Metabolic engineering to simultaneously activate anthocyanin and proanthocyanidin biosynthetic pathways in *Nicotiana spp*.

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Abstract

Proanthocyanidins (PAs), or condensed tannins, are powerful antioxidants that remove harmful free oxygen radicals from cells. To engineer the anthocyanin and proanthocyanidin biosynthetic pathways to *de novo* produce PAs in two *Nicotiana* species, we incorporated four transgenes to the plant chassis. We opted to perform a simultaneous transformation of the genes linked in a multigenic construct rather than classical breeding or retransformation approaches. We generated a GoldenBraid 2.0 multigenic construct containing two *Antirrhinum majus* transcription factors (*AmRosea1* and *AmDelila*) to upregulate the anthocyanin pathway in combination with two *Medicago truncatula* genes (*MtLAR* and *MtANR*) to produce the enzymes that will derivate the biosynthetic pathway to PAs production. Transient and stable transformation of *Nicotiana benthamiana* and *Nicotiana tabacum* with the multigenic construct were respectively performed. Transient expression experiments in *N. benthamiana* showed the activation of the anthocyanin pathway producing a purple color in the agroinfiltrated leaves and also the effective production of 208.5 nmol (-) catechin/g FW and 228.5 nmol (-) epicatechin/g FW measured by the *p*-dimethylaminocinnamaldehyde (DMACA) method. The integration capacity of the four transgenes, their respective expression levels and their heritability in the second generation were analyzed in stably transformed *N. tabacum* plants. DMACA and phoroglucinolysis/HPLC-MS analyses corroborated the activation of both pathways and the effective production of PAs in T0 and T1 transgenic tobacco plants up to a maximum of 3.48 mg/g DW. The possible biotechnological applications of the GB2.0 multigenic approach in forage legumes to produce “bloat-safe” plants and to improve the efficiency of conversion of plant protein into animal protein (ruminal protein bypass) are discussed.
Introduction

The colorless flavonoid polymers called proanthocyanidins (PAs) or condensed tannins (CTs), and their monomeric building blocks, the (epi)-flavan-3-ols (+) catechin and (-)-epicatechin, are important bioactive compounds both for human health and agriculture, and their production in crop plants using metabolic engineering would permit the design of à-la-carte nutraceutical foods. Oligomeric and polymeric PAs are end products of the flavonoid biosynthetic pathway. Flavonoids constitute one of the largest groups of plant secondary metabolites derived from the phenylpropanoid and acetate/malonate metabolic pathways [1–3]. Several types of transcription factors (TFs), including basic helix-loop-helix (bHLH), MYB and WD-40, control the expression of genes coding for enzymes of this pathway [4]. These TFs act together to regulate the expression of genes encoding enzymes such as chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS) and UDP glucose-flavonoid 3-O-glucosyltransferase (UFGT), [5–12]. One approach to genetically modified plants to produce PAs is to transform first plants with a transcription factor or factors which presence increase the availability of upstream precursors of PAs therefore inducing anthocyanidin production [13]. Then, in a second step the co-expression of one or more genes coding for PA-specific biosynthetic enzymes might lead to PAs accumulation. For example, the conversion of cyanidin to the flavan-3-ol (-)-epicatechin, a building block of PAs, has been accomplished by such a strategy [1, 14].

The anthocyanin branch of flavonoid biosynthesis is activated in Antirrhinum majus by two TFs, AmRosea1 (encoding a MYB-related transcription factor) and AmDelila (encoding a bHLH transcription factor), [15, 16]. These TFs have been used to produce high levels of anthocyanin in the tomato fruit by their expression under the control of the fruit-specific E8 promoter [17]. Once the biosynthesis of anthocyanins is activated in a plant tissue to direct the flavonoid pathway to PAs production, the activity of leucoanthocyanidin reductase (LAR) to produce (+)-catechin and (+)-gallocatechin and anthocyanidin reductase (ANR), to produce (-)-epicatechin and (-)-epigallocatechin are necessary [18–21].

In the last decades, technical hurdles limiting the number of genes transferred to plants have introduced a significant bottleneck to progress in plant biotechnology [22–24]. The genes are stacked in transgenic plants using iterative processes, such as successive rounds of crosses between different transgenic lines [25, 26], or the sequential transformation of transgenic plants with additional transgenes [27–29]. Both approaches have two major drawbacks: the long and labor-intensive processes involving several breeding generations and the fact that the different transgenes are unlinked, leading to segregation in subsequent generations [30]. The alternative to unlinked transformation is the simultaneous transfer to the plant of several genetic units assembled into the same DNA string (Multigene transfer; MGT). This approach facilitates not only the inheritance of all the transgenes among generations but also the selection of a successful transformation event, because only one selection marker is required. Several methods have been implemented to facilitate the assembly of more than two genes in the same plasmid but most of them are limited in the number of parts to be assembled (Multisite Gateway) [31, 32], and others require de novo synthesis of parts in each assembly reaction to add overlapping regions limiting the standardization and reusability [33]. Therefore, genetic engineering has progressively evolved from single-gene intervention to multigene transformation to tackle increasingly ambitious objectives [23]. An alternative approach is the modular construction of genetic devices using standardized DNA parts. Modular design facilitates combinatorial engineering, as standard DNA parts can be easily exchanged improving the possibilities of the building process. A good example for this is the GoldenBraid 2.0 (GB2.0) cloning...
system a standardized DNA assembly platform developed to facilitate multigene engineering in plants by the simultaneous incorporation of several transgenes (https://gbcloning.upv.es/). GB2.0 allows the binary combination of multipartite assemblies using an extremely simple set of rules. This cloning system facilitates the reusability of DNA parts and assembled devices to efficiently build complex constructs and includes a collection of pre-assembled genetic devices that facilitate the generation of multigenic constructs [34–36]. This multigenic cloning system has been proved by transient expression (agroinfiltration) in Nicotiana benthamiana leaves and in stable transformation of N. benthamiana and Nicotiana tabacum [37, 38].

An interesting challenge in plant metabolic engineering is the introduction of PAs into alfalfa (Medicago sativa L.) forage, which would be particularly important to ruminant livestock producers to combat “pasture bloat”, a digestive pathology caused by the production of greenhouse gases in the rumen due to excessive fermentation of dietary protein from forages [2]. Currently, the livestock diet should be complemented with surfactants to break down the protein foams or mixed with forage known to contain moderate levels of PAs, unfortunately both are costly options. The presence of PAs into alfalfa could help to fight pasture bloat and improve the efficiency of conversion of plant protein into animal protein (ruminal protein bypass). The lack of PAs in the leaves of the major forage legume such as alfalfa has prompted studies for the understanding of the molecular and cellular biology of PA polymerization, transport and storage helped by the functional genomics tools available in Medicago truncatula [3, 4, 20, 21].

Here we report the engineering of the flavonoid biosynthetic pathway to produce the monomeric building blocks of PAs by multigene transfer using the GoldenBraid2.0 (GB2.0) cloning system. The GB2.0 construct was designed to carry two A. majus sequences coding for TFs (AmRosea1 and AmDelila) that activate the anthocyanin biosynthetic pathway and two sequences coding for the PA-specific biosynthetic enzymes (MtANR and MtLAR) from M. truncatula. Transient expression experiments in N. benthamiana and stable transformation of N. tabacum showed the activation of both pathways and the production of monomeric components of PAs (+) catechin and (-) epicatechin in the transgenic plants, therefore supporting the use of the GoldenBraid 2.0 cloning system to modify this metabolic pathway in plant species.

**Materials and methods**

**Plant material**

Plants of N. benthamiana were grown from seeds in a greenhouse at 24°C day/20°C night in a 16 h light/8 h dark cycle. M. truncatula 2HA plants were grown in a growth chamber with 16 h light/8 h dark photoperiod and temperature range of 22°C day/18°C night. Tobacco plants (N. tabacum cv Petite Havana SR1) were grown in a mixture (1:1) of sphagnum:vermiculite. Plants were irrigated with Hoagland N’1 solution supplemented with oligoelements [39] and cultivated under 16 h photoperiods in a greenhouse at 25°C day/18°C night. Supplementary lighting was provided by 400 W Phillips HDK/400 HPI [R] [N].

**GoldenBraid 2.0 reactions**

Restriction-ligation reactions were performed as described previously [35, 40]. Briefly, 75 ng of the destination vectors and the parts/modules to be assembled were mixed with 1μL of T4 Ligase (Promega) and the appropriate Type IIs restriction enzyme (i.e., BsaI for the alfa assemblies and BsmBI for the omega assemblies and the domestication of GBparts) in a final volume of 10 μL. These reaction mixes were generally incubated for 25 Golden Gate cycles (2 min at 37°C, 5 min at 16°C). One microliter of each reaction was transformed into Escherichia coli
DH5α electrocompetent cells. Positive clones were selected on LB agar plates supplemented with appropriated antibiotics. For the GPparts domestication reactions, ampicillin at 100 μg ml⁻¹, 0.5 mM IPTG and X-Gal (20 mg/ml) were used. For Alpha and Omega assemblies we used kanamycin and spectinomycin at 50 μg mL⁻¹, respectively. The binary vectors generated by the GB2.0 multigenic approach were transformed into Agrobacterium tumefaciens strains GV3101 and LBA4404, which were used for plant transformation experiments. Plasmid DNA was extracted using the E.Z.N.A. Plasmid Mini Kit I (Omega Bio-Tek, Norcross, GA, USA). Correct assemblies were confirmed by restriction analysis and sequencing when appropriate.

Domestication of the MtANR and MtLAR GPparts

The coding sequences of MtANR and MtLAR were adapted to the GB2.0 grammar as described earlier [35]. This process is known as domestication, and involves the addition of the appropriate flanking overhangs to the DNA parts, and also the occasional removal of internal Type II restriction sites by introducing silent mismatches to disrupt the enzyme target sites. MtANR and MtLAR were isolated by RT-PCR from RNA of M. truncatula seeds 10 days after pollination using specific primers designed from the available sequences (NCBI: XM_013601695.1 and XM_003591782 respectively). Both MtANR and MtLAR coding sequences included internal restriction sites that had to be removed. MtANR was amplified using the primers MtANR-F1 and MtANR-R1 for the first fragment, and MtANR-F2 and MtANR-R2 for the second one (S1 Table). MtLAR was amplified using the primers MtLAR-F1 and MtLAR-R1 for the first fragment, and MtLAR-F2 and MtLAR-R2 for the second one (S1 Table). Amplified bands were purified using the QIAquick PCR purification Kit (Qiagen) and quantified in a Nano Drop Spectrophotometer 2000. The restriction-ligation reaction was performed as described above. The correct assemblies of the GPparts pMtANR and pMtLAR were confirmed by restriction analyses and sequencing.

Transient expression assays in N. benthamiana

For transient plant transformation assays, plasmids were transferred to A. tumefaciens strain GV3101 by electroporation. Agroinfiltration was performed as previously described [41]. Briefly, cultures were inoculated in LB medium and incubated for 18-22h at 28˚C and 250 rpm. Bacterial cultures were centrifuged for 20min at 4,000rpm and the pellets resuspended into agroinfiltration medium (10 mM MES pH 5.6, 10 mM MgCl₂, 200 mM acetosyringone) to an optical density of 0.5 at 600 nm. Agroinfiltration was carried out using a needle-free syringe in leaves of 4–5 weeks old N. benthamiana plants. The vector pEGB 35S:DsRed:TNos [34], which product shows red fluorescence under UV light, was used as control to verify that agroinfiltration was functional. Two rounds of 10 plants were agroinfiltrated. Control plants were agroinfiltrated only with the reporter gene DsRed. Leaves were harvested 5 to 7 days post-infiltration and assayed for transgene expression.

Stable genetic transformation of N. tabacum

The binary vector pDGB2-Hyg-35S:AmRosea1:TNos-35S:AmDelila:TNos-35S:MtANR:TNos-35S:MtLAR:TNos was electroporated into A. tumefaciens strain LBA4404. Tobacco plants were transformed according to standard procedures [42, 43] using leaf disks in co-culture with A. tumefaciens strain LBA4404. Transgenic tobacco plants were selected on organogenic induction medium IKZ containing 25 μg mL⁻¹ hygromycin and 0.8% phytagar. Positive transgenic plants were confirmed by amplifying the AmRosea1, AmDelila, MtANR and MtLAR genes using genomic PCR. Specific primers for the AmRosea1 (Rosea 244 Dir and Rosea 618 Rev), AmDelila (Delila Dir and Delila Rev), MtANR (MtANR 39 Dir and MtANR 419 Rev) and
MtLAR (MtLAR 39 Dir and MtLAR 356 Rev) were used (S1 Table). Hygromycin resistant plants were acclimated and maintained in a greenhouse. Expression of the transgenes was confirmed in the positive transformants by qRT-PCR with RNA extracted from leaves. Plants with the complete set of transgenes in a single copy were retained for further analyses.

RNA extraction

Immature seeds were collected from *M. truncatula* pods at developmental stage 10 days after anthesis using a stereomicroscope (Leica, MZ16F). Immature seeds were pulverized and resuspended in ice-cold RNA lysis solution of Plant RNA Purification Reagent (Invitrogen). Purified RNA was obtained as described in the Turbo DNA-free instructions (Ambion) after removing traces of contaminating DNA. The cDNA samples were synthesized from 1 μg of total RNA using the Primerscript™ RT Reagent Kit (Perfect Real Time, Takara) and random primers in 20 μL total volume reaction. This cDNA was used to isolate the MtANR and MtLAR genes. RNA was also isolated from *N. benthamiana* and *N. tabacum* leaves using the same protocol described above. Total RNA was purified from tissue samples using the E.Z.N.A. Plant RNA Kit, following the manufacturer’s recommendations (Omega) from 100 mg of tissue. RNA was quantified using absorbance at 260 nm, whereas its purity was assessed based on absorbance ratios at 260/280 nm. The integrity of purified RNA was confirmed by denaturing agarose gel electrophoresis and ethidium bromide staining. Then, 1 μg of total RNA was reverse transcribed with a Primerscript™ RT Reagent Kit.

RT-PCR analyses

All RT-PCR reactions were performed and analyzed on a Thermo Scientific Arktik Thermal Cycler (Thermo Fisher Scientific). The reaction mix was prepared in a 25 μL total volume containing 2 μL of cDNA solution, 0.2 mM of each dNTP, 3 mM MgCl₂, 1x reaction buffer, 1 unit of Taq DNA Polymerase (Biotools) and 0.8 mM of the appropriate pair of primers (S1 Table).

PCR conditions for amplification of *AmRosea1*, *AmDelila*, *MtANR*, *MtLAR*, *CHS*, *CHI*, *F3H*, *DFR1* and *ANS* consisted of initial denaturation at 94˚C for 5 min, 30 amplification cycles of 94˚C/30 sec, 55˚C/30 sec, and 72˚C/60 sec, and a 7 min final extension at 72˚C. The PCR program was limited to 25 cycles for the housekeeping genes (*i.e.*, *NtACT*; [44]). 20 μL of the PCR products were separated on 1% ethidium bromide-stained agarose gels. PCR fragments were visualized under UV using a Syngene GBOX (Syngene) and captured with the GeneSnap program (Syngene). The analyses were carried out in duplicate using biologically independent material with similar results. The possibility of genomic DNA contamination in the RT-PCR assays was controlled with the No Reverse Transcriptase control (No RT control). Oligonucleotides for the RT-PCRs were designed using the corresponding sequences of the *NtCHI*, *NtF3H*, and *NtANS* genes of *N. tabacum* and the *NbCHS* and *NbDFR1* genes of *N. benthamiana* (S1 Table).

Extraction and quantification of soluble proanthocyanidins by the dimethylaminocinnamaldehyde (DMACA) reagent

The extraction of soluble proanthocyanidins from *N. benthamiana* leaves was performed as described by [45]. Briefly, 2 g of fresh material were ground in liquid nitrogen and extracted with 8 mL of extraction solution (70% acetone and 30% of 1% acetic acid in water). The extracts were vigorously vortexed for 2 min, sonicated at 30˚C for 30 min, and then centrifuged at 8,000 rpm for 10 min to remove cell debris. The pellet was resuspended in 4 mL of extraction solution, repeating the same procedure. Supernatants of both extractions (12 mL) were collected and transferred to 25 mL glass flasks and acetone was evaporated under reduced
pressure. The remaining aqueous phase was extracted twice with 10 ml of dichloromethane and three times with hexane. Samples were finally evaporated to dryness and dissolved in 1 mL of the first extraction solution for colorimetric reaction. Soluble PAs were quantified by reaction with the p-dimethylaminocinnamaldehyde (DMACA) reagent using catechin standards, as described previously [46, 47]. The DMACA reagent gives coloured adducts with flavanols showing maximum absorption between 632 and 640 nm, thus preventing the interference of other coloured compounds that might be present in the same extracts, such as anthocyanins. A 20 μL aliquot from the final extract with a concentration of 2 g of fresh N. benthamina leaves/mL, was mixed with 980 μL of DMACA reagent (0.2% [w/v] in a mixture of methanol and 3N HCl [1:1]) in spectrophotometer cuvettes. The reference blanks contained 20 μL of extraction solution instead of the aliquot samples. The absorbance at 640 nm was measured after 5 min using a Pharmacia Biotech U1000E UV/Visible spectrophotometer and PAs content was calculated, after subtracting the blanks, as catechin equivalents.

Analysis of PAs by phloroglucinolysis and HPLC-MS

The phloroglucinolysis analysis allowed the quantification of proanthocyanidins after degradation in the presence of phloroglucinol that leads to the monomers (catechin and epicatechin as the terminal units of the oligomers) and the catechin and epicatechin adducts that show the extension units in the PAs. Proanthocyanidins were quantified as previously reported [48] using an acid catalysis in the presence of phloroglucinol. Briefly, 50 mg of lyophilized sample were dissolved in 800 mL of phloroglucinol (50 mg mL⁻¹) added with ascorbic acid (10 mg mL⁻¹) dissolved in methanol acidified with 0.1 N HCl. The reaction mix was vortexed and incubated at 50˚C for 20 min. The reaction tube was placed in ice and 1 mL of 40 mM sodium acetate was added to stop the reaction. The sample was centrifuged, filtered with a 0.22 μm PVDF filter, and injected in an HPLC/MS apparatus. The identification and quantification of catechin, epicatechin and their adducts was carried out by Agilent 1100 Series apparatus equipped with detector MSD Trap 1100 Series (Agilent), as previously described [49, 50]. Briefly, the column used was an Atlantis C18 (250 mm x 4.6 mm, 5 μm particle size; Water, Milford, MA, US) operating at a flow rate of 1 mL min⁻¹; the injection volume was 8 μL. The solvents were 2.5% acetic acid in water (A) and acetonitrile (B) with a separation gradient starting with 3% B in A at 0 min, 9% at 5 min, 16% at 15 min, 50% at 45 min followed by washing and conditioning steps. The phenolic compounds were quantified at 280 nm with a calibration curve of catechin (1–300 μg L⁻¹). The MS detector operated in negative ion-mode. The Trap interface and ion optics settings were the following: spray potential 65 psi; nebulization gas (nitrogen) relative flow value 11; capillary temperature 325˚C. Full-scan mass spectra were acquired scanning the range 100–800 m/z. For calculation of the degree of polymerization (mDP), the flavan-3-ol monomers (catechin and epicatechin) present endogenously in the plant material, were analyzed by the same chromatographic method used for the phloroglucinolysis analysis, before the phloroglucinolysis degradation and therefore the monomers present endogenously were subtracted in the calculation of mDP.

Results

Generation of the GoldenBraid 2.0 multigenic construct

We have generated a multigenic construct using the GoldenBraid 2.0 modular cloning system to simultaneously activate the anthocyanin and proanthocyanidin pathways in plants. For this purpose we have combined the AmRosea1 and AmDelila transcription factors of A. majus with the anthocyanidin reductase (MtANR) and the leucoanthocyanidin reductase (MtLAR) genes of
Multigene engineering of the anthocyanin and proanthocyanidin pathways

M. truncatula to produce the two enzymes that function at branches between anthocyanin and PA biosynthesis (Fig 1A).

To generate the multigenic construct, first the MtANR and MtLAR genes were domesticated as GBparts, as described in the Materials and Methods section (Fig 1B). Next, two transcriptional units (TUs) were assembled from its basic parts (i.e., promoter+coding sequence...
+terminator) into the Alfa level plasmids (35S:MtANR:TNos into the pDGB1 Alfa1 and 35S: MtLAR:TNos into the pDGB2 Alfa2, see Assemblies 1 and 2 in Fig 1C). The GBparts GB0030 (pCaMV35S, the Cauliflower Mosaic Virus 35S Promoter) and GB0035 (pTNos, the Nopaline synthase terminator) were used in the constructs with the pMtANR and pMtLAR genes. Both assembled TUs were further binary combined into the pDGB1 Omega2 vector (see Assembly 3 in Fig 1C), so that the generated construct is compatible with the preassembled 35S:AmRosea1:TNos-35S:AmDelila:TNos module, that was already available in the GBCollection (GB0129; https://gbcloning.upv.es/search/). The four genes were later combined into the pDGB1 Alfa2 vector (see Assembly 4 in Fig 1C). This four-gene construct was moved together with the hygromycin resistance gene (GB246) into the pDGB2 Omega1 Vector. This final multigeneric construct was electroporated into A. tumefaciens strains GV3101 and LBA4404 and used for transient and stable transformation experiments.

Functional validation of the multigeneric construct by transient expression assays in N. benthamiana
To test the functionality of the complete multigeneric construct AmRosea1:AmDelila: MtANR: MtLAR, we performed transient expression assays in agroinfiltrated leaves of N. benthamiana plants. Our results showed purple pigmentation (anthocyanin production) in the infiltrated areas (Fig 2A) in comparison with the infiltrated control with the DsRed reporter gene and the non-infiltrated WT control (Fig 2B and 2C respectively). The purple pigmentation was evident 5 days after infiltration and increased progressively.

To determine the expression levels of AmRosea1, AmDelila, MtANR and MtLAR, we performed RT-PCR analyses using RNA isolated from the N. benthamiana agroinfiltrated leaves. The housekeeping gene Actin-8 (NbACT8) was used as control of a constitutive expression. Our results indicated that the CaMV35S constitutive promoter induced high expression of the four transgenes after 25 amplification cycles in leaves collected 5 days after infiltration (Fig 2D). In addition, a RT-PCR assay demonstrates that the increased expression of both AmRosea1 and AmDelila transcription factors is able to upregulate the expression of the genes involved in the anthocyanin biosynthetic pathway (CHS, CHI, F3H, DFR and ANS). Our results show that an increase in the expression levels both TFs specially activates the F3H, DFR1 and ANS genes when compared with control leaves infiltrated with DsRed, in which these genes were not activated (Fig 2E). This analysis corroborated the regulatory effect of both AmRosea1 and AmDelila TFs in the expression of three central enzymes of the flavonoid biosynthetic pathway: CHS, F3H and ANS [17].

Detection of flavan-3-ols and proanthocyanidins in agroinfiltrated N. benthamiana leaves
DMACA assays showed increased PA levels in the N. benthamina plants infiltrated with the AmRosea1:AmDelila:MtANR:MtLAR multigenic construct compared with the non-infiltrated WT control. Total flavonoid content was expressed as catechin or epicatechin equivalents [228.51 ± 24.17 nmol (-)-epicatechin/g FW and 208.56 ± 31.19 nmol (-)-catechin/g FW respectively], (Fig 2F and S2 Table).

Experimental validation of the multigeneric construct by stable transformation of N. tabacum: Integration and expression of transgenes
The validated multigeneric construct AmRosea1:AmDelila:MtANR:MtLAR was used to perform stable genetic transformation experiments in N. tabacum. Two important aspects were
evaluated in the regenerated T0 transgenic plants: the integration capacity of the four transgenes (which is especially important for multigenic constructs) and their respective expression levels.

Anthocyanin pigmentation was a visual sign for the selection of hygromycin resistant tobacco plants. We detected high levels of anthocyanin pigmentation in the young developing *N. tabacum* transgenic calli, however some of them never regenerated shoots (Fig 3A). This effect is probably due to the toxic effects of the excess in anthocyanin production during the regeneration process [51, 52]. In the pool of regenerated plants, we selected 10 plants from different transgenic lines. Most of them showed more or less severe purple pigmentation in all plant tissues (stem, leaves, flowers) when compared with control plants (Fig 3B–3F, 3H and 3I). One plant (Nt#5) showed small purple spots in the leaves (Fig 3G, in comparison with a fully purple leaf from plant Nt#7 in panel F). We observed that plant growth was affected in those plants where higher purple pigmentation occurred. These findings suggest that high levels of anthocyanin accumulation, mainly produced by the ectopic expression driven by the 35S promoter, have a deleterious effect on plant growth and development.
The integration capacity of the multigenic construct into the plant genome was proved by PCR amplification of genomic DNA isolated from young leaves of *N. tabacum* hygromycin-resistant plants using specific oligonucleotides (S1 Table). Our results showed the presence of the four transgenes in six (Nt#5, #6, #7, #8, #9 and #10) of the 10 *N. tabacum* transgenic plants analyzed (S1A Fig). These results suggest that the integration capacity of the complete set of transgenes into the *N. tabacum* genome is high.

### Transgene expression levels in T0 *N. tabacum* plants

The expression levels of the four transgenes were evaluated by quantitative RT-PCR (qRT-PCR) in young leaves of *N. tabacum*, establishing as constitutive expression the
endogenous Actin-8 gene (NtACT). Three N. tabacum plants were selected according to their phenotype: one plant presenting only small purple spots in their leaves (Nt#5), one plant showing a partial purple pigmentation (Nt#6) and one plant showing strong purple pigmentation in all tissues (Nt#7). Our results showed that the expression level of the four transgenes varies between the different transgenic lines (greater in Nt#7 and lower in Nt#5, Fig 4A). This finding also correlated with the different intensity in anthocyanin accumulation, which depends on the expression levels of the AmRosea1 and AmDelila transcription factors.

We also analyzed the expression levels of these genes encoding the main enzymes required for anthocyanin biosynthesis in the T0 N. tabacum plants, which should have been upregulated by the expression of AmRosea1 and AmDelila. RT-PCRs were performed using RNA from leaves of transgenic N. tabacum plants Nt#6 and Nt#7. The endogenous NtACT gene was used as control of constitutive expression. In N. tabacum, there was a correlation between the expression levels of the transgenes (Fig 4B) and the severity of the coloured phenotypes observed. In addition, our results showed that AmRosea1 and AmDelila upregulated the expression of genes encoding the enzymes acting in the central route of flavonoids biosynthesis (NtF3'H, NtDFR1 and NtANS) in the N. tabacum transgenic plants, when compared with control plants.

Detection of flavan-3-ols and proanthocyanidins in transgenic N. tabacum leaves

We analyzed whether the constitutive expression of the four transgenes AmRosea1, AmDelila, MtANR and MtLAR were able to activate the route of biosynthesis of PAs in two N. tabacum transgenic plants that showed partial and severe purple pigmentation (Nt#6 and Nt#7 respectively). Colorimetric analysis of leaf extracts with DMACA showed that both plants produced more PAs when compared with a WT control plant. The maximum levels of PAs expressed as catechin or epicatechin equivalents were found in the plant Nt#7 (633 ± 27.5 nmol (-) epicatechin/g FW and 577.79 ± 25.09 nmol (-) catechin/g FW), (Fig 5A and S3 Table). Plant Nt#6 showed seven-fold less production of PAs than plant Nt#7 (86.56 ± 4.91 nmol (-) epicatechin/g FW and 79 ± 4.48 nmol (-) catechin/g FW). These results corroborate the active role of the
enzymes MtLAR and MtANR in the transgenic plants catalyzing the conversion of their respective intermediate substrates (leucoanthocyanidin and anthocyanidin) in proanthocyanidins.

The PAs content was determined by phloroglucinol derivatization (phloroglucinolysis). Phloroglucinolysis allowed the quantification of PAs after separation of the different products (terminal units and adducts) in the presence of phloroglucinol, followed of HPLC-MS analysis. This analysis showed that only epicatechin was present as extension unit, and also that epicatechin is more relevant than catechin. As expected, in plants Nt#6 and 7 we detected the presence of PAs when compared with a WT control plant. The high level of PAs was detected in plant Nt#7 (3.48 mg/g DW), in contrast with plant Nt#6 (0.15 mg/g DW) (Fig 5B). We also detected differences in the average degree of polymerization (mDP) in plants Nt#6 and Nt#7. Plant Nt#6 presents the highest value (2.32 ± 0.12), compared with plant Nt#7 (1.76 ± 0.56). This result indicates that PAs in Nt#7 are mainly composed of monomers and dimers, whereas in Nt#6 is mainly composed of dimers but in small quantities (S4 Table).

All the exposed results revealed that there is a correlation between the expression levels of AmRosea1 and AmDelila, the activation of genes encoding the enzymes involved in the anthocyanin biosynthetic pathway, the purple pigmentation of the different plant tissues and the de novo production of PAs in the N. tabacum transgenic plants. All together indicate that the multigenic construct is fully functional and simultaneously activate the anthocyanin and proanthocyanidin biosynthetic pathways.

Heritability and functionality of the four transgenes in the T1 tobacco plants

All the tobacco T0 plants selected were fertile and produced seeds. The heritability of the four transgenes was evaluated in the second generation (T1) of N. tabacum Nt#6 and Nt#7 transgenic plants (S1B Fig). In both cases the lineages obtained showed different degrees of coloured leaf phenotypes (Fig 6A–6D). The purple plants showing a strong phenotype (high anthocyanin content) grew more slowly than those showing middle or weak purple phenotypes probably due to the excessive accumulation of anthocyanins in their vacuoles. About the 60% of the T1 plants incorporated the full set of transgenes. In the lineage of plant Nt#6, the
unique expression of AmRos1 in the Nt#6.2 plant was sufficient to induce anthocyanin production, whereas plants Nt#6.1 and Nt#7.5 showed a green phenotype due to the absence or low expression of AmRos1. The unique presence of AmDel was not capable to induce anthocyanin production in the Nt#6.1 plant. The purple plants showing a strong phenotype grew more slowly during the first stages of development than those showing middle or weak purple phenotypes, probably due to the excessive accumulation of anthocyanins in their vacuoles as we indicated previously. In the lineage of plants Nt#6 and Nt#7 we analyzed by semi-qRT-PCR three plants with the complete set of transgenes showing a weak (Nt#6.8), a middle (Nt#6.11) and a strong purple phenotype (Nt#7.6). In the three plants the four transgenes were properly expressed. To normalize the samples the constitutive NtACT8 gene was used. F. The Nt#6.11, Nt#6.8 and Nt#7.6 transgenic plants produced PAs as demonstrated by HPLC-MS analysis of leaf extracts when compared with the WT.

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Discussion

Genetic modification of secondary metabolic pathways to produce desirable natural products is an attractive approach in plant biotechnology. The ability to manipulate the biosynthesis of flavonoids in plants is of high interest in the areas of nutraceuticals and human and animal feed. Proanthocyanidins are active principles of plants with demonstrated antioxidant properties to provide benefits to the health and contribute as anti-aging resources [53, 54].

Different MYB, bHLH and WD-40 complexes regulate the expression of genes encoding enzymes catalysing each step of the anthocyanin and PA biosynthetic pathways [55–57]. Butelli et al. [17] set out to produce tomato fruits with substantially elevated levels of anthocyanins by harnessing of two selected A. majus transcription factors (AmRosea1 and AmDelilala) under the control of the E8 fruit-specific promoter. The use of both TFs to induce anthocyanin production and the co-expression of leucoanthocyanidin reductase (MtLAR) and anthocyanidin reductase (MtANR) from M. truncatula, might then lead to PA biosynthesis in plants. The DNA assembly platform GoldenBraid2.0, developed for multigene engineering, could be a good instrument to incorporate more than two genes in the same plasmid and therefore to stack genes in plants. Currently, there are no reports on the use of multigenic constructs to introduce a set of genes to activate the anthocyanin and PA pathways in the same transgenic plant.

The GB2.0 multigenic construct transitory activates the anthocyanin and PA biosynthetic pathways in N. benthamiana

Using the AmRosea1 and AmDelilala TFs, in combination with two M. truncatula (MtLAR and MtANR) genes, we have generated a multigenic GoldenBraid2.0 construct to simultaneously activate both the anthocyanin and proanthocyanidin biosynthetic pathways. Transient expression experiments in N. benthamiana showed the activation of the anthocyanin pathway producing a purple color into the infiltrated leaves. Our results indicated that the increased expression of both AmDelilala and AmRosea1 TFs, is able to upregulate the expression of genes involved in the anthocyanin biosynthetic pathway CHS, CHI, F3H, DFR1 and ANS, activating the F3H, DFR1 and ANS genes coding for three central enzymes of the flavonoid biosynthetic pathway [58]. In addition, DMACA assays showed the de novo production of PAs in the N. benthamina plants infiltrated with the multigenic construct.

The multigenic construct AmRosea1:AmDelilala:MtANR:MtLAR simultaneously activates the anthocyanin and PA biosynthetic pathways in stably transformed N. tabacum plants

N. tabacum plants were stably transformed with the above described multigenic construct. One of the interesting aspects evaluated in the transgenic plants was the integration capacity of the four transgenes present in the construct. Full insertion of the four transgenes occurred in 60% of the tobacco plants. This information is relevant to the GoldenBraid 2.0 cloning system community of users, since few of the publications that have reported stably transformed plants analyze in depth the integrity of the multigenic construct through generations [37, 38, 40]. Our results suggest that there is a relationship between the integration capacity of the complete set of transgenes present in the multigenic construct and the plant species in question. The process of integration of the T-DNA often results in deletion, inversion or duplication of the DNA portion between the right and the left borders [59, 60]. Studies which demonstrate the relationship between the species and the loss of transgenes after the insertion of the T-DNA have been conducted in the last years. For example, the nptII gene showed different levels of
deletion, being 12% in tobacco, 30% in watermelon and 60% in carrot [61]. In our multigenic construct the transgenes closest to the borders of the T-DNA are the gene that confers resistance to hygromycin (hpt) (left border, LB) and the MtLAR gene (right border, RB). In T0 N. tabacum plants, we observed always the deletion of MtLAR when located besides the RB. However, T1 AmRosea1:AmDelila;MtANR:MtLAR transgenic plants tend to lose neighbour transgenes located near the LB (AmRosea1 or AmDelila). We never documented the loss of alternate transgenes. This deleterious effect could be connected to the repetitive use of the same promoter and terminator in all the transcriptional units (i.e., CaMV35S promoter and TNos terminator) so favouring DNA recombination that results in the loss of portions of the multigenic construct. The use of different promoters and terminators for multigenic constructs is highly suggested, and this could be easily implemented by browsing the GBCollection and using the different characterized regulatory regions [35, 40].

Regardless the percentage of plants that carry out the four transgenes and the deletion patterns of these in tobacco, the use of multigenic constructs generated by the GoldenBraid 2.0 cloning system is a suitable option for the stable transformation of plants. In this way, it is possible therefore the co-expression of multiple transgenes, indicating that this system is appropriate to modify polygenic characters in different plant species.

We evaluated whether the TFs AmRosea1 and AmDelila were able to activate the route of biosynthesis of flavonoids in N. tabacum, with the consequent production of anthocyanins. Most of the transgenic plants presented purple pigmentation in all vegetative and reproductive tissues, with a greater accumulation into the abaxial side of the leaves and vascular tissues. Tobacco plants with an intense purple pigmentation in all tissues due to accumulation of anthocyanins, showed a delayed development. This fact can be explained because cells transport and store the produced anthocyanins in the vacuoles, which have a limited storage capacity. The accumulation of anthocyanins into the cells resulted in toxicity. The consequence is a delay in plant development and even plant death [51, 52]. After these results, we decided to investigate if the genes encoding the enzymes involved in the biosynthesis of flavonoids pathway were induced in the transgenic plants. Our results showed that the expression of genes encoding the enzymes CHS, CHI, F3’H, DFR1 and ANS, were induced in the transgenic plants indicating a correlation exists between the expression levels of the TFs AmRosea1 and AmDelila, the purple phenotype and the expression of genes involved in the anthocyanin biosynthetic pathway. Thus, the TFs of Antirrhinum, AmRosea1 and AmDelila, are able to recognize cis regulatory elements present in the promoters of N. benthamiana and N. tabacum genes encoding enzymes involved in the biosynthesis pathway of anthocyanins in both species.

The biosynthesis of PAs shares common steps with the anthocyanin biosynthetic pathway until the leucocyanidin step. Leucocyanidin is converted into flavan-3-ols catechin and epicatechin through either a single-step reaction catalyzed by LAR or a two-step reaction catalyzed by leucoanthocyanidin dioxygenase (LDOX) and ANR, respectively [4]. Genetic engineering of catechin-based PAs requires operation of the anthocyanin pathway only as far as leucoanthocyanidin, whereas epicatechin-derived PAs require a source of anthocyanidin in addition to expression of ANR [19]. When Xie et al. [14] co-expressed the MtANR gene and the MYB transcription factor AtPAP1 in tobacco, no free (+)-catechin was detected in the AtPAP1-MtANR transgenic plants. Ectopic expression of the tea (Camellia sinensis) genes CsANR2 or CsLAR led to the accumulation of low levels of PA precursors and their conjugates in Medicago truncatula hairy roots and anthocyanin-overproducing tobacco. Surprisingly, the expression of CsLAR in tobacco overproducing anthocyanin led to the accumulation of higher levels of epicatechin and its glucoside than of catechin, again highlighting the potential importance of epimerization in flavan-3-ol biosynthesis [21].
We used the colorimetric DMACA reaction to evaluate the presence of flavanols and PAs in leaf extracts from the *AmRos1*: *AmDel*: *MtANR*: *MtLAR* transgenic tobacco plants. In addition, the analysis by HPLC coupled to a mass spectrometer is the ideal method to analyze their content in PAs after phloroglucinolysis. Our results showed that the production of PAs (catechin and epicatechin) varied between the analyzed transgenic plants, being higher in the plant presenting more purple pigmentation which is associated with a greater accumulation of anthocyanins. It is also likely that the presence of PAs in *N. tabacum* is due to the enzymatic action of *MtANR* and *MtLAR*, being able to convert their relevant intermediate substrates in PAs. Leucoanthocyanidin reductase has been shown to convert leucocyanidin to (+)-catechin. *M. truncatula* has a single LAR gene that is highly expressed in the seed coat, but the PAs present in the seed coat are composed almost exclusively of epicatechin [27]. It has been reported that *MtLAR* has a role in the extension of proanthocyanidins, and a loss of function of this gene unexpectedly leads to loss of soluble epicatechin-derived PAs, increased levels of insoluble PAs, and accumulation of 4β-(S-cysteinyl)-epicatechin [62]. In the stable transformed tobacco plants with our multigenic construct, the catechin levels measured by phloroglucinolysis/HPLC-MS were lower when compared with the epicatechin ones. Likewise, the expression of other LARs in transgenic plants also failed to result in the accumulation of catechin or produced more epicatechin than catechin [21, 27, 63–65].

Our results indicate that there is a correlation between the expression levels of *AmRosea1* and *AmDelila* and the activation of genes encoding the enzymes involved in the biosynthetic pathway of flavonoids, anthocyanin accumulation and production of PAs. In addition, we have corroborated the expression of the four transgenes in the T1 plants and the existence of a correlation in the anthocyanin accumulation and PAs production in the transgenic plants. All the exposed results lead to validate our multigenic construct to simultaneously activate the anthocyanin and proanthocyanidin pathways in two *Nicotiana* spp.

### Possible biotechnological applications of the multigenic construct: Activation of anthocyanin and PA biosynthetic pathways in forage legumes

Transcriptional regulation of flavonoid biosynthesis is poorly understood in legumes. Major forage legumes like clovers, alfalfa and lupine do not contain appreciable amounts of PAs in the leaves, where they only accumulate in glandular trichomes [66]. This is insufficient to prevent “pasture bloat” in ruminant animals, which is caused by the production of methane gas in the rumen due to excessive fermentation of dietary protein from forages. Modest levels of PAs in forages reduce the occurrence of bloat and at the same time promote increased dietary protein nitrogen utilization in ruminant animals [2, 66, 67]. The alfalfa (*M. sativa*) has high protein content but lacks PAs in their vegetative organs. The presence of PAs into alfalfa could eliminate help to fight pasture bloat, improve the efficiency of conversion of plant protein into animal protein (ruminal protein bypass), reduce greenhouse gases, reduce gastrointestinal parasites and inhibit insect feeding [68–74].

Classical breeding approaches have failed to introduce PAs into alfalfa foliage, and this problem is likely to require a biotechnological solution. Different genetic and metabolic engineering approaches to induce PAs production in forages have been reported in the last decade but have not been fully successful [21]. The majority of studies so far reported indicate that the regulation of PAs is more complex compared with anthocyanin biosynthesis and that provision of sufficient substrate and high expression of the ANR and LAR genes are still insufficient to support high levels of PAs accumulation in transgenic alfalfa plants [21, 58, 63, 75–78].
The concentration and structure of the PAs strongly affect the palatability (bitter taste) and nutritional value of forage legumes being in the order of 20–45 g/kg or 2–4.5% of dry weight the suitable quantities [75]. It has been suggested that a PAs concentration of about 20 mg/g DW in the forage might be sufficient to prevent frothy bloat in cattle [66, 68]. In the *N. tabacum* transgenic plants produced in this work the PAs concentration was lower with respect to the recommended concentration in forages. In any way, the production of PAs using our multigenic construct should be probed in transgenic alfalfa plants where the Medicago genes *MtANR* and *MtLAR* could be more efficient than in *Nicotiana spp.* due to the high degree of synteny existing between both legumes.

We have proved that the *AmRosea1* and *AmDelila* transcription factors of *A. majus* are completely functional in two *Nicotiana spp.* and, in combination with the two Medicago genes (*MtANR* and *MtLAR*), are able to induce PAs production in agroinfiltrated leaves and in stably transformed plants. This multigenic approach could be useful to generate transgenic alfalfa plants producing PAs. The versatility of the GB2.0 cloning system allows to easily incorporate new combinations of different transcription factors with other ANR and LAR genes to the multigenic scheme that could help to increase PAs production in the transgenic plants.

**Conclusions**

1. We generated a GoldenBraid 2.0 multigenic construct containing two *A. majus* transcription factors (*AmRosea1* and *AmDelila*) to upregulate the anthocyanin pathway in combination with two *M. truncatula* genes (*MtLAR* and *MtANR*) to produce the enzymes that will derivate the biosynthetic pathway to PAs production.

2. Transient expression experiments by agroinfiltration of *N. benthamiana* leaves showed the activation of the anthocyanin pathway, producing a purple color in the infiltrated areas, the activation of genes involved in the anthocyanin biosynthetic pathway, and the effective production of PAs in comparison with non-infiltrated control plants.

3. The integration capacity of the four transgenes, their respective expression levels and their heritability were verified in T0 and T1 stably transformed *N. tabacum* plants.

4. DMACA and phoroglucinolysis/HPLC-MS analyses corroborated the activation of both pathways and the effective production of PAs in T0 and T1 transgenic tobacco plants in comparison with non-transformed control plants.

**Supporting information**

S1 Fig. Transgene integration and heritability in T0 and T1 *Nicotiana tabacum* transgenic plants. (A) Detection by PCR of the presence of the *AmRosea1*, *AmDelila*, *MtANR* and *MtLAR* transgenes in T0 *N. tabacum* transgenic plants. 6 out of 10 plants incorporated the complete full set of transgenes. Plants Nt#1 to Nt#4 lacked the *MtLAR* transgen. (B) Detection by PCR of the presence of the *AmRosea1*, *AmDelila*, *MtANR* and *MtLAR* transgenes in some plants of the T1 lineage of *N. tabacum* Nt#6 and Nt#7 transgenic plants. About the 60% of the T1 plants incorporated the full set of transgenes. The unique expression of *AmRosea1* in the Nt#6.2 plant was sufficient to induce anthocyanin production, whereas plants Nt#6.1 and Nt#7.5 showed a green phenotype due to the absence or low expression of *AmRosea1*. The unique presence of *AmDelila* was not capable to induce anthocyanin production (plant Nt#6.1). In T0 *N. tabacum* plants, there was always the deletion of *MtLAR*, located beside the RB. However, T1 *AmRosea1-AmDelila-MtANR-MtLAR* transgenic plants tend to lose neighbour transgenes located
near the LB (AmRosea1 or AmDelila).

S1 Table. Primers used in this work.

S2 Table. Quantification of PAs in leaf extracts of Nicotiana benthamiana agroinfiltrated leaves by the dimethylaminocinnamaldehyde (DMACA) colorimetric reaction. Data represent the mean of three replicates ± the standard error of data for each sample.

S3 Table. Quantification of PAs in leaf extracts of T0 Nicotiana tabacum transgenic plants by the dimethylaminocinnamaldehyde (DMACA) colorimetric reaction. Data represent the mean of three replicates ± the standard error of data for each sample. Different letters indicate statistically significant differences, according to the analysis of variance ANOVA (p<0.05).

S4 Table. PA levels and degree of polymerization (mDP) in leaf extracts of T0 Nicotiana tabacum transgenic plants estimated by phloroglucinolysis and HPLC/MS. WT: control, non-transformed plant. The results are expressed as mg/g of lyophilized leaf material (dry weight).

S5 Table. PA levels and degree of polymerization (mDP) in leaf extracts of three T1 Nicotiana tabacum transgenic plants estimated by phloroglucinolysis and HPLC/MS. WT: control, non-transformed plant. The results are expressed as mg/g of lyophilized leaf material (dry weight).

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