild-type alfalfa genome (regions R-I–R-IV) and the superevent PL1 genome showing the insertion of a t-DNA fragment containing the BAR transgene between genomic regions R-III and R-IV and its associated inversion into the region R-II. The primer sites for genome walking, RT-qPCR, southern blot and event-specific PCR assays and the expected BAR protein codified by the BAR transgene for Western blot assay are highlighted. **b** Southern blot assay demonstrating the integrity of the heritability of the BAR supertransgene from superevent PL1. T0 = superevent PL1 under C23 genomic background. T1 = transgenic progeny of superevent PL1 under C23 background crossed with wild-type elite germplasm SIMA 1728. C1 = alfalfa clone containing multiple copies of the BAR transgene. C2 = binary vector pPZP200BAR. wd = without digestion. H = Hind III. **c** Western blot analysis showing the integrity of the heritability of the BAR protein from superevent PL1, using wild-type (SIMA 1728) and different individual transgenic plants from the T0 and T1 generations as described in the Southern Blot experiments. The Coomassie blue-stained gel of plant proteins is shown below. **d** Event-specific PCR assay for the specific detection of superevent PL1 (PL1). To show the specificity of this amplification, an independent event (PL2) of alfalfa transformed with the binary vector pPZP200BAR was added as an additional control. The BAR transgene present in transgenic plants PL1 and PL2 and the ATT housekeeping gene present in all alfalfa plants were also amplified to demonstrate the high quality of the template (genomic DNA) used in event-specific PCR assays

forage. Thus, we analyzed the expression of the herbicide-tolerant trait in alfalfa-BAR in young seedlings and young, mature and flowering plants under chamber and greenhouse conditions, respectively. During all these growth stages, and in contrast to the wild-type cultivar CW 1010, alfalfa-BAR transgenic plants showed complete tolerance to glufosinate without apparent toxicity effects in all vegetal tissues (Fig. 5), suggesting a high-level, constitutive and ubiquitous expression of the herbicide-tolerance trait.

The elite performance and the high expression of the transgenic trait of alfalfa-BAR was confirmed by productivity (Fig. 6a) and glufosinate tolerance (Fig. 6b) assays

A

Generation	Parental plants [transgenic genotype] {copies of the BAR transgene}		Glufosinate % tolerant	Glufosinate % expected	Transgenic genotypes produced in this generation	Transgenic genotypes selected [methods for selection]
	♀	♂				
T1	SIMA1728 [xxxx]{0}	superevent PL1 [Axxx]{1}	26.4	33.3	xxxx, Axxx	Axxx [Glufosinate]
T2	Super Sonic [xxxx]{0}	Selected T1 plants [Axxx]{1}	26.9	33.3	xxxx, Axxx	Axxx [Glufosinate]
T3	Milonga II [xxxx]{0}	Selected T2 plants [Axxx]{1}	27.2	33.3	xxxx, xxx	Axxx [Glufosinate]
T4	Bacana II [xxxx]{0}	Selected T3 plants [Axxx]{1}	27.9	33.3	xxxx, Axxx	Axxx [Glufosinate]
T5	CW1010 [xxxx]{0}	Selected T4 plants [Axxx]{1}	27.6	33.3	xxxx, Axxx	Axxx [Glufosinate]
T6	Random selected T5 plants intercrossing [Axxx]{1}		54.9	55.5	xxxx, Axxx, AAxx	AAxx [Glufosinate + RT-qPCR]
syn	Random selected T6 plants intercrossing [AAxx]{2}		93.8	93.7	xxxx, Axxx, AAxx AAAx, AAAA	without selection

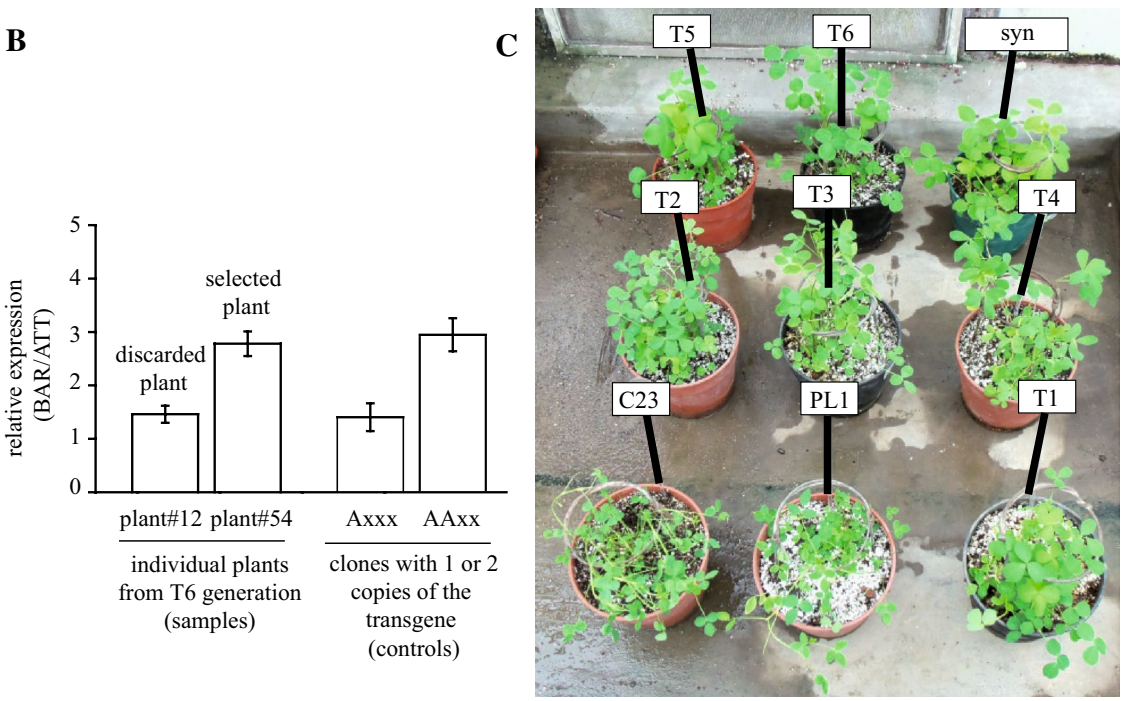

Fig. 4 Herbicide-tolerant alfalfa variety developed from superevent PL1 using phenotypic and molecular selections. **a** The production of a synthetic variety of transgenic alfalfa (syn) comprises five generations of backcrosses of superevent PL1 containing the BAR transgene with wild-type alfalfa (from T1 to T5) for the introgression of the transgenic trait (herbicide tolerance) into elite germplasm and an additional generation of random intercrossing transgenic individual plants for the increase in the transgene copy number per plant (T6). Glufosinate tolerance and RT-qPCR assays were applied for the selection of transgenic plants produced during these generations. The transgenic alfalfa variety developed can generate five transgenic genotypes: non-transgenic plants (xxxx), hemizygous simplex (Axxx), hemizygous duplex (AAxx), hemizygous triplex (AAAx) and homozygous (AAAA) for the dominant BAR transgene (allele "A"). **b** Representative qRT-PCR assay showing its ability to discriminate individual plants containing one copy (plant#12) or two copies (plant#54) of the BAR transgene per genome. In these molecular assays, individual clones derived from random intercrossing of T1 and genotyped by inheritance assays were used as "Axxx" and "AAxx" genotype controls. **c** Phenotypic analysis of the wild-type regenerative clone C23 (C23), superevent PL1 into C23 genomic background (PL1), and representative individual plants from different transgenic generations (T1-syn), showing the increase in the vigor during the breeding program

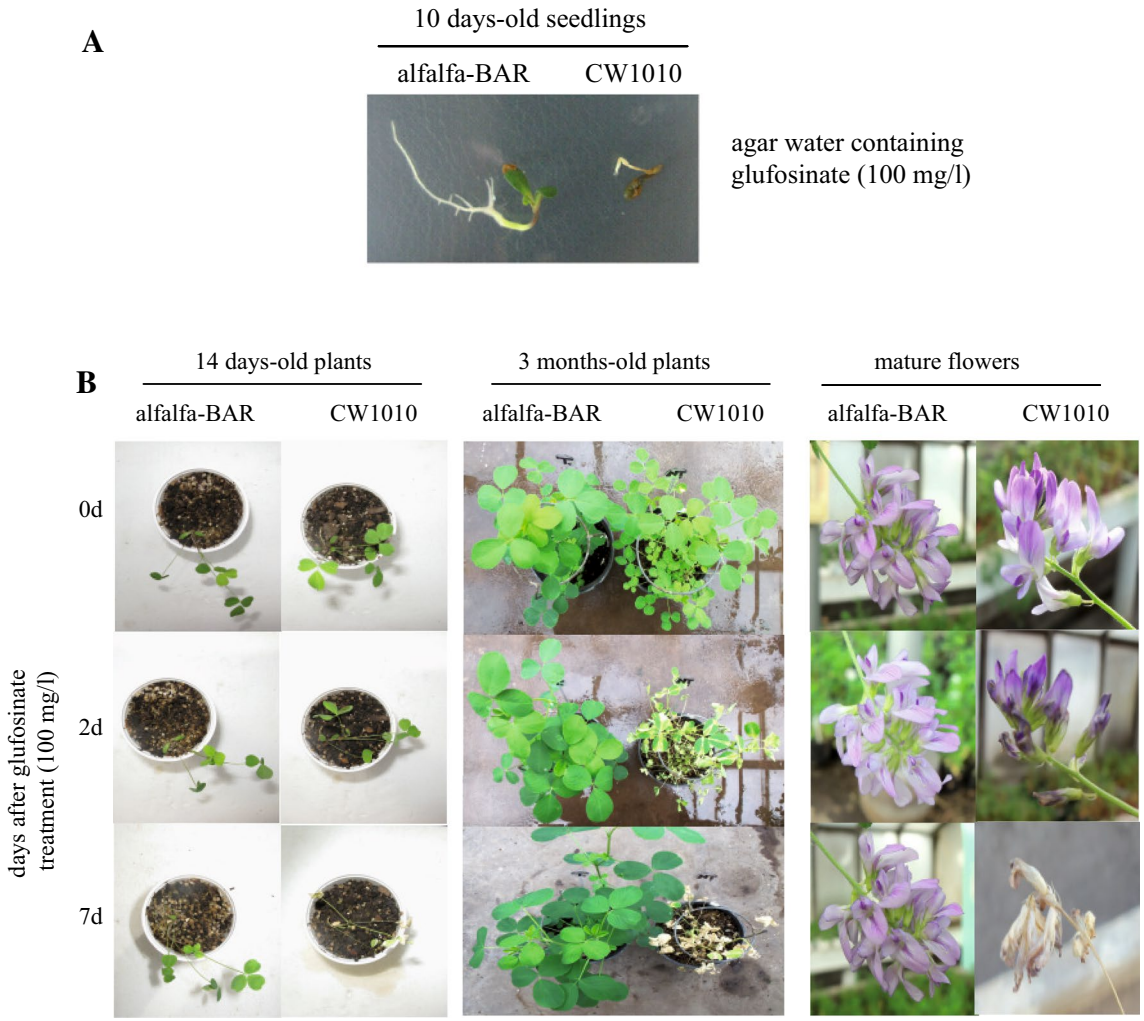

Fig. 5 Expression of the transgenic trait in alfalfa-BAR at different growth stages. **a** Glufosinate tolerance of alfalfa-BAR transgenic plants was firstly analyzed in agar-water petri dishes. For this analysis, transgenic (alfalfa-BAR) and non-transgenic (CW 1010) alfalfa seeds were treated with sulfuric acid for 10 min, washed three times with sterile water, placed in Petri dishes with 1% agar water supplemented with 100 mg/l glufosinate and incubated in growth chambers (16 h light at 23 °C). Under this herbicide treatment, alfalfa-BAR young seedlings showed a good germination, vigorous growth and complete emergence of the two cotyledons, while the traditional cultivar CW 1010 showed a dramatic delay in its germination, followed by seedlings that died almost immediately. **b** The expression of the transgenic trait in alfalfa-BAR was also analyzed in 1-L pots containing a mixture of soil:vermiculite (3:1). For these studies, transgenic (alfalfa-BAR) and non-transgenic (CW 1010) young, mature and flowering alfalfa plants were challenged with three herbicide applications of 100 mg/l glufosinate for three consecutive days. A week after the first herbicide application, alfalfa-BAR plants showed vigorous growth without toxicity effects, while all CW 1010 plants died by the application of the herbicide under greenhouse conditions (15–16 h light at 20–28 °C)

under field conditions comparing this herbicide-tolerant transgenic alfalfa variety with the individual conventional elite germplasm used in the introgression process (SIMA 1728, Super Sonic, Bacana II and CW 1010) or with a mix of these wild-type varieties (WT), respectively. The levels of yield, regrowth and survival of alfalfa-BAR were similar to those of its genetic-related wild-type varieties (Fig. 6a), suggesting that this transgenic germplasm is a high-yielding, winter hardiness, healthy variety of alfalfa. These results further support the success of the introgression of superevent PL1 into elite alfalfa germplasm. In contrast to that observed for weeds and wild-type elite alfalfa plants, alfalfa-BAR transgenic plants showed high tolerance to glufosinate without toxicity effects, even in high doses of herbicide (Fig. 6b). In addition, exposure to increased doses of glufosinate (0, 2 and 3.5 l/ha) led to enhanced yields in alfalfa-BAR, showing that the use of this herbicide not only does not damage the transgenic plant but also improves its standard performance (Fig. 6b).

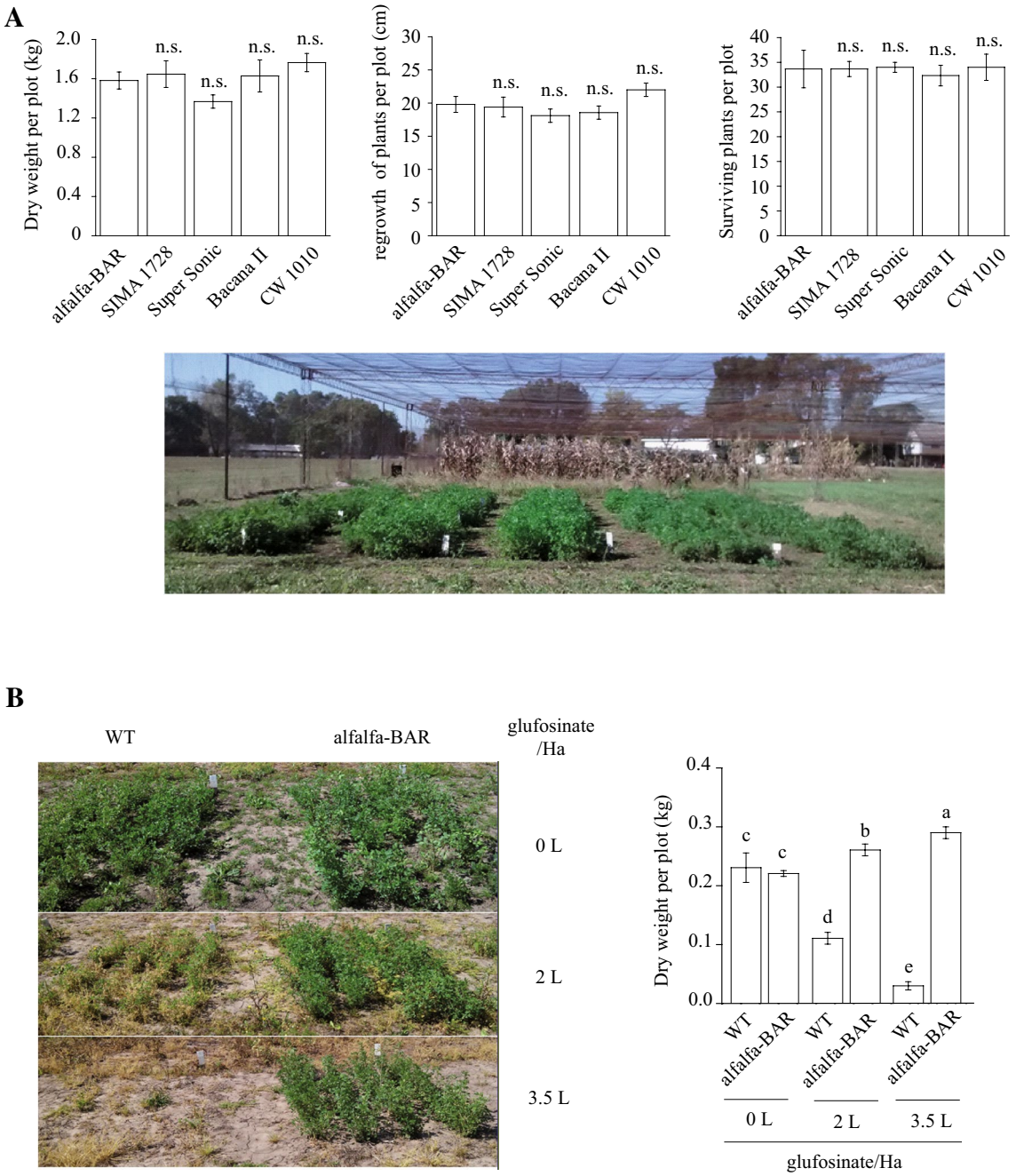

Fig. 6 Evaluation of the germplasm quality and transgenic trait expression in alfalfa-BAR under field conditions. **a** Yield, regrowth and survival of alfalfa-BAR plants were analyzed to determine the germplasm quality of this novel transgenic variety under field conditions. The genetically related wild-type elite varieties SIMA 1728, Super Sonic, Bacana II and CW 1010 were included as high-quality performance germplasm controls. **b** Alfalfa-BAR plants were grown without herbicide or exposed to two different doses of glufosinate to determine the expression of the transgenic trait under field conditions. A mix of wild-type elite varieties (WT) containing plants from the SIMA 1728, Super Sonic, Bacana II and CW 1010 cultivars was included as an herbicide-sensitive control

Discussion

Alfalfa is the major source of vegetable protein to meat and milk production systems worldwide. Due to its ability to establish efficient symbiosis with nitrogen-fixing rhizobial strains, the production of animal proteins from alfalfa culture does not require the use of nitrogen fertilizers derived from fossil fuel, mitigating our dependence on non-renewable energy sources and reducing the emission of greenhouse gases. This ancestral and sustainable system for animal

production is particularly vulnerable to weed infections and weed management activities. Although conventional varieties of alfalfa with varying degrees of tolerance to fungal pathogens and abiotic stress have been developed, conventional approaches are unable to produce alfalfa cultivars with high tolerance to herbicides. The supertransgene process generated a transgenic alfalfa variety using one transgenic event. This is in contrast to the traditional two independent events for production of transgenic alfalfa varieties, using the dihomogenic process. Accordingly, this new technique can minimize the cost of production of a transgenic alfalfa cultivar, improving the current ability to add new transgenic traits in elite alfalfa germplasm. In addition to its direct application to alfalfa breeding programs, the supertransgene process could be an attractive strategy to produce transgenic crops in other polyploid and outcrossing species. This is because this process uses events associated with inversions (superevents), which offer stackable and highly expressed transgenes. Considering previous reports of monocot and dicot transformations showing the stable incidence of inversion associated with t-DNA insertions in transgenic libraries (Castle et al. 1993; Laufs et al. 1999; Nacry et al. 1998; Takano et al. 1997; Zhu et al. 2010) and the transgenic silencing as a natural occurrence in transgenic plants (Schubert et al. 2004; Vermeersch et al. 2013), the high expression of the transgene in superevents can also be an alternative approach to bypass the transgenic silencing in almost all crop species. Inversions are commonly known to suppress recombination between differently oriented chromosomal regions. Recombination in heterokaryotypes is reduced, causing non-Mendelian inheritance of the genes associated with inversions (Alves et al. 2014; Ayala et al. 2011). In addition, when an alternative chromosomal arrangement has been established in a diverging population, it can contribute to reproductive isolation and speciation (Feder and Nosil 2009; Noor et al. 2001; Rieseberg 2001). Related to this last point, and in the context of agricultural biotechnology, it could be interesting to evaluate the containment of transgene flow using the supertransgene process in future studies.

Previous studies and the results reported here show that the BAR gene can be used as a non-antibiotic-selectable marker for both plant transformation and tolerance to the broad-spectrum non-systemic post-emergent herbicide glufosinate. Moreover, the use of BAR gene—glufosinate herbicide technology displays increased advantages, including (1) the heterologous functional activity of the BAR protein in both monocot and dicot species (ILSI 2017), (2) the complete innocuity of commercial transgenic crops containing the BAR gene (CERA 2011), (3) the fact that the non-systemic nature of glufosinate decreases the selection pressure as compared to systemic herbicides (e.g., glyphosate) and considerably reduces the occurrence of herbicide-resistant weeds (Heap 2014), and (4) the fact that the most recent patents of the BAR gene (US5648477) and agrochemical formulations containing glufosinate as the active ingredient (Rely® 280—Bayer Crop Science) for weed control have expired and thus generics of this herbicide are in the market (e.g., Reckon 280 SL Herbicide—Solera, Glufosinate 280SL—WILLOWOOD USA, FORFEIT 280—Loveland Products and Refer 280SL—Summit Agro), providing complete freedom to operate with the BAR gene and increased competition for lower value of glufosinate.

Considering the extremely high cost of production and release of transgenic cultivars and that almost all commercial transgenic crop developments begin with the transformation of a non-elite background and then need the incorporation of the transgenic trait into elite germplasm, the combination of the supertransgene process and the BAR gene can be used by public breeding programs as a free framework to generate new commercial glufosinate-tolerant cultivars in any plant species or to facilitate the production of novel commercial transgenic crops containing additional transgenic traits. This can be done through the incorporation of additional genes into BAR-t-DNA regions, the use of glufosinate as selective marker for both plant transformation and event introgression, and the increase of event copies per plant via BAR-RT-qPCR analysis, regardless of the ploidy level and reproductive behavior of the crops to be transformed.

Interestingly, some works have reported that the transient inhibition of cytosolic glutamine synthetase by glufosinate application increases the ammonia accumulation and activates the chloroplastic isoform of glutamine synthetase, triggering the systemic acquired resistance to multiple pathogens (Ahn 2008; Uchimiya et al. 1993) and increasing biomass production (Dragićević et al. 2012; Nikolić et al. 2013), respectively. Although these indirect functions of glufosinate are widely accepted, they have never been tested in variable and complex environments such as under field conditions. In this article, field experiments for the evaluation of glufosinate tolerance in alfalfa-BAR showed a positive and significant association between increases in the glufosinate doses and plant productivity, providing evidences, for the first time, of the plant growth-promoting actions of glufosinate under field conditions. Further studies involving the evaluation of alfalfa-BAR exposed to different pathogens will show whether the BAR gene can also incorporate indirectly the broad-spectrum disease resistance under field conditions.

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Compliance with ethical standards

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

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