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Collard and cauliflower as a base for production of recombinant antigens

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Abstract

Plants have emerged as a modern production system to produce recombinant proteins—antigens that can be used as subunit vaccines. The ideal plant candidate for this purpose should be capable to sustain high levels of expression of foreign proteins without adverse effects on its growth and development. It is also essential that it has large biomass, is edible and suitable for long-term storage and delivery.

This work is a part of an effort to develop Cruciferae-based production system using transgenic vegetable plants collard and cauliflower. Several parameters were tested and optimized to achieve an efficient stable transformation of these recalcitrant species with constructs containing expression cassettes for the known viral antigens. Using the original procedure we obtained transgenic collard cv Morris Heading that express high levels of smallpox vaccine candidate (B5) in leaves and retain its normal phenotype. Transgenic cauliflower plants cv Early Snowball were obtained in similar procedure and have shown detectable amounts of SARS coronavirus spike-protein (SARS-CoV S1) in floret tissue of mature curd.

To our knowledge, this is the first report on generation of transgenic collard plants ever and the first successful attempt to use these vegetables for production of pharmaceutical proteins.

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Keywords: Plant biotechnology; Cruciferous vegetables; Transgenic collard; Transgenic cauliflower; Recombinant viral antigen

1. Introduction

Plant genetic transformation technology has opened a new avenue to producing complex recombinant pharmaceutical proteins [1–6]. This approach in plants has become an attractive alternative to other technologies, since it is associated with low production cost, overall safety and scalability potential [4,7,8]. However, despite numerous studies, there are only a few reports of actual production of immunologically functional recombinant subunit vaccines in plants [9], and even fewer plant-derived vaccine candidates have reached clinical trials [10].

The major benefit of using plants for vaccine production is that they allow direct oral and other needle-free routes of immunization [11,12]. The plant system also avoids costly purification processes and allows simple downstream processing of transgenic material in its natural or modified forms such as powder, tablets, creams, etc. [2,8]. Currently, the ideal plant for such use is considered one that is edible, suitable for long-term storage and delivery, easily grown and processed and able to sustain high levels of expression of foreign recombinant proteins without adverse effects on its growth and development [4,7].

Plant transgenic biotechnology has provided successful transformation techniques for a variety of dicot and monocot plants [13,14]. However, efforts in pharmaceutical production have been limited mainly to those model plant species that are easily transformed such as tobacco and Arabidopsis [reviewed in 3,9] Among the crop species used for production of recombinant vaccines are tomato [15–18], potato [19–24] and alfalfa [25–27]. Stand-alone examples also include lettuce [28] and carrot [29]. Monocot plants have also been used for production of recombinant antigens, in most cases presented in maize seeds [30] and recently reported for rice [31]. Most of these studies were done as proof-of-concept [9,10] and it has since become clear that some plants are not suitable for this purpose. In tomato, for example, antigen
expression levels in the fruits can be very low and/or vary dramatically in the pool of fruits originating from the same transgenic line [15,16]. Our own recent experiments revealed severe degradation of recombinant protein in the ripened tomato fruits as compared to immature green fruits [18]. Potato tubers, which were once considered promising for vaccine production purposes, have shown very low levels of antigen expression and, for oral delivery, would have required heat treatment that destroys the recombinant protein [32]. In most cases reported for crops, the overall yields of recombinant proteins expression were relatively low and/or plant material was not suitable for oral administration or storage [8–10].

To address the modern requirements for production and delivery of recombinant vaccines, we are developing a Cruciferae-based system comprised of collard, cauliflower and other vegetables. Collard is a large green leafy crop that is easy to grow, survives ambient temperatures, and produces kilogram amounts of rough leaf material from a single plant. It is convenient for production of recombinant proteins in large leaf tissues using strong tissue-specific promoters and specific intracellular targeting signals. Cauliflower has an edible overgrown inflorescence (curd or head) that is also convenient for long-term storage, transportation and delivery. Like other Cruciferous vegetables, cauliflower also has proven anti-cancer bioactive sulfur-containing compounds known as glucosinolates (isothiocyanates and terpenes) [reviewed 33,34]. Both collard and cauliflower are close relatives of Arabidopsis, a well-studied plant model system [35]. Thus, advances in Arabidopsis research, especially regarding identification of suitable mutants/knockouts and specific genetic elements, will help greatly in developing the Cruciferae-based system.

To date, production of transgenic collard plants has not been reported. Despite many attempts to transform cauliflower, only a few studies describe successful transformation events [36–39,41]. This species is considered recalcitrant for genetic transformation due to low regeneration efficiency, high sensitivity to Agrobacterium, and difficulties with selection procedures [39–41]. None of the published reports describe the use of transgenic cauliflower for production of bio-pharmaceutical proteins.

Here we describe generation of stable transgenic vegetable plants of the Cruciferae family, collard and cauliflower, as a part of plant-based system for production of pharmaceutical proteins. Multiple parameters were tested and optimized to achieve an efficient stable transformation of these recalcitrant species with constructs containing expression cassettes for the known viral antigens. Efficient transformation procedures were developed for these species based on the nptII and bar genes as selectable markers. Use of our original procedure led to the generation of transgenic collard that express B5 recombinant vaccine candidate against smallpox at high levels with no adverse effect on its phenotype. In the case of cauliflower, transgenic plants were obtained expressing the S1-fragment of SARS-CoV spike protein in transgenic florets.

2. Materials and methods

2.1. Transformation of collard

2.1.1. Plant material and shoot regeneration

Seeds of collard (Brassica oleracea var acephala) cvs Morris Heading, Yates and Georgia (obtained from the Carolina seeds Co., Hartford, CT) were sterilized in 70% ethanol for 1 min followed by 2% sodium hypochlorite for 20 min. After rinsing three times in sterile distilled water, seeds were plated in germination medium MS-1 containing MS macro- and microelements [42], 1% sucrose and 0.8% agar (see Table 1 for details on media composition). Germination and in vitro culture were carried out at 24 °C at 16 h-light/8 h-dark photoperiods with light intensity of 40 μE/m²/S. Cotyledons and hypocotyls of the three collard cultivars were cut from 4-, 7- and 10-day-old seedlings and placed on MSR-1 regeneration medium. Ten to twelve explants per Petri dish were cultured for 5 weeks and tested for shoot regeneration efficiency.

2.1.2. Transformation procedures

Four-day-old cotyledon and hypocotyl explants were inoculated with Agrobacterium suspension (OD600=0.5, 0.3, or 0.1) for 10 min. After blotting dry with sterile filter paper, explants were transferred to MS-2 co-cultivation medium supplemented with acetosyringone (Table 1) and incubated in the dark for 2 or 3 days at 24 °C. To determine the effect of pre-culture on transformation efficiency, explants were cultured for 2 or 4 days on MSC callus induction medium before inoculation with Agrobacterium. In another set of experiments, explants were placed on a feeder layer of tobacco cells during co-cultivation [43]. After co-cultivation explants were transferred to MS-3 regeneration medium without selection for 0–12 days and then transferred to MS-5 regeneration selection medium containing 2-mg/l phosphoinotinic (PPT) (Sigma, St. Louis, MO). After 4–5 weeks in selection medium, regenerated green shoots (putative transformants) were formed. Healthy green

| Name | Media composition |
|------|-------------------|
| MS0  | Basic Murashige-Skoog basal medium* (MS) with 3% sucrose and 0.8% agar^2 |
| MSC  | MS0 with 0.5-mg/l NAA, 0.5-mg/l 2,4-D, 0.5-mg/l BAP |
| MSR-1| MS0 with 20-μM AgNO₃, 1-mg/l BAP, 1-mg/l zeatin, 0.1-mg/l NAA |
| MSR-2| MS0 with 20-μM AgNO₃, 1-mg/l BAP, 1-mg/l zeatin, 0.05-mg/l NAA |
| MS-1 | MS medium with 1% sucrose and 0.8% agar |
| MS-2 | MS0 with 100-μM acetosyringone |
| MS-3 | MS0-1 with 300-mg/l timentin |
| MS-4 | MSR-2 with 300-mg/l timentin |
| MS-5 | MS-3 with 2-mg/l phosphoinotinic |
| MS-6 | MS-4 with 20-mg/l kanamycin |
| MS-7 | MS0 with 3-mg/l phosphoinotinic and 300-mg/l timentin |
| MS-8 | MS0 with 30-mg/l kanamycin and 300-mg/l timentin |

* Full composition of MS medium according to original recipe [42].
^ The pH of all of the media used was adjusted to 5.7 before autoclaving.
shoots (1–2 cm) were excised and transferred to MS-7 selection medium supplemented with increased concentration of PPT (3 mg/l) for rooting. Selected plantlets with roots (~3–5 cm long) were transferred to soil [Metromix, K. C. Schoefer, York, PA] in greenhouse conditions. Just before soil transfer, the transgenic status of the plants was confirmed by PCR. For seed production, collard plants were placed in a cold room (4 °C) for 1 month and then transferred to a plant growth chamber (24 °C). Transgenic plants were self-pollinated for production of T₁ seeds.

2.2. Optimization of transformation parameters for cauliflower

2.2.1. Plants material and in vitro shoot regeneration

Seeds of three cauliflower (B. oleracea var. botrytis) cvs Early Snowball, Snowball, and All Year Around (obtained from Carolina Seeds Co., Hartford, CT) were sterilized and germinated as described above for collard. Cotyledons and hypocotyls were cut from 4-, 7- and 10-day-old seedlings, placed in MSR-2 regeneration medium, and evaluated 5 weeks later for regeneration efficiency.

2.2.2. Transformation procedure

The procedure established for transformation of collard was used for cauliflower cv Early Snowball, with some modifications. Cotyledons and hypocotyls were excised from 7-day-old seedlings, pre-cultivated for 2 days on MSC callus induction medium and inoculated with Agrobacterium suspension at several concentrations (OD600-0.1, -0.05, -0.02) for 10 min. After blotting dry with sterile filter paper, explants were transferred to MS-2 co-cultivation medium supplemented with acetosyringone and incubated in the dark for 2 or 3 days at 24 °C. Explants were then transferred to MS-4 regeneration medium without selection for 0–12 days, followed by transfer to MS-6 selection regeneration medium supplemented with 20 mg/l kanamycin (Km) (Sigma, St. Louis, MO). Four to five weeks after selection, putative transgenic green shoots that developed were excised and transferred to MS-8 medium supplemented with 30 mg/l Km for root induction. Cauliflower plantlets that formed visible roots in selection medium and confirmed by molecular analyses were transplanted to soil and grown in a growth chamber (21 °C).

2.3. Viral antigens expression cassettes

Agrobacterium tumefaciens strain LBA4404 was used in all experiments and grown overnight in LB medium supplemented with appropriate antibiotics at 28 °C. It was applied in several dilutions for plant transformation.

A binary vector harboring an expression cassette of vaccinia virus extracellular enveloped glycoprotein B5 driven by the CaMV-35S promoter was engineered using the pB002 vector [44]. The expression cassette comprises the full-size 255-amino acids (aa) antigenic region without the N-terminal 20-aa signal peptide and the C-terminal transmembrane domain containing C-terminal tags (c-myc-[ISEEDL]) for possible purification needs [45]. The vector also contained the expression cassette for the bar gene selection of transgenic plants in PPT supplemented medium. Plasmid pB002-37B5 was introduced into A. tumefaciens for use in collard transformation.

The pBI-based binary vector pE1801-79SHDEL [18] contains the expression cassette for the N-terminal fragment of human SARS coronavirus spike 700-aa glycoprotein with major antigenic epitopes, plant-derived 23-aa [MIMASSKLL-SLALFALLSHANS], signal peptide, and a histidine tag [RGSHHHHHHH] at the N-terminus, resulting in a 79-kDa polypeptide with addition of the plant-specific endoplasmic reticulum retention signal [HDEL] under the synthetic OCS3MAS promoter (kindly provided by S. Gelvin, Purdue University, West Lafayette, IN). The vector also contained the nptII gene for Km selection of transgenic plants. The plasmid was introduced into A. tumefaciens and used for cauliflower plant transformation.

2.4. PCR analyses of transgenic plants

PCR of genomic DNA isolated from leaf tissues by REDExtract-N-Amp Plant PCR Kit (Sigma, St. Louis, MO) was used to confirm the presence of foreign gene material in transgenic plants essentially as described [18]. The presence of vaccinia virus-specific B5 genetic material in transgenic collard plants was detected with the gene-specific primers [5'-tgt-act-gta-ccc-act-atg-aat-aac-g-3' and 5'-atg-ata-agt-tgc-ttc-taa-cga-ttc-3'] with the expected size product of 777 bp. For detection of SARS-CoV S-protein gene in transgenic cauliflower, the gene-specific primers [5'-atg-gac-tca-ctg-gta-ctg-gtg-tgt-taa-cctc-ctt-c-3' and 5'-aca-tgc-tca-gct-ctc-cct-ata-aga-cag-cct-gct-tg-3'] were used to yield the expected 338-bp product.

2.5. Western blot analysis of transgenic plants

Protein extracts were prepared essentially as described [18] and resolved by 4–20% gradient SDS-polyacrylamide gel electrophoresis. After electro-blotting, viral-specific antigen was detected in transgenic collard, cauliflower and tobacco plants (as control) with B5-specific mouse antibody MAB-206C5-F12 at 1/1000 dilution (obtained from Dr. S. Isaacs, University of Penn, Philadelphia, PA) or SARS S-protein-specific Sn + Sm rabbit antibodies (cat# AP600b and cat# AP600a) (Abgent, San Diego, CA) at 1/1000 dilution. Secondary detection was done using the corresponding horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (1/10,000 dilution).

3. Results

3.1. Selection of collard explant material for optimal regeneration efficiency

Preliminary experiments using different media compositions and types of explants indicated that shoots from several commercial collard cultivars regenerated most efficiently on MS medium supplemented with the hormones zeatin, BAP, and...
NAA, with addition of 20 μM silver nitrate (MSR-1 medium; Table 1), and that cotyledon and hypocotyl explants had significantly better regeneration potential, compared to leaf and stem segments. During 5 weeks 70–85% of cotyledons produced shoots in MSR-1 medium and regeneration occurred directly from surface tissues, without callus formation (Fig. 1A, right). Hypocotyls also showed an acceptable regeneration potential (30–40%); however, they also tended to form callus tissues which are unable to regenerate shoots (Fig. 1A, left). Comparison of 4-, 7- and 10-day-old explants demonstrated that regeneration capacity tended to decrease sharply with age (data not shown).

Comparison of regeneration capacity of three commercial collard cultivars (Georgia, Vates and Morris Heading revealed the highest regeneration efficiency in Morris Heading cotyledons and hypocotyls, reaching 85% and 40%, respectively (Fig. 2A)). Based on these data, we chose 4-day-old cotyledons and hypocotyls of cv Morris Heading for transformation experiments.

3.2. Agrobacterium-mediated transformation of collard

Preliminary transformation experiments revealed several problems associated with inoculation, co-cultivation and selection procedures. One of the most important was the necrosis of collard tissues after exposure to Agrobacterium, leading to low transformation efficiency. Indeed, the exposure of explants to overnight Agrobacterium culture at OD₆₀₀-0.5 caused severe necrosis in most of the treated collard explants and, in turn, a transformation efficiency of less than 1%; however, inoculation of explants with suspension diluted to OD₆₀₀-0.1 increased the overall transformation efficiency to 4–11% (Fig. 2B). In the same set of experiments, we have showed that 2 days of co-cultivation led to higher transformation efficiency than after 3 days (Fig. 2B).

In an effort to further reduce necrosis of explants in response to agrobacteria and thus improve overall transformation efficiency we did the following: (i) pre-cultivating of cotyledon and hypocotyl explants in MSC induction medium for 2 or 4
days before inoculation with agrobacteria; and (ii) using a feeder layer of tobacco cells during co-cultivation. Both were unsuccessful and actually led to a decrease in transformation efficiency (Fig. 2C).

No transgenic plants were recovered when selection was started immediately after the co-cultivation. Therefore, we tested the effect of delay period, during which infected explants were kept in MS-3 non-selective medium supplemented with timentin for Agrobacterium elimination for 4, 8, or 12 days. Transgenic shoot regeneration from explants was highest (12%) when infected explants were left on MS-3 medium for 8 days before selection (Fig. 2D). A prolonged delay period (12 days) led to a higher percentage of regenerants but to lower transformation efficiency (6%) due to the larger number of escapes.

Collard transgenic plants were selected on medium supplemented with the selective agent PPT (Fig. 1B). Use of low concentrations of PPT (2 mg/l) for first selection (medium MS-5) revealed the survival of a high percentage of non-transgenic shoots. Thus, a second round of selection, root induction on selection medium MS-7, was carried in the presence of increased concentration of PPT (3 mg/l). Under these conditions, transgenic shoots developed roots (Fig. 1C, right panel), whereas non-transgenic collard shoots were unable to form roots on this selection medium and eventually died (Fig. 1C, left panel).

Green plants with developed roots were confirmed by PCR and transferred to soil and grown to maturity under greenhouse conditions (Fig. 3A). Transgenic 3–4-month-old Morris Heading plants were moved from the greenhouse (24 °C) to cold conditions (4 °C) for 1 month and then returned to 24 °C. Three to four weeks after cold treatment, collard plants started to flower (Fig. 3E).

Together, the data indicate a simple and efficient protocol for collard cv Morris Heading transformation with a final efficiency of 11%. Transgenic plants grew to maturity and produced T1 seeds.

3.3. Optimization of transformation parameters for cauliflower

The transformation procedure developed for collard was found to be suitable for production of transgenic cauliflower. Three commercial cauliflower cultivars were tested in experiments for regeneration potential similar to those described for collard. Explants from cv Early Snowball showed the best regeneration potential. Here we describe only the adjustments made to the original procedure that allowed generation of transgenic cauliflower plants.

Explants from cauliflower showed better regeneration capacity (~80%) when excised from 4-day-old seedlings and placed on MSR-2 medium with decreased concentrations of NAA compared to those in MSR-1 (see Table 1). However, cauliflower explants at this age were still tiny, fragile and extremely sensitive to Agrobacterium exposure, developing a strong necrosis response. Therefore explants excised from 7-day-old seedlings were used. These were more robust and still retained a good regeneration capacity. The concentration of the Agrobacterium inoculum was also decreased to OD600-0.02. A 2-day pre-cultivation step was found to be important for
cauliflower and an extended delay period of at least 10 days (as compared to 8 days for collard) was required for development of regenerable compact green tissues. With these few changes, the overall explant survivability and subsequent transformation/regeneration efficiency was increased for this recalcitrant species.

Kanamycin was used as the selection agent. The Km concentration for primary shoot formation was established at 20 mg/l, where some of the non-transgenic shoots were still able to survive. Thus, for root induction selection medium, the Km concentration was increased to 30 mg/l. Plants that developed roots under these conditions were confirmed by PCR and transferred to soil until development of a head (Fig. 4A). Together the data indicate that the procedure developed for collard with a few modifications was effective for cauliflower cv Early Snowball with transformation efficiency 2.4%.

### 3.4. Production of recombinant subunit vaccines in transgenic plants

#### 3.4.1. Transgenic collard plants expressing viral coat protein B5 (smallpox antigen)

Constitutive expression of Smallpox antigen in transgenic collard was achieved by stable *Agrobacterium*-mediated transformation of cv Morris Heading with a binary vector carrying an expression cassette with B5 gene driven by CaMV-35S promoter (see Section 2). Almost all putative transgenic lines that produced roots on MS-7 medium containing phosphinotricin (Fig. 3A) were confirmed by PCR for the presence of antigen-specific DNA (examples are shown in Fig. 3B, lanes 1–4), whereas non-transgenic wild-type (wt) collard revealed none. Leaf tissues of transgenic lines were tested for level of expression of Smallpox antigen. Western blot analysis with antigen-specific antibodies revealed a single
protein band of the expected molecular size in the leaf tissue of transgenic plants (shown for the best expressing line in Fig. 3C and D). These plants had no visible morphological changes as compared to a non-transgenic wild-type plant of the same age (Fig. 3C). Once in soil, plants produced large green leafy biomass amounts with the total weight of fresh tissue of more than 1 kg, and after 3 months reached a height of 50 cm and rosette diameter of 60 cm. Upper, medium and lower leaves were tested and confirmed for the presence of the antigen (data not shown).

3.4.2. Transgenic cauliflower plants expressing viral spike protein (SARS antigen)

Expression of the 79-kDa fragment of the SARS-CoV spike protein in transgenic cauliflower was achieved using a binary vector with nptII gene for selection of transgenic plants on kanamycin-containing medium [18]. Km-resistant putative transgenic shoots were generated within 5–6 weeks in MS-6 medium and placed in the MS-8 selection medium with increased Km content for root induction (Fig. 4A). Rooted KmR plants tested by PCR analysis revealed the antigen-specific product of correct 338-bp size in genomic DNA of transgenic plants and control plasmid DNA (Fig. 4B, lanes 1–4 and /+/ lane, respectively). This product was not present in the DNA sample of non-transformed wild-type plants (Fig. 4B). PCR-positive transgenic plants were placed in soil to grow and form heads. Western blot analysis using SARS antigen-specific Sn + Sm antibodies [18] revealed the antigen-specific band of expected molecular weight in florets of several cauliflower transgenic lines and not in the wild-type floret sample (Fig. 4D, right panel, arrowhead). A second specific band of lower molecular weight was present in all transgenic floret samples (Fig. 4D, right panel, double-arrowhead). In control experiments, only one band was detected in the leaf tissue of transgenic tobacco plants transformed with the same construct (Fig. 4D, left panel, lanes 1 and 2). A protein product of almost the same size was detected in E. coli extracts with induction of the same expression cassette (Fig. 4D, left panel, lanes +). Some transgenic cauliflower plants showed slight inhibition of growth as compared to non-transformed plants. SARS-CoV S1 antigen was easily detected in transgenic floret samples stored for as long as 5 months at −80 °C.

4. Discussion

Accumulated data, including our own, have clearly demonstrated the feasibility of recombinant protein expression in plants. Most of the recombinant subunit vaccine components are of viral origin. However over-expression of large full-size viral antigens, such as rabies G-protein or SARS spike protein, appears to perturb the normal growth and development of the plants (our unpublished data). That is probably due to the induction of natural plant defense mechanism against some viral pathogens [46–48]. Our goal is to overcome this problem and develop plant expression system that is capable to produce large amounts of viral antigens.

Toward this goal, we consider the following steps. First, the expression cassettes used must allow the appropriate intracellular localization, folding and posttranslational modifications of the recombinant antigens, making them suitable for abundant accumulation in targeted plant cells/tissues as well as being immunologically functional. Second, the suppression of plant defense mechanisms can be examined using readily available Arabidopsis knockout/mutant plant lines with the capability for sustained high-level expression and accumulation of viral glycoproteins. Finally, for production purposes we are developing crop plants of the same Cruciferae family as the model Arabidopsis plant into an efficient transformation-production system (Fig. 5). Our criteria for choosing these crops include: easy scalability for production, overall consumption safety, large production biomass, long-term storage capacity, and ease of processing and delivery. It is expected that results obtained for Arabidopsis are also likely in the related plants. These steps, serve to identify a strategy for the generation of a unique plant-based system for production of large recombinant viral proteins, i.e. components of subunit vaccines, in amounts sufficient for immunization of humans and/or animals preferably via the oral and possibly via other mucosal routes [6,15,18,28].

Fig. 5. Schematic diagram of Cruciferae-based plant system for production of pharmaceutical proteins. Transgenic plants for three Cruciferae species (Arabidopsis, collard and cauliflower) are currently used for testing and production purposes (green circles). Transformation procedures for two other cruciferous vegetables (cabbage and broccoli) are currently under development (gray circles). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)
Here we focused on developing an efficient transformation procedure for production of transgenic vegetables from the *Cruciferae* family (collard and cauliflower). Transformation conditions were optimized by altering multiple parameters, such as type of explant, concentration of *Agrobacterium* inoculum, duration of co-cultivation period, and selection/regeneration schemas.

As a first step, we developed an efficient regeneration system for commercial collar and cauliflower cultivars, based on MS medium supplemented with hormones BAP, zeatin, NAA and for commercial collard and cauliflower cultivars, based on MS regeneration schemas.

Inoculum, duration of co-cultivation period, and selection/Agrobacterium conditions were optimized by altering multiple parameters, *Cruciferae* procedure for production of transgenic vegetables from the *Agrobacterium* culture used for inoculation was very important. Dilution of *Agrobacterium* led to a significantly reduced necrosis in explant tissues. A pre-cultivation period proved useful only for cauliflower, consistent with previous studies indicating positive results with pre-cultivation for other *Cruciferous* species [41,49]. For both collar and cauliflower we were not able to regenerate transgenic shoots when selection was carried out immediately after co-cultivation. Therefore, we applied the combination of a delay period followed by low selection pressure in regeneration medium and increased selection pressure in root induction medium. This schema has proven to be very efficient and allow for fast generation of transgenic plants with total elimination of large number of escapes. This approach was efficient for both selection agents used (Km and PPT). Altogether, optimized selection and regeneration procedures for collar and cauliflower yield transgenic plants in a relatively short time of 8–10 weeks from the beginning of the experiment until transfer of transgenic plants to soil. Overall transformation efficiency of collar cv Morris Heading was very high (11%). For cauliflower cv Early Snowball transformation efficiency was 2.4% that is comparable with the one obtained for this recalcitrant species by other authors [reviewed in 41].

For collar transformation, we used the binary vector carrying expression cassette of smallpox antigen vaccinia virus B5 coat protein driven by a strong CaMV-35S promoter. The 37 kDa B5 extracellular envelope protein is required for formation of infectious virus and for the cell-to-cell and long-range dissemination of the virus *in vitro* and *in vivo*. Transgenic collar plants expressing this antigen showed no morphological changes or anomalies compared to control plants. Even the highest expressing line (B5*) had the same growth characteristics as control plants. High and stable expression levels of smallpox antigen were obtained through all stages of collar development in leafy tissues. The expression levels of this antigen in collard were not decreased after several months’ growth in greenhouse conditions.

In cauliflower, we used a construct containing a SARS-CoV S1 gene driven by the strong Ocs3, Mas promoter. Presently the SARS-CoV S protein and its truncated versions are considered the best candidates for generation of a recombinant vaccine against this disease. Our recent study describes the successful constitutive expression of SARS S1 antigen in *Solanaceae* plants [18]. In this study, the vaccine candidate against SARS was expressed in transgenic cauliflower. Apparently, the Ocs3, Mas promoter worked well in florets, though we observed some deterioration (second band of lower molecular weight) of this particular protein that was not observed in tobacco plants transformed with the same construct.

To our knowledge, this is the first report of a successful transformation of collar plants. This is also the first report on production of pharmaceutical proteins in cruciferous transgenic vegetables collar and cauliflower. This work opens new possibilities to use these species in transgenic biotechnology.

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