TITLE:

Neutralizing antibodies against rotavirus produced in transgenically labelled purple tomatoes.

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Edible fruits are inexpensive biofactories for human health promoting molecules that can be ingested as crude extracts or partially purified formulations. We show here the production of a model human antibody for passive protection against the enteric pathogen rotavirus in transgenically labelled tomato fruits. Transgenic tomato plants expressing a recombinant human immunoglobulin A (hIgA_2A1) selected against the VP8* peptide of rotavirus SA11 strain were obtained. The amount of hIgA_2A1 protein reached 3.6 ± 0.8% of the total soluble protein (TSP) in the fruit of the transformed plants. Minimally-processed fruit-derived products suitable for oral intake showed anti-VP8* binding activity and strongly inhibited virus infection in an *in vitro* virus neutralization assay. In order to make tomatoes expressing hIgA_2A1 easily distinguishable from wild type tomatoes, lines expressing hIgA_2A1 transgenes were sexually crossed with a transgenic tomato line expressing the genes encoding *Antirrhinum majus Rosea1* and *Delila* transcription factors, which confer purple colour to the fruit. Consequently, transgenically labelled purple tomato fruits expressing hIgA_2A1 have been developed. The resulting purple-coloured extracts from these fruits contain high levels of recombinant anti-rotavirus neutralizing human IgA in combination with increased amounts of health-promoting anthocyanins.

Introduction

Fleshy fruit have been traditionally exploited as natural biofactories for orally-active, health-promoting metabolites (Cipollini and Levey, 1997). Recently, they have also been envisioned as an excellent vehicle for nutritional enhancement and considerable efforts have been made to enrich their content of indigenous and/or exotic human health-promoting compounds such as carotenoids, folate, flavonols or anthocyanins through molecular breeding and metabolic engineering (Apel and Bock, 2009; Bovy et al., 2002; Bovy et al., 2007; Butelli et al., 2008; Davuluri et al., 2005; Giuliano et al., 2008). Unlike other plant-derived products, edible fruit and their derivatives are considered safe and palatable for human consumption in unheated, minimally processed forms, and their content of anti-nutrients and toxic compounds sharply decreases upon ripening. This feature makes edible fruits ideal biofactories for exotic heat-labile compounds with mucosal activity such as health-promoting metabolites and mucosal active proteins like oral vaccines, immune modulators, biocides and/or antibodies for artificial passive immunotherapy.

Transgenic crops have been proposed as advantageous platforms for recombinant antibody production due to their productivity, scalability, and the low risk of contamination with mammalian pathogens (De Muynck et al., 2010). The technical feasibility of an artificial passive immunization approach based on a plant-made antibody was first demonstrated by the pioneering work of Ma and colleagues who expressed in tobacco a hybrid murine/human secretory immunoglobulin G/A (Guy´s 13 sIgG/A) which is effective against tooth decay (Ma et al., 1995; Ma et al., 1994; Ma et al., 1998). The Guy´s 13 monoclonal antibody produced in tobacco provided effective protection against *Streptococcus mutans* infections in healthy

volunteers. The technology to express Guy's 13 sIgG-A in plants was licensed by Planet Biotechnology Inc (USA). This was the first plant-made antibody that was evaluated in phase I and II clinical trials (Weintraub et al., 2005) as CaroRx™. In the United States, CaroRx™ is an Investigational New Drug and in the European Union, it is a registered Medical Device. More recently, the anti-HIV monoclonal IgG 2G12 was successfully produced in corn with the aim of conferring passive protection of vaginal mucosa against HIV infection (Rademacher et al., 2008). Plant-derived IgG 2G12 was shown to be effective in viral inhibition assays and has become a promising cost-effective source for vaginal gel formulations (Ramessar et al., 2008).

Antibody purification is still a major component of the total cost of goods in plant-made recombinant antibody production (Hood et al., 2002). Although biosafety of any therapeutic/prophylactic approach involving complex botanical mixes should be exhaustively investigated, it is expected that minimally processed extracts from antibody-producing edible fruits can be used as safe formulations for mucosal delivery, therefore reducing downstream processing costs. Tomato fruits have been studied as successful production platforms for recombinant oral vaccines (Walmsley et al., 2003; Zhang et al., 2006b). However, issues such as the capacity of fleshy fruits to accumulate functional antibodies, the evolution of antibody activity throughout the process of fruit ripening and the formulations that retain neutralizing activity have not been addressed before. To address these issues, we have assessed the recombinant production in tomato fruit of a phage display-derived human IgA antibody that is effective against the model rotavirus strain SA11, and tested the ability of minimally processed fruit-derived products to neutralize rotavirus infection *in vitro*.

Besides antibody activity and formulation, an important concern in using edible fruits to produce recombinant pharmaceuticals stems from the possibility that the fruit expressing pharmaceuticals are mixed with fruit used for food consumption. To address this issue, it has been recommended that transgenic fruit should be labelled with distinctive features that help to preserve their identity, facilitate traceability and avoid contamination of the food supply. As a

proof of this concept, we engineered purple, IgA-producing tomatoes, where *Antirrhinum majus Rosea1* and *Delila* transcription factors, known to ectopically activate anthocyanin biosynthesis in tomato fruits (Butelli et al., 2008), were incorporated into the transgenic IgA background by sexual crossing to obtain tomato-made antibodies in transgenically labelled fruit and fruit derived products.

Results

Design and selection of human IgA genes for expression in tomato fruits

Antibody repertoires displayed in phage format provide a flexible and adaptable source of neutralizing antibody fragments that can later be transferred to plant expression systems, either as antibody fragments or as full-length antibodies (Wieland et al., 2006). An important limitation of this approach is the significant heterogeneity of expression levels in the plant, apparently due to differences in stability conferred by the antibody variable regions, which might be exacerbated by the use of synthetic or semi-synthetic libraries. A possible solution to this is the introduction of a quick selection step for *in planta* stability using transient expression analysis, preferably performed in the same plant organ used for the production of the compound. Based on this rationale, we designed a set of shuttle vectors to facilitate the transfer of the coding sequences for V regions from a phagemid library into plant expression vectors, and then took advantage of a fruit transient expression assay (Orzaez et al., 2006) to test the expression levels of a subset of the plant produced antibodies against the model rotavirus strain SA11.

DNA fragments which encoded single chain antibody fragments (scFv) that had been selected for their recognition of the VP8* peptide of rotavirus VP4 protein were inserted into plant expression cassettes carrying the constant regions of human IgA (Supplementary Figure 1a, b). To gain versatility in the cloning strategy, a Gateway-based set-up was established. Two

Gateway pENTR vectors (pENTR_IgH and pENTR_IgL) were generated containing the constant regions of IgHα1 and Igλ respectively. Convenient restriction sites were engineered at the 5´ ends of each constant region to allow for the direct cloning of VH and VL derived from the Griffin.1 phage display library (Supplementary Figure 1a). The variable regions from three different anti-VP8* scFv (namely 2A1, 2E4 and 2B3) were introduced into the pENTR vectors and transferred to the binary vector pKGW7 through site-specific LR recombination. As a result, plant expression constructs were generated containing either the heavy or the light chain of a fully reconstituted IgA antibody under the control of the constitutive 35S promoter.

The *in planta* expression of each reconstituted antibody was initially tested using an *Agrobacterium*-mediated transient expression assay. Plant expression vectors carrying the genes encoding the heavy and light chains for each antibody to be analyzed were transferred to *Agrobacterium* and infiltrated into tomato fruits. The high co-transformation efficiency provided by the fruit agroinjection approach allowed for facile monitoring of the formation of full-length IgA in fruit cells. As shown in Figure 1a, only the variable regions derived from scFv 2A1 yielded co-expression of heavy chain (HC) and light chain (LC) proteins *in planta* as detected in western analysis under non-reducing conditions. When IgA_2E4 and IgA_2B3 were transiently co-transformed, only light chains were detected, whereas their respective heavy chains remained undetectable. A 35SCaMV:GFP construct was included in all transformations and used to normalize the transformation efficiency in the samples. Transient expression analysis using KDEL-tagged versions of the IgA_2A1 heavy and light chains demonstrated that ER retention resulted in higher antibody yields (Supplementary Figure 2). The transient expression data encouraged us to continue with the stable plant transformation of the IgA_2A1 idiotype using HC and LC fused to a KDEL peptide as an ER-retention signal. For this purpose, an *in trans* co-transformation strategy was followed (referred as 35S_IgA): tomato explants were simultaneously co-inoculated with two *Agrobacterium* cultures carrying 35S:IgH2A1-kdel and 35S:IgL2A1-kdel expression cassettes respectively.

In addition to the above mentioned *in trans* co-transformation experiment, an *in cis* co-transformation approach was also carried out (referred as NH_IgA). In this case, in order to prevent duplication of the 35S promoter, LC was placed under the control of the tomato NH promoter. The NH promoter drives high levels of gene expression predominantly in fruits at mature-green stage (Estornell et al., 2009). Therefore a dual construct was made (as depicted in Supplementary Figure 1b) carrying 35S:IgH2A1-kdel and NH:IgL2A1-kdel expression cassettes located within the same T-DNA.

Transgenic fruits accumulate high levels of mAb

Primary transformants from *cis* and *trans* co-transformation approaches were selected by kanamycin resistance and the expression of HC and LC was confirmed by western blots. IgA expression levels in T1 generation were evaluated by ELISA and the best performing lines were self-pollinated up to T3 for high antibody titers and homozygocity. The description of two selected T3 lines per construct (NH_1A-13-2 and NH_III6-2-6 from the NH_IgA approach and 35S_20A7 and 35S_17A4 from the 35S_IgA approach) is presented here.

Western analysis of NH_IgA lines showed low IgA levels in leaves when compared with fruits (Figure 1b). Interestingly, when samples were analyzed under reducing conditions, it was found that not only LC but also HC was nearly absent in leaves, suggesting that HC does not accumulate at significant levels in the absence of its cognate LC. Notably, a significant portion of the total antibody content in NH_IgA samples under non-reducing conditions was present in the form of a 45 kDa band, which reacted both with anti-HC and anti-LC antibodies. Furthermore, reducing conditions clearly showed a 25 kDa degradation band that was recognized by anti-HC antibody, while the LC remained intact. Together, these observations indicate that a significant proportion of total IgA was present in the form of Fab' fragments, probably as a result of the cleavage of the full IgA perhaps in the hinge region. IgA production

levels were quantified by ELISA in fruits of the NH_1A-13-2 homozygous line, reaching 15.6 ± 1.2 µg IgA equivalents /g FW (1.1 ± 0.1% TSP) (Table 1).

The *in trans* co-transformation strategy (35S_IgA) resulted in 20% (4 out of 20) of the T1 kanamycin-resistant plants showing both HC and LC expression in vegetative tissues. Following the same mini-breeding strategy as for the NH_IgA plants, lines 35S_20A7 and 35S_17A4 were selected for further characterization. In contrast to the NH_IgA lines, 35S_IgA plants showed similar expression levels in fruits and vegetative tissues by western blot analysis, when gel loads were equalized per total protein content in the sample (Figure 2b). As in the NH_IgA approach, a significant part of the total antibody content in leaves and fruits of the 35S_IgA plants was present in the form of Fab' fragments, resulting from the association between partially degraded HC (Figure 2b, R/HC panel) and intact LC (R/LC panel). In absolute terms, 35S_IgA approach resulted in higher levels of recombinant protein production, reaching 41.2 ± 2.8 µg of IgA equivalents/g FW in fruits of elite T3 line 35S_20A7, equivalent to 3.6 ± 0,8% of TSP (table 1). This remarkable antibody accumulation is partially due to the low total protein content in fruits. By comparison, IgA levels of up to 90 µg IgA/g FW were achieved in leaves of the same elite plant, representing only 1% of TSP in those organs. Accumulation of IgA in fruits is illustrated in Figure 1c, where the full-size LC and the HC fragments are clearly observable as 25 kDa bands in Coomassie stained gels, while the full-size HC band remains masked by the co-migrating 55 kDa bands in crude fruit extracts.

Anti-VP8* activity is maintained in late ripening fruits in the form of Fab' fragments

The important physiological changes taking place during fruit ripening are likely to affect the accumulation of recombinant antibodies in the fruit. To learn about the effect of ripening on antibody accumulation, activity and stability, 35S_20A7 and NH_1A-13-2 tomatoes at different ripening stages (mature green, breaker, red and red ripe) were analysed by antigen specific-ELISA to assess the specific VP8* binding activity, and by western blot to check for

antibody integrity. In addition, red ripened tomatoes which had been air-dried at room temperature during one month were also analysed (dried tomato samples).

As shown in Figure 2a, the anti-VP8* activity, expressed relative to fruit fresh weight, declines stepwise during ripening, with mature green tomatoes showing maximum activity. This decline is stronger in NH_1A-13-2 than in 35S_20A7 tomatoes. Remarkably, 35S_20A7 fresh red ripened tomatoes, and notably dried tomatoes, still maintain 1/3 of the maximum activity. Ripening-associated loss of anti-VP8* activity in 35S_20A7 tomatoes apparently proceeds at slower pace than the overall decrease in protein content associated with normal ripening, as anti-VP8* specific activity (referred to total protein content) is maintained even in the late ripening stages (Figure 2a). When these samples were analysed by western blot (Figure 2b), it became clear that a significant fraction of the full-length IgA is converted into Fab' fragments during late ripening. In contrast to full length IgA, Fab' seems highly resistant to further degradation as it is highlighted by the strong Fab' band observed in 35S_20A7 tomatoes at the late ripe stage (Figure 2b, lanes RR and D).

Minimally-processed tomato-based products show strong anti-VP8* activity

The anti-VP8* binding activity of fruit-derived extracts was determined using fruits from the elite line 35S_20A7. The assays used antigen-ELISA plates coated with VP8* or with BSA as a control. In order to test the resistance of the antibody activity to downstream processing, three tomato formulations compatible with long-term conservation and oral delivery were assayed, referred to as "juice", "powder" and "dried juice". "Juice" consisted in a clarified extract from frozen fruit samples ground in liquid nitrogen with PBS (1:3) (w:v). The "powder" format consisted in freeze-dried ground tomato powder, later reconstituted in PBS using 3 volumes of the original fresh weight. "Dried juice" consisted in freeze-dried "juice" sample, later reconstituted in water using 3 volumes of the original fresh weight. All samples were clarified by centrifugation and incubated in serial dilutions in an antigen-ELISA test. Control

"juice", "powder" and "dried juice" samples from wild type Moneymaker fruits also were prepared as described above. For all three products, a strong anti-VP8* binding activity was observed, which was not detected in formulations prepared from wild type samples or with control assays that used BSA. Antibody binding titers showed consistent behaviour, as all three samples reached endpoint titers at 1/512 dilutions (w/v) (Figure 3). The full conservation of antibody binding activity in "powder" and "dried juice" formats is particularly promising for oral delivery, as dried formulations also ensure an increase in antibody dosage. In the case of 35S_20A7 tomatoes, because dry weight represents 6% of the total fresh weight, the recombinant antibody levels in the "powder" samples reach remarkable levels up to 0.68 mg of IgA equivalents per gram of dry weight (Table1).

Minimally processed fruit samples show strong rotavirus neutralization activity

Once the VP8* binding activity of the IgA_2A produced in the fruit was determined, the next steps consisted of (i) testing the rotavirus neutralizing activity of the recombinant antibody expressed in fruit, and (ii) evaluating the possible effect of the context conferred by the remaining components of the tomato extract in the neutralization capacity. Affinity purified recombinant IgA was obtained from 35S_20A7 mature green fruits using SSL7-agarose chromatography columns. The SSL7 protein specifically binds the hydrophobic interface between the Cα2 and Cα3 domains of the Fc portion of the HC. The purification steps were monitored by antigen-ELISA and western blot, and the presence of contaminant proteins in the final elution was tested with silver-stained gels. Single-step affinity purification with SSL7-agarose resulted in moderate 3.5 x enrichment in antibody activity with respect to the crude starting extracts (Figure 4a). A significant part of the total activity was detected in the flow-through fraction. This is probably because the Fab' fragments do not bind SSL7 due to the lack of Fc domain, as observed in the western blot analysis in Figure 4b. Silver-stained PAGE of

eluted fractions in Figure 4b illustrate the level of purification of the full-size recombinant IgA obtained after a single-step affinity purification with the SSL7 agarose.

Neutralization activity against SA11 rotavirus was determined by means of immunofluorescence assays on MA104 cell monolayers. The assays were performed by a previous incubation of rotavirus with serial dilutions of the IgA samples, followed by the infection of the formed monolayer with the resulting mixture. In a first assay, serial dilutions of the SSL7-purified IgA were compared with samples treated with PBS as mock treatment controls. The neutralization capacity of the sample is represented in Figure 5a as the percentage of foci reduction with respect to the number of foci produced by the control treatment. IgA dilution series started with 30 μg/mL, with minimum significant titers (>60%) obtained with 3.75 μg/mL of IgA, and a calculated IC_{50} value of 2.5 μg/mL.

Once the neutralization capacity of the recombinant IgA produced in tomato fruit was demonstrated, different assays were done using different minimally-processed tomato fruit extracts, all of which were suitable candidates for therapeutic delivery. For this purpose, the IgA content of "juice" and "powder" samples from the 35S_20A7 tomatoes was estimated by ELISA. Serial dilutions of the same samples were assayed for rotavirus neutralization along with equivalent "juice" and "powder" samples from wild type tomatoes as reference controls. Both "juice" and "powder" samples of IgA fruits showed similar neutralization curves, with IC_{50} values of 1.8 and 2.0 μg/mL respectively (Figure 5b). In these samples, neutralization titers >60% were obtained with 3.7 μg/mL of IgA equivalents. This approximately corresponds to a 1/12 dilution of the original IgA content in fresh fruits and 1/192 (w:w) of the IgA abundance in the fruit powder. Although IC_{50} values of purified and un-purified IgA samples are very similar, it is interesting to notice that "powder" and to a lesser extent "juice" samples, when employed at low dilutions (equivalent to 7.5 and 15 μg IgA/mL) are more effective in foci reduction than

equivalent concentrations of purified IgA (Figure 5a). An image of the effect of tomato IgA on the reduction of rotavirus infective foci is shown in Figure 5c.

Rosea1 and *Delila* transgenes can be used to confer identity preservation to IgA-expressing tomatoes

Recombinant biofactory strategies involving food crops may benefit from the introduction of labelling traits. Recently, transgenic *Del/Ros1* MicroTom tomato fruits with an intense purple colour have been described (Butelli et al., 2008). Several traditionally red tomato cultivars are known to display a bronze coloration due to accumulation of anthocyanins in the peel. In contrast, *Del/Ros1* accumulates large amounts of anthocyanins not only in the peel of the fruit, but also in the pericarp and jelly tissues. This feature makes *Del/Ros1* tomatoes ideal for transgenic labelling and makes traceability easier for both fruit biofactories and their derived "juice" and "powder" products.

Del/Ros1 genotype was initially introduced into the dwarf, cherry tomato MicroTom cultivar. However, large globe-type tomatoes are preferable in fruit biofactory strategies to maximize yields of fruit material. With this goal in mind, purple-fruited MicroTom *Del/Ros1* plants (kindly provided by Prof. Martin) were crossed with globe-type wild type Moneymaker plants. The offspring showing combined globe-type and purple phenotypes were single-seed selected up to the F7 generation. A plant from the F7 generation was sexually crossed with a T1 35S_IgA expressing line (35S_3), and the offspring of this cross were screened in an antigen-ELISA for IgA expression. Eight out of thirteen plants were found to be IgA positive and were grown to maturity (referred as CR lines). All of them resulted in a purple-coloured fruit phenotype. Moreover, this colour trait was maintained during fruit processing steps, as "juice" and "powder" samples showed a clearly distinguishable purple colour, in contrast with the light red colour of wild type derived products (Figure 6a). Fruits from the F1 generation CR lines were then tested for IgA expression, showing maximum antibody levels of 6.2 µg/g FW (0.8%

TSP). CR lines were then self-pollinated to F3 generation. As a result, an elite F3 plant with purple fruit, called CR2-3-8, showed maximum IgA expression levels of 33.5 ± 4.2 µg IgA/g FW, equivalent to 1.9 ± 0.3% TSP, which is a level comparable to that found in the red fruited T3 35S_20A7 line (shown in Table 1). Endpoint ELISA titers of tomato powder were also conducted, showing titering curves consistent with the estimated IgA levels and confirming the conservation of the biological activity of the IgA expressed in the purple fruit (Figure 6B). Finally, the neutralization activity of IgA powder from purple fruit was confirmed in VN assays (Figure 6C). In these assays, disruption of the cell monolayer was observed when samples derived from purple powder were incubated at low dilutions (up to 1:12 w:v, not shown). To dissect any possible effect of the anthocyanin background on the cell monolayer, VN titers of the CR2-3-8 "powder" samples were performed at dilutions above the cell disruption threshold (1:24 w:v onwards) using an IgA-free sibling purple fruited line CR2-3-5 as a control. Purple IgA expressing tomato fruit samples also strongly neutralized rotavirus, with a calculated IC_{50} value of 1.98 µg/mL of IgA (Fig 6c), a value similar to that observed previously in the red tomato "powder".

Discussion

Diarrheas caused by enteric pathogens kill an estimated 2.5 million infants worldwide per year (Kosek et al., 2003), and 500.000 of the fatalities are caused by rotavirus alone (Parashar et al., 2003). Recently, two jennerian vaccines against rotavirus have been successfully launched. However challenges remain regarding the potential effectiveness of oral live vaccines in developing countries in view of the prevalence of competing intestinal flora in children, the occurrence of mixed infections, the high levels of maternally transmitted antibodies and general malnutrition. Recombinant production of rotavirus antigens in plants has been proposed as an alternative to traditional production platforms (Birch-Machin et al., 2004; Choi et al., 2005). In addition, passive oral immunization emerges as a complementary strategy,

as this approach has repeatedly been shown effective against enteric infections (Corthesy, 2002). In this context, the availability of an inexpensive source of neutralizing antibodies for mucosal protection might serve as a valuable complement to current vaccination strategies. This work shows that recombinant antibodies against the model rotavirus strain SA11 accumulate to considerable levels in tomato fruits and that neutralizing antibody preparations can be obtained from edible fruit tissues using extremely simple processing steps. Therefore, these results suggest that the production of antibodies in edible fruits can serve as an economically viable complement for combating enteric diseases, particularly in developing countries.

Oral delivery of minimally-processed products derived from edible plant organs has been successfully assayed both in active and passive immunization models. In active vaccination strategies, the context provided by the plant tissue has been eventually shown to positively influence protection. This might be related to the natural antigen encapsulation provided by plant cells and the adjuvant effect provided by some of the components of the plant extract. The alkaloid tomatine has been proposed as an adjuvant, potentiating the response observed when tomato-made norovirus VLPs were used for oral delivery (Zhang et al., 2006a). In another example, oral boosts with chloroplast-made plague fusion antigen F1-V without standard adjuvants but delivered as crude plant material, performed as well as adjuvated sub-cutanous boosts (Arlen et al., 2008). More recently, a chloroplast-based strategy using lettuce as production platform conferred dual protection against cholera and malaria in mice (Davoodi-Semiromi et al., 2010). In the veterinary field, oral passive protection using plant extracts has been sucessfully reported in pigs for protection against transmisible gastroenteritis virus (Monger et al., 2006) and in poultry for protection against coccidiosis (Zimmermann et al., 2009). In the latter case, pea-derived scFv against *Eimera* protozoa showed an enhanced prophylactic activity. It is likely that protease inhibitors present in pea seeds might contribute to the higher stability of the protein and, therefore, enhanced protection. Herein we show that preparations of IgA-expressing tomato fruit are at least as efficient as purified recombinant IgA

in neutralizing rotavirus infection, indicating that (i) neutralizing activity is not affected by processing and (ii) the Fab' fragments predominant in fruit tissues are efficient for virus neutralization. Moreover, the "juice" and especially the "powder" formulations seemed even more effective than purified IgA in preventing rotavirus infection. This could be explained by a protective role of the extract in the stability of the antibody, or by the contributors of additional components of the extract acting cooperatively with the antibody in neutralizing the infection. The effect of the edible plant extracts to enhance virus neutralization needs further investigation, as it may boost the effectiveness of passive protection strategies.

Tomato is a highly productive largely self-pollinated crop that is well adapted to green-house cultivation under the confined conditions required for molecular farming. Having low protein content per fresh weigh, fruits have sometimes been discarded as potential biofactories. However, as demonstrated here, once water is removed from the fruit by drying, the antibody production in fruit approaches highly competitive levels of gram per Kilogram of dry weight. Considering an average production of 6 Kg of tomato (FW) per plant, current IgA yields in fruits would lead to production levels reaching 0.5 grams of antibody (IgA + Fab's) per tomato plant, which can be administered as semi-crude formulations where the remaining components are Generally-Regarded-As-Safe. Moreover, downstream processing may benefit from "state of the art" tomato food/beverage processing technology. For instance liquid spry-drying, a process increasingly used in antibody formulation (Maa et al., 1998), is also employed in the food industry for the production of tomato powder (Santos de Sousa et al., 2008). Furthermore, the availability of single-step affinity purification systems as the SSL7-agarose described here offers an economically relevant tool to obtain partially-purified formulations with increased concentration and/or safety profile.

Zhou and co-workers (Zhou et al., 2008) reported an almost complete disappearance of HIV recombinant antigens upon ripening in transplastomic tomato fruits, and therefore

proposed the use of green-fruited tomato varieties for molecular farming. Ripening seems to affect the protein biosynthesis capacity in plastids more drastically than in the nucleus/cytoplasm. We found only a moderate decrease in activity levels of nuclear encoded antibody in ripened fruit. Moreover, the stability of Fab' fragments during ripening and post-harvest seems key in the maintenance of the neutralizing activity. The use of green-fruited tomatoes is nevertheless an interesting proposal also for nuclear-encoded transgenes, as it may contribute to further increases in yields. Further pyramiding of ripening-related genes could lead to new tomato varieties better adapted to molecular farming. For example, by disrupting ripening-associated disadvantageous traits (e.g. protease activity or chromoplast transition), while preserving others (e.g. reduction of anti-nutrients or responsiveness of engineered promoters), the deleterious effects of ripening on pharmacological production may be obviated.

IgA_2A1 made in tomato fruit was engineered for *in planta* production by inserting the variable regions of scFv_2A1 into the constant regions of human IgA, and placing the resulting antibody coding regions under the control of plant promoters. A single-chain 2A1 antibody fragment was previously isolated in a phage display screening against VP8* peptide, a rotavirus surface antigen known to induce neutralizing antibody responses (Higo-Moriguchi et al., 2004). It is generally accepted that broad-spectrum passive immunization against intestinal pathogens including rotavirus will benefit from polyclonal mixes of neutralizing antibodies. This could be achieved either by combining different monoclonal transgenic lines or by engineering single transgenic lines producing a combination of monoclonal antibodies. In both cases, phage display constitutes an interesting source of antigen-binding variable regions for *in planta* production. However, we and others (Ballester et al., 2010; Verma et al., 2010; Wieland et al., 2006) have repeatedly observed strong differences in the stability of different phage-display derived scFv, or scFv-reconstituted full-size antibodies when expressed *in planta.* Therefore, when using phage display as a source for variable antibody regions, it is important to identify stable antibody idiotypes before undertaking stable transformation. For this it is crucial to

establish (i) flexible tools for scFv grafting and (ii) fast *in planta* expression systems, preferably in the same tissues in which the final production is intended. In our lab we previously developed a transient expression system for tomato fruits known as fruit agroinjection, which allows facile construct-testing in fruit tissues such as placenta, gel and inner pericarp (Orzaez et al., 2006). In addition, we have adopted a grafting procedure based on restriction/ligation and Gateway recombination that facilitates the cloning of new variable regions into IgA constant regions. The need for an *in planta* selection step is illustrated in this work. We took advantage of the aforementioned tools to select among three anti-VP8* full-length antibodies, two of which had to be discarded prior to stable transformation as they showed very low levels of heavy chain accumulation.

In the final design of the recombinant antibody, a number of additional decisions were made. The endogenous signal peptide was substituted with the signal peptide of a pectate lyase expressed in fruit to ensure processing of the IgA protein in the fruit tissues. IgA was chosen as full antibody isotype, as it will facilitate, if required for increased efficiency, further conversion into secretory IgA (Crottet and Corthesy, 2001; Ma et al., 1995). Two transformation approaches were undertaken, involving *in trans* and *in cis* co-transformation, the latter using an additional fruit-operating NH promoter (Estornell et al., 2009). The NH_IgA approach resulted as expected, in very low expression in vegetative tissues compared with fruits. Fruit-specific expression may be appreciated as a biosafety measure, and may avoid the diversion of synthetic capacity of the crop towards unprofitable protein production in vegetative tissues. However, in this case, the *in trans* strategy employing the same 35S promoter for both HC and LC yielded higher IgA levels in fruits. The 35S promoter has been previously shown to operate at high levels in fruits during development and ripening (Estornell et al., 2009) and may have contributed to the high IgA expression levels in spite of a general decline in transcriptional activity that takes place during late ripening.

The high accumulation of ER-retained IgA_2A1 observed in transient fruit expression assays prompted us to use ER-retention in stable versions of IgA expression. This effect has been reported repeatedly for plant-made antibodies (Orzaez et al., 2009) and may be indicative of a lower level of protease activity in the ER milieu. ER-retention prevents complex glycosylation in the Golgi apparatus, particularly the addition of fucose and xylose plant-specific residues. It has been argued that the presence of immunogenic fucose and xylose in plant-made antibodies could lead to allergic reaction, and ER retention could be used as a preventive measure (Sriraman et al., 2004). However, it is unlikely that these residues are allergenic through the mucosal route given the continuous exposure of mucosa to plant glycosylation patterns in food. Also, in the design of hIgA_2A1 we incorporated the HCα1 coding region. IgA1 has an extended shape compared with IgA2, as a result of an elongated hinge region. It has been proposed that the IgA1 shape facilitates the cross linking of viral particles, and, therefore might provide specialized functions in the protection against viral pathogens (Bonner et al., 2009).

Edible plant organs provide clear advantages in particular molecular farming approaches. However the concerns about contamination of the food chain with engineered contents is often reasonably raised when pharmaceuticals are produced in food crops. We propose here the use of natural coloured plant compounds for labelling transgenic crops by means of metabolic engineering. A relatively small amount of otherwise health-promoting compounds such as anthocyanins, a natural component in many edible berries, is sufficient to label IgA-producing plants. These compounds confer an intense purple colour ensuring the traceability of transgenic fruits and their derivatives. F1 plants coming from the initial crossing of purple and IgA expressing plants showed considerably lower IgA levels than those measured in the elite T3 35S_IgA expressing lines. This raised the question of a possible dilution effect of the recombinant protein production in the anthocyanin background. However, when purple IgA plants were bred up to F3, a considerable increase of IgA content was observed, reaching levels

of 33.5 μg IgA/g FW, close to those reported for red IgA plants (41.2 μg/gFW). This indicates that the anthocyanin background has a minimal effect on the levels of IgA accumulation in fruits. Moreover, IgA from the transgenically labelled purple tomatoes also showed anti-VP8* binding and rotavirus neutralizing activities similar to those described for red tomatoes, again indicating that IgA activity is not affected by the presence of anthocyanins. Disruption of cell monolayers was observed in VN assays when samples derived from purple "powder" were incubated at low dilutions. It has previously been reported that some phenolic compounds may react with constituents of certain cell culture media (Long et al., 2010). These reactions can result in the oxidation of phenol to produce a range of toxic products such as H_2O_2. These effects can be regarded as artifactual as they result from the interaction with the culture media, and are not likely to take place *in vivo*. Moreover, when VN assays were performed at dilutions above the cell disruption threshold, the neutralizing effect of recombinant IgA was evident.

A further degree of safety can be achieved by genetically linking the antibody and the *Del/Ros1* modules (e.g. including both modules in the same T-DNA). The same synthetic biology rational can be used to include additional safety modules as male sterility as well as any other with relevance to antibody production as glycoengineering, decreased protease activity, etc. leading to the design of highly optimized platforms for molecular farming (Sarrion-Perdigones et al., 2011).

This is, to our knowledge, the first report showing viral neutralization activity of a plant produced antibody in minimally processed edible fruit extracts. The results support the need for a detailed investigation of the biosafety and *in vivo* effectiveness of fruit-based passive immunization. Moreover, we created the first example of a biofortified plant crop additionally displaying prophylactic activity. The model plant designed here is an example of how gene stacking, either by sexual crossing or by other means, can lead to new agricultural products with added-value and new applications. The introduction of four transgenes (HC, LC, *Ros1*, and *Del*)

in a single tomato plant resulted in a completely new product: a transgenically labelled, orally safe, inexpensive fruit juice with a health-promoting (antioxidant) activity (Butelli et al., 2008) and anti-rotavirus prophylactic potential.

Experimental procedures

DNA constructs and vectors

The DNA sequences corresponding to the constant regions of human alpha heavy (HCα) and lambda light (LCλ) antibody chains were obtained from the human library clones EHS1001-9024643 and EHS1001-9024145 respectively (Open Biosystems, Huntsville, USA). LCλ was PCR amplified in a nested reaction using D06mar01 and D06mar07 oligos as overlapping forward primers and D06mar08 as reverse primer. D06mar01 and D06mar07 overlapping primers were used to fuse to the 5′end of LCλ a number of elements, namely an attB1 recombination site, a Kozak sequence, the signal peptide (SP) from the tomato pectate lyase and a small polylinker containing ApaLI, and NotI restriction sites. In another construct, the D06mar08 primer incorporated a KDEL peptide, a stop codon and an attB2 recombination site in the 3′ end of the LCλ coding region (Supplementary Table 1). The resulting LCλ expression cassette, still lacking the sequence corresponding to the variable region of the light chain (VL) was BP-cloned in pDNOR221 vector, generating an ENTRY vector named as pENTR_LCλ. In a similar fashion, a pENTR_HCα vector was created for the convenient cloning of variable regions of the heavy chain into an IgCα frame. In this case, cloning of HCα was preceded by a step of directed mutagenesis designed to silently remove NcoI and XhoI sites from HCα coding sequence. For this purpose, HCα was pre-amplified in three contiguous fragments using oligonucleotide pairs D03Mar00/ D03Mar19, D06Mar03/D03Mar04 and D03mar20/D03Mar06 respectively, and subsequently joined by overlapping PCR. The resulting mutagenized HCα was re-amplified in a nested reaction with D06mar01 and D06mar02 as forward primers, which incorporated attB1, the pectate lyase SP and NcoI/XhoI cloning sites.

At the 3' end, the D06Mar06 primer incorporated a stop codon, the KDEL peptide and attB2. The resulting HCα expression cassette was BP cloned into pDONR221 vector yielding pENTR_HCα vector.

The sequences of the variable antibody regions reacting against VP8* were cloned from a previously described anti-VP8 scFv collection selected from the phage display human antibody library Griffin.1 (Monedero et al., 2004). The Griffin.1 library is a large naïve human scFv phagemid library (total diversity of 1.2×10^9) constructed from synthetic V-gene segments made by recloning the heavy-chain and light-chain variable regions from the lox library vectors (Griffiths et al., 1994) into the phagemid vector pHEN2. VL fragments (2A1, 2B3 and 2E4) were ApaLI/NotI digested and cloned into pENTRY_LCλ ApaLI/NotI sites, generating pENTR_IgL vectors. In parallel, VH fragments (2A1, 2B3 and 2E4) were NcoI/XhoI excised from Griffin.1 vector and cloned in the equivalent sites of pENTR_HCα, generating pENTR_IgHα vectors. To harbour the expression cassettes of the individual antibody chains, pBINJITGW and pKGW7.0 were used as destination vectors. pKGW7.0, containing 35S promoter and terminator was kindly provided by Dr Karimi (Karimi et al., 2002). pBINJGW was engineered by introducing a Gateway cassette (Invitrogen, Carlsbad, USA) in the SmaI site of pBINJIT plasmid, following manufacturers procedures. Binary expression vectors for heavy and light antibody chains were obtained by performing LR reactions between ENTRY vectors (e.g. pENTR_IgL_2A1, pENTR_IgHα_2A1) and binary destination vectors, generating plant antibody expression clones (e.g. pEXP_ 35SIgL_2A1 and pEXP_ 35SIgHα_2A1).

Heavy and light chains expression cassettes were also combined in a single binary vector under the constitutive 35S and the fruit-operating NH promoters respectively. For this purpose, the IgL_2A1 coding region was PCR-amplified using primers L07Nov07 and L07Nov08 containing attB4R and attB3R extensions and BP-recombined into pDONR221P4r-P3r (Invitrogen), generating pENTR4R3R_IgL2A1. A triple gateway recombination reaction

was then performed between vectors pENTR1-4_NH, pENTR3-2_Tnos, both belonging to the previously described pENFRUIT collection of combinatorial vectors (Estornell et al., 2009), and pENTR4r3r_IgL2A1, using pKGW.0 as a destination vector. In this way, an expression cassette was generated comprising the NH promoter fused to IgL-2A1 and the Tnos terminator. The whole 4.9 Kb expression cassette was PCR-amplified using primers L07Jun05 and L08Jan01, containing AscI and SalI recognition sites respectively. The resulting PCR fragment was digested with AscI/SalI and cloned into pEXP_35SIgHα_2A1, generating the binary plasmid pEXP_35SIgH_NHIgL, which combines heavy and light antibody chains under the control of two different promoters both operating in the tomato fruit.

Tomato transformation, plant material and sample preparation

Plasmids were transferred to *Agrobacterium tumefaciens* LBA4404 strain by electroporation and used for tomato stable transformation (var. Moneymaker), as described previously (Ellul et al., 2003). For transient expression, plant expression plasmids were transferred to *Agrobacterium tumefaciens* strain C58 and assayed transiently in MicroTom fruits, as described previously (Orzaez et al., 2006). For sample preparation, plant tissues (leaves and tomato fruit) were ground with a mortar and pestle to a fine powder under liquid nitrogen and stored at -80º until used. For "Juice" samples, proteins were extracted with three volumes (w/v) of phosphate-buffered saline (PBS) pH 7,4. After mixing, the suspension was centrifuged twice at 4º C at maximum speed and the supernatant was immediately used for further analysis. For "Dried Juice" samples, total protein was extracted in the same manner, followed by a 24 h freeze-drying step. The sample was reconstituted in three volumes of water (w/v) for analysis. The "Powder" samples were obtained from fine tissue powder after a 24 h freeze-drying treatment; total protein was extracted with three volumes of PBS pH 7,4 (w/v), centrifuged twice at 4º C at maximum speed and the supernatant was used for the analysis.

VP8* Rotavirus surface protein production

Recombinant VP8* was obtained from pQEVP8* transformed *Escherichia coli* M11. Plasmid pQEVP8* was kindly provided by Dr. Monedero from Instituto de Agroquímica y Tecnología de Alimentos (IATA, Valencia). VP8* expression was performed using the QIA*expressionist* protocol (www.qiagen.com). Frozen cell pellets were thawed in 20 mM Tris-HCl pH 8.0. Cells were lysed by sonication on ice (6 x 25 sec) and washed with buffer A (20 mM Tris-HCl, 0.5 M NaCl, 2 M Urea and 2% Triton X-100) to isolate inclusion bodies. After 15 min 18.000 rpm spin, cells were sonicated (6 x25 sec) one more time and washed again with the same buffer. A last wash with urea-free buffer A was made.

Inclusion bodies were solubilised with 20 mM Tris-HCl, 0.5 M NaCl, 6M Guanidine HCl, 5 mM Imidazole and 1 mM 2-β-mercaptoethanol at pH 8.0. The sample was stirred at room temperature for 1 hour and then centrifuged for 15 min at high speed. The remaining particles were removed by passing the sample through a 0.22 µm filter. Purification was performed using HisTrap HP affinity columns (GE Healthcare, Buckinghamshire, UK) following manufacturers procedures. Protein refolding in column was performed with 30 mL of a lineal 6-0 M urea gradient at a flow rate of 1mL/min, starting with buffer B (20 mM Tris-HCl, 0.5 M NaCl, 6 M urea, 1 mM 2-mercaptoethanol) and finishing with urea-free buffer B. The refolded protein was eluted using a 10 mL lineal gradient starting with buffer C (20 mM Tris-HCl, 0.5 M NaCl, 20 mM Imidazole, and 1 mM 2-mercaptoethanol) pH 8.0 and ending with the same buffer C supplemented with 500 mM imidazole.

ELISAs for detection of VP8* binding activity and recombinant immunoglobulin A determination

Plates (Corning, New York, USA) were coated overnight with 10 µg/ mL of recombinant VP8* for detection of VP8* binding activity or 2 µg/ mL of anti-human IgA α specific (Sigma Aldrich St. Louis, USA) for IgA determination, in coating buffer (50 mM carbonate buffer pH 9,8) at 4º C. Plates were then washed 4 times in PBS and blocked with a

2% (w/v) solution of ECL Advance™ Blocking agent (GE Healthcare) in PBS-T (0.1% (v/v) Tween 20 in PBS). Samples were diluted in PBS as required for each assay and incubated for 1 hour at room temperature. After incubation, plates were washed 4 times in PBS and the anti-human IgA α specific-HRP 1:5000 (Sigma Aldrich) in 5% blocking buffer (GE Healthcare) in PBS-T was added and incubated for 1 h at room temperature. After 4 PBS washes, the substrate (*o*-phenilenediamine from Sigma Aldrich) was added and the reactions were stopped with 3M HCl. Absorbance was determined at 492 nm. As a control for specificity, plates were coated with 10 μg/ mL of bovine serum albumin (BSA). A sample was considered positive when its absorbance was at least 3 times higher than the absorbance value of the same sample in the control. Specific VP8* binding activity was estimated using serial sample dilutions, dividing the absorbance at 492 nm of those dilutions falling within the lineal range, by the total protein content of each sample (mg). A standard curve from 0.8 μg/mL to 0.1 μg/mL of commercial IgA from human colostrum (Sigma Aldrich) was made to calculate the concentration of IgA in the different samples. IgA concentration in each sample was obtained by interpolation with the IgA standard curve. For IgA quantification in elite lines, three samples from each elite line were analyzed and the mean ±SD for each line was calculated.

SDS-PAGE and western blot analysis

Proteins were separated by SDS/PAGE in 10% and 3-8% denaturing gels (Invitrogen). Gel staining was carried out either with Coomassie blue or silver staining following standard procedures. For western blot analysis, blots were incubated with 1:20.000 anti-human IgA α specific-HRP (Sigma Aldrich) for the detection of the heavy chain (HC) and 1:10.000 Anti human λ light chain (Sigma Aldrich) followed by 1:10.000 μg/mL HRP conjugated anti-rabbit IgG (GE Healthcare) for the detection of the light chain (LC). Blots were developed with ECL Plus western blotting Detection System (GE Healthcare).

Protein SSL7 affinity purification

Protein extracts prepared as explained previously were further clarified using a 0.22 µm Stericup (Millipore, Massachusetts, USA) on ice. The clarified extract was directly used for purification with staphylococcal superantigen-like protein 7 (SSL7) agarose columns from InvivoGen following manufacturer´s protocol with minor modifications. Wash Buffer was substituted by PBS (0.1 M phosphate, 0.1 M NaCl pH 7.4). Dialysis was not needed as the samples were already in PBS and Elution neutralization was carried out mixing with PBS 1:1 (v/v).

Neutralization assays

Tomato-IgA neutralization of rotavirus SA11 strain was assessed by immunofluorescent focus reduction assays on MA104 (Health Protection Agency Culture Collection) cell monolayers in 96 well cell culture microplates (Corning) infected with rotavirus (Asensi et al., 2006). The plates were coated with 2 x 10^4 cells per well in MEM (minimum essential medium from Gibco-Invitrogen) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM glutamine, 0.7% CO_3H and 10% fetal bovine serum (FBS) and incubated for 24 h at 37ºC in a CO_2 stove until the monolayers were confluent. Rotavirus suspensions (at two different concentrations, 2 x 10^3 and 1 x 10^4 fluorescing cell-forming units per mL [f.f.u./mL]) in MEM without FBS were activated with 10 µg/mL of trypsin type IX (Sigma Aldrich) prior to the incubation with serial dilutions (1:2 to 1:256) of IgA samples in MEM (FBS-free), and the resulting mixes were used to infect the monolayers (1 h incubation at 37ºC). After incubation, the monolayers were washed with MEM and incubated for 18 h in MEM containing 1 µg/mL trypsin at 37ºC in a CO_2 stove. Four different IgA samples were used in the different assays: ssl-7 purified recombinant IgA in PBS, 35S_20A7 clarified fruit extract ("juice"), freeze-dried tissue samples from 35S_20A7 and CR2-3-8 fruits reconstituted in PBS ("powder"). PBS, "juice", and reconstituted "powder" from fruits of wild type Money Maker or IgA-free purple-fruited CR2-3-5 plants were used as mock solutions for comparisons. All fruit samples were

collected and processed between 2 and 4 days after reaching the breaker stage. After incubation, monolayers were fixed with 100 µL/well of 1:1 (v:v) methanol-acetone for 15 min and then washed with PBS. An anti-rotavirus VP6 monoclonal antibody in PBS 1% BSA (1:80) was applied as the primary antibody for 1 h at 37ºC, followed by three washes with PBS. The secondary antibody was an anti-mouse IgG FITC-labeled (SIGMA) (1:400). The monolayers were washed 3 more times with PBS and stained with Evans blue for 5 min, washed again with PBS and 20 µL of mounting fluid (Millipore) were applied to each well. Images were obtained by excitation with 480 nm light and acquired at 6,3x magnification factor with a Leica MZ16F binocular equipped with a Leica DFC300FX digital camera and Leica application suite software. The 50% inhibiting concentrations (IC_{50}) were calculated by the method of Reed and Muench (Reed and Muench, 1938), using the concentrations present during the antibody-virus preincubation step.

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Tables

ELITE LINES (T3)	GENOTYPE	TPS (mg/gFW)	IgA (% TSP)	IgA (µg/gFW)	IgA (µg/gDW)
35S_20A7	IgH/IgL	1,2 ± 0,2	3,6 ± 0,8	41,2 ± 2,8	686,9 ± 46,7
NH_1A-13-2	IgH/IgL	1,4 ± 0,1	1,1 ± 0,1	15,6± 1,2	259,6 ± 19,7
CR2-3-8	IgH/IgL/Ros1/Del	1,7 ± 0,1	1,9 ± 0,3	33,5± 4,2	558,3 ± 70,3

Table 1. Total soluble protein (TSP) and IgA levels referred to TSP, fresh weigh (FW) and dry weight (DW) of diferent tomato elite lines.

Figure legends

Figure 1. Western blot analysis of IgA expression in tomato. (a) Transient expression analysis of scFV-derived 2E4, 2B3 and 2A1 antibodies using direct fruit-agroinjection. Fruits were co-agroinjeted with ER-retained heavy and light antibody chains, together with GFP as internal control. (b) Western blot analysis of leaves and fruits from best-permorfing T3 lines from *in cis* (NH) and *in trans* (35S) transgenic approaches. Three fruits from each elite T3 plant were pooled in each representative sample. All fruits were labelled at breaker stage and collected bewteen 48 and 72 hours later (c) Comparison between fruit extracts from of 35S_20A7 and wild type (WT) plants. Samples were resolved under either reducing (R) or non-reducing (NR) conditions and decorated using anti-heavy chain antibody (HC), anti-light chain antibody (LC), anti-GFP antibody (GFP) or by Coomasie staining (CS). HS lane contains control human serum.

Figure 2. IgA accumulation profile during tomato fruit ripening. (a) Evolution of anti-VP8* activity in fruit extracts of 35S_20A7 and NH_1A-13-2 elite plants referred to fresh weight (light grey) and total protein content (dark grey columns). (b) Western blot analysis of the same

fruit samples as in (a). Samples were resolved under either reducing (R) or non-reducing (NR) conditions and decorated using anti-heavy chain (HC) or anti-light chain antibodies (LC). The selected fruit developmental stages were: Mature green fruits (MG), corresponding to fully expanded green fruits (4-5 weeks after anthesis); Breaker fruits (BK) turning yellow/red, Red fruits (R), harvested between 5 and 12 days after breaker stage; red ripe fruit (RR), harvested between 14 and 21 days after breaker stage; and dry (D) tomatoes, harvested at the red ripe stage and stored at room temperature for one month. HS is a sample of human serum. All samples were obtained by pooling three fruits from each developmental stage and analyzed in triplicate with bars representing standard error.

Figure 3. End-point antigen-ELISA titering of minimally-processed fruit-derived products. (a) "Juice" is a clarified fruit extract. (b) "Powder" is freeze-dried tissue reconstituted in PBS. (c), "Dried Juice" is a freeze-dried "juice"reconstituted in PBS. Binding activity refers to the absorbance at 492 nm after 15 min incubation. Samples consisted in 2-3 pooled fruits from each elite or control plant, harvested 2-4 days after the breaker stage. All samples were titered in triplicate against VP8* or BSA, with bars representing standard error.

Figure 4. SSL7-based affinity purification of IgA from tomato fruits. (a) Specific Anti-VP8* activity measured in the different fractions of the purification process. (b) SDS-PAGE and western analysis of the starting tomato crude extract (CE) and the final SSL7-agarose eluted fraction (EL). SDS-PAGE gels were silver-stained (SS) to asses purity, and western blot analysed to detect heavy chain (HC) and light chain (LC) under both reducing (R) and non-reducing (NR) conditions.

Figure 5. *In vitro* rotavirus neutralization assays. Trypsin-activated rotavirus of SA11 strain (10.000 ffu/mL) were pre-incubated for 1h with serial dilutions of IgA-containing samples or equivalent control samples and used to infect MA104 cell monolayers (2 x 10^4 cells/well). Infective foci were detected after 18h by immunofluorescence and analyzed in triplicate with

bars representing standard error. (a) Neutralization titers of affinity purified tomato-made IgA_2A1. (b) Neutralization titers of minimally processed products. IgA content in “Juice” and reconstituted “Powder” samples was estimated by sandwich ELISA and equalized with the addition of PBS. Equivalent wild type “juice” and “powder” samples were equally diluted in PBS and used as controls to estimate the percentage of neutralization of each sample. All samples consisted of at least three pooled fruits from either elite 35S-20A7 or wild type Moneymaker plants (c) Example of foci reduction conferred by incubation with tomato-made IgA. Left: wild type “Juice”; right: transgenic “Juice”.

Figure 6. Purple tomatoes anti-VP8*. (a) Tomato fruit, “powder” and “juice” samples from WT (right) and Del/Ros1/IgH/IgL tomatoes (left); (b) Endpoint anti-VP8* ELISA titering of purple and red tomato “powder”. Samples were obtained by pooling three fruits from each plant, namely a wild type plant (WT), the IgA-free control purple plant CR2-3-5 (Del/Ros1), the elite IgA-containing 35S-20A7 plant (IgA) and CR2-3-8 (IgA/Del/Ros1) respectively. Samples were titered in triplicates against VP8* or BSA (only CR2-3-8 line), with bars representing standard error. (c) Virus neutralization assay of purple IgA tomatoes. The IgA content in a “powder” sample consisting of three pooled fruits from the elite line CR2-3-8 was estimated by sandwich ELISA. “Powder” samples from CR2-3-8 and its Del/Ros1-positive, IgA-negative sibling line CR2-3-5 were reconstituted in PBS in equivalent dilution series and pre-incubated with rotavirus SA11 prior to infect MA104 cell monolayers. Samples were analyzed in triplicate and neutralization is represented as the mean percentage of the reduction of foci number with respect to the CR2-3-5 control line, with bars representing the standard error.

FIGURE 1

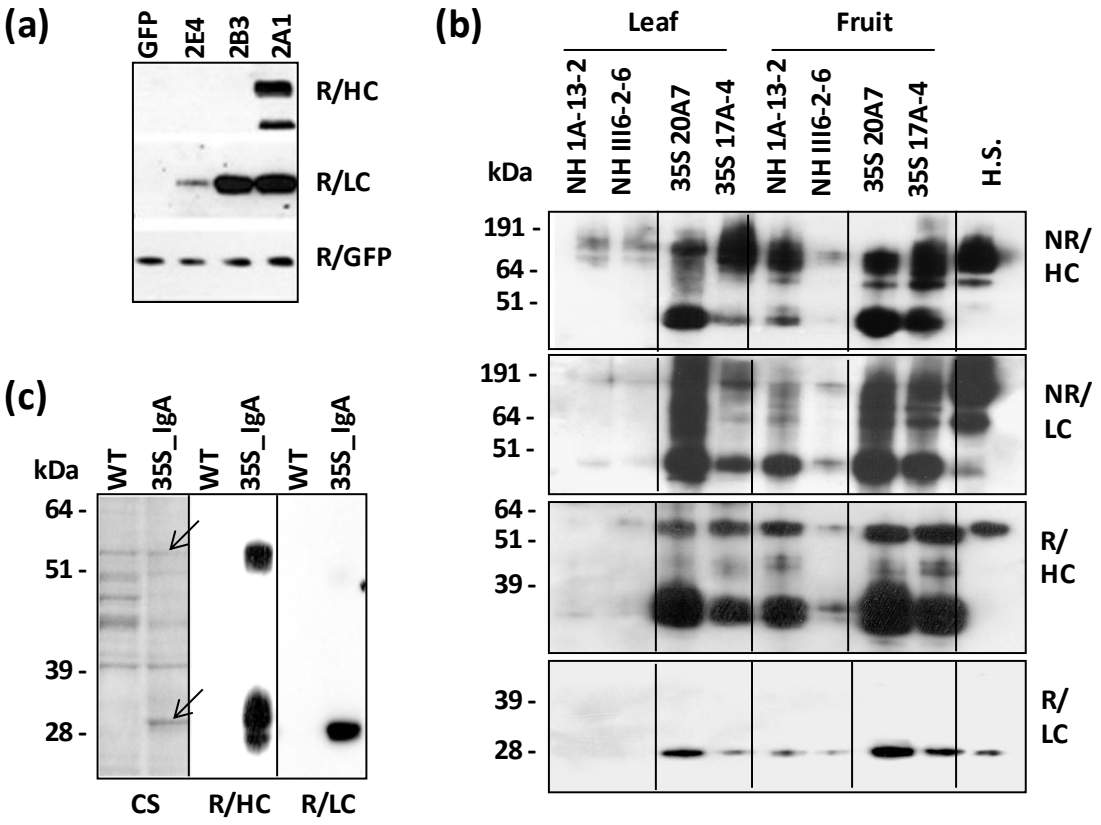

(a)
GFP
2E4
2B3
2A1
R/HC
R/LC
R/GFP
(b)
Leaf
Fruit
kDa
NH 1A-13-2
NH III6-2-6
35S 20A7
35S 17A-4
NH 1A-13-2
NH III6-2-6
35S 20A7
35S 17A-4
H.S.
191 -
64 -
51 -
NR/ HC
NR/ LC
R/ HC
R/ LC
39 -
28 -
(c)
kDa
WT
35S_IgA
WT
35S_IgA
WT
35S_IgA
64 -
51 -
39 -
28 -
CS
R/HC
R/LC

FIGURE 2

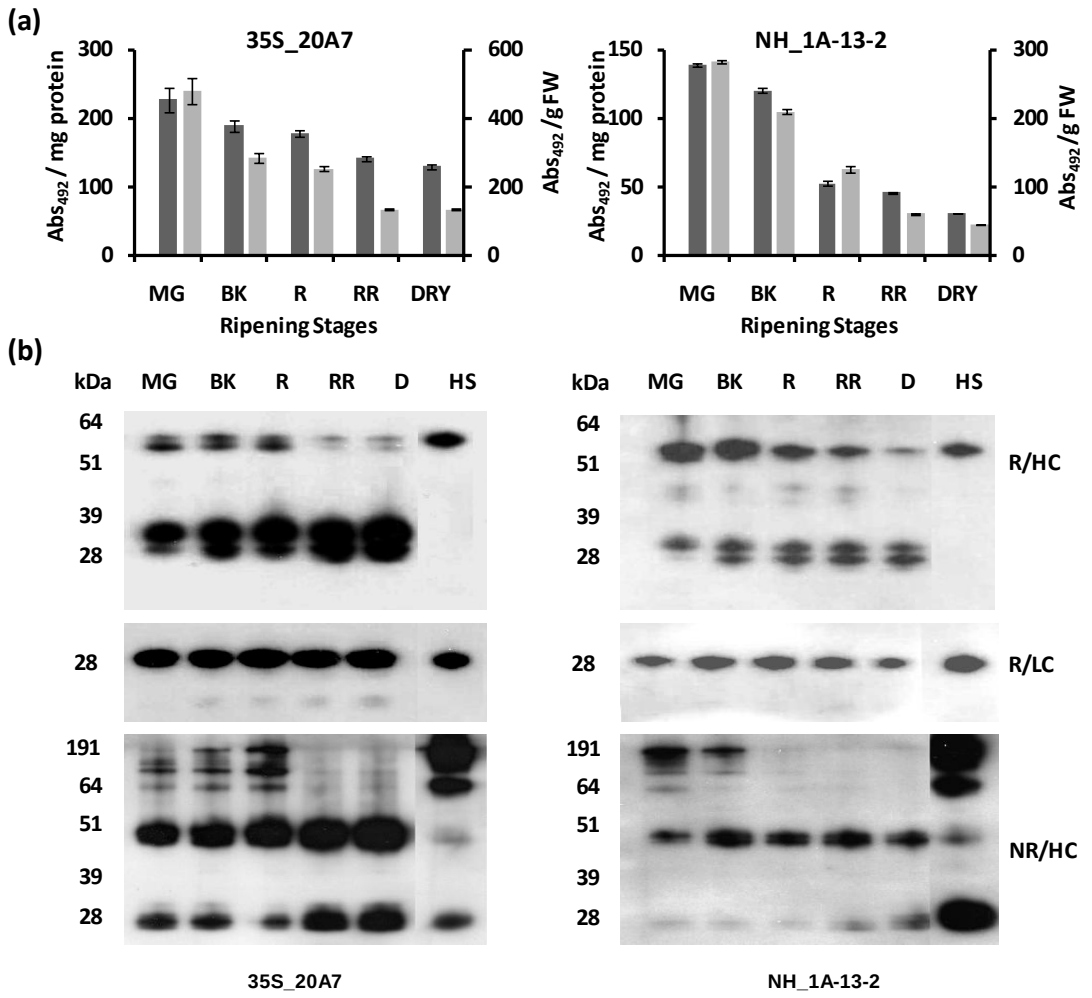
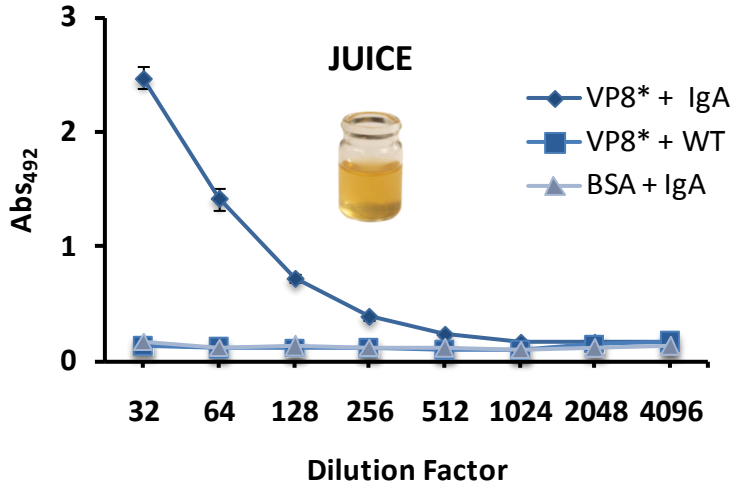
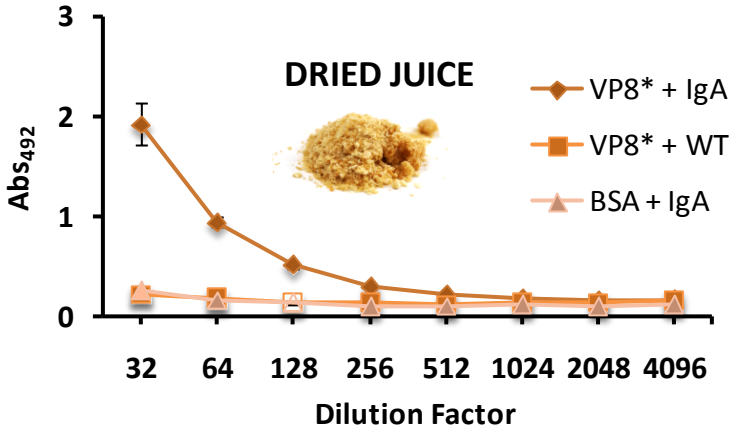

(a)
35S_20A7
NH_1A-13-2
Abs492 / mg protein
Abs492 /g FW
MG
BK
R
RR
DRY
Ripening Stages
(b)
kDa
MG
BK
R
RR
D
HS
64
51
39
28
191
R/HC
R/LC
NR/HC
35S_20A7
NH_1A-13-2

FIGURE 3


JUICE
VP8* + IgA
VP8* + WT
BSA + IgA
Abs492
3
2
1
0
32 64 128 256 512 1024 2048 4096
Dilution Factor


DRIED JUICE
VP8* + IgA
VP8* + WT
BSA + IgA
Abs492
3
2
1
0
32 64 128 256 512 1024 2048 4096
Dilution Factor

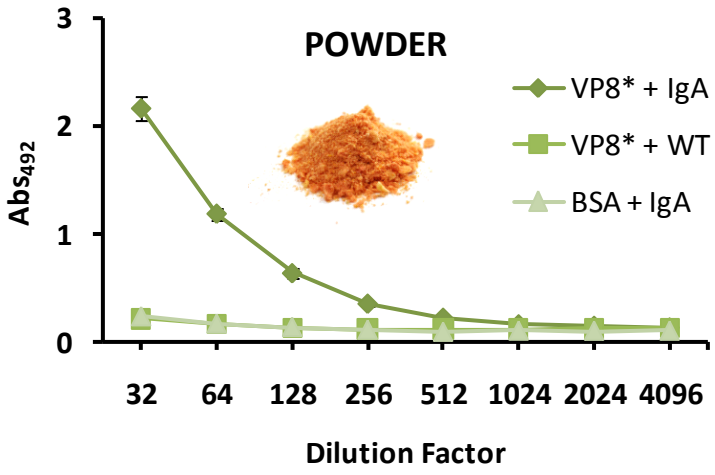

POWDER
VP8* + IgA
VP8* + WT
BSA + IgA
Abs492
3
2
1
0
32 64 128 256 512 1024 2024 4096
Dilution Factor

FIGURE 4

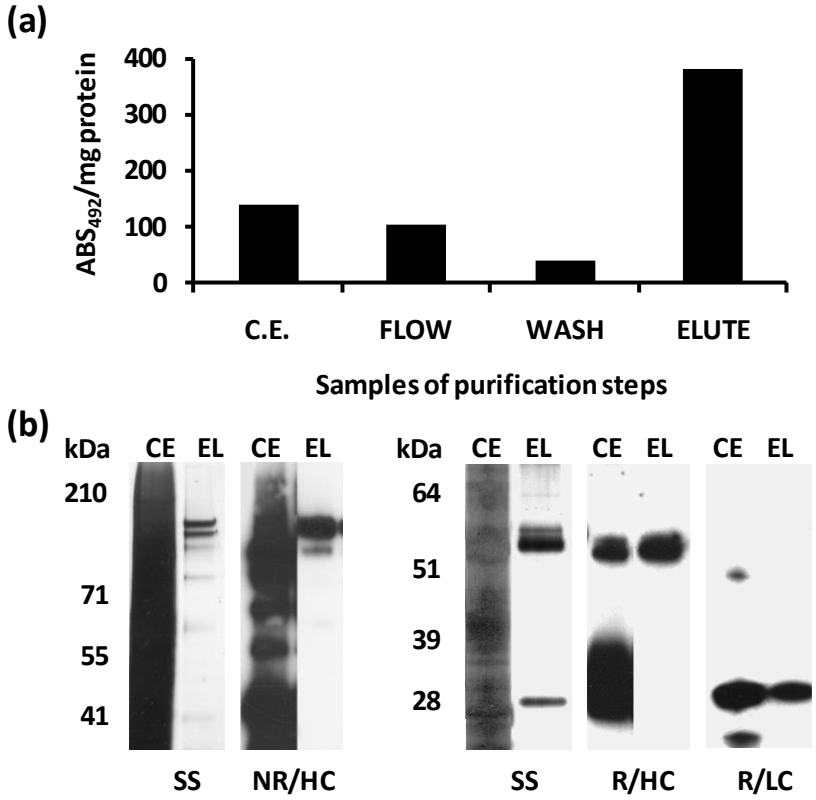

(a)
400
300
200
100
0
ABS492/mg protein
C.E.
FLOW
WASH
ELUTE
Samples of purification steps
(b)
kDa
CE
EL
CE
EL
210
71
55
41
SS
NR/HC
kDa
CE
EL
CE
EL
CE
EL
64
51
39
28
SS
R/HC
R/LC

FIGURE 5

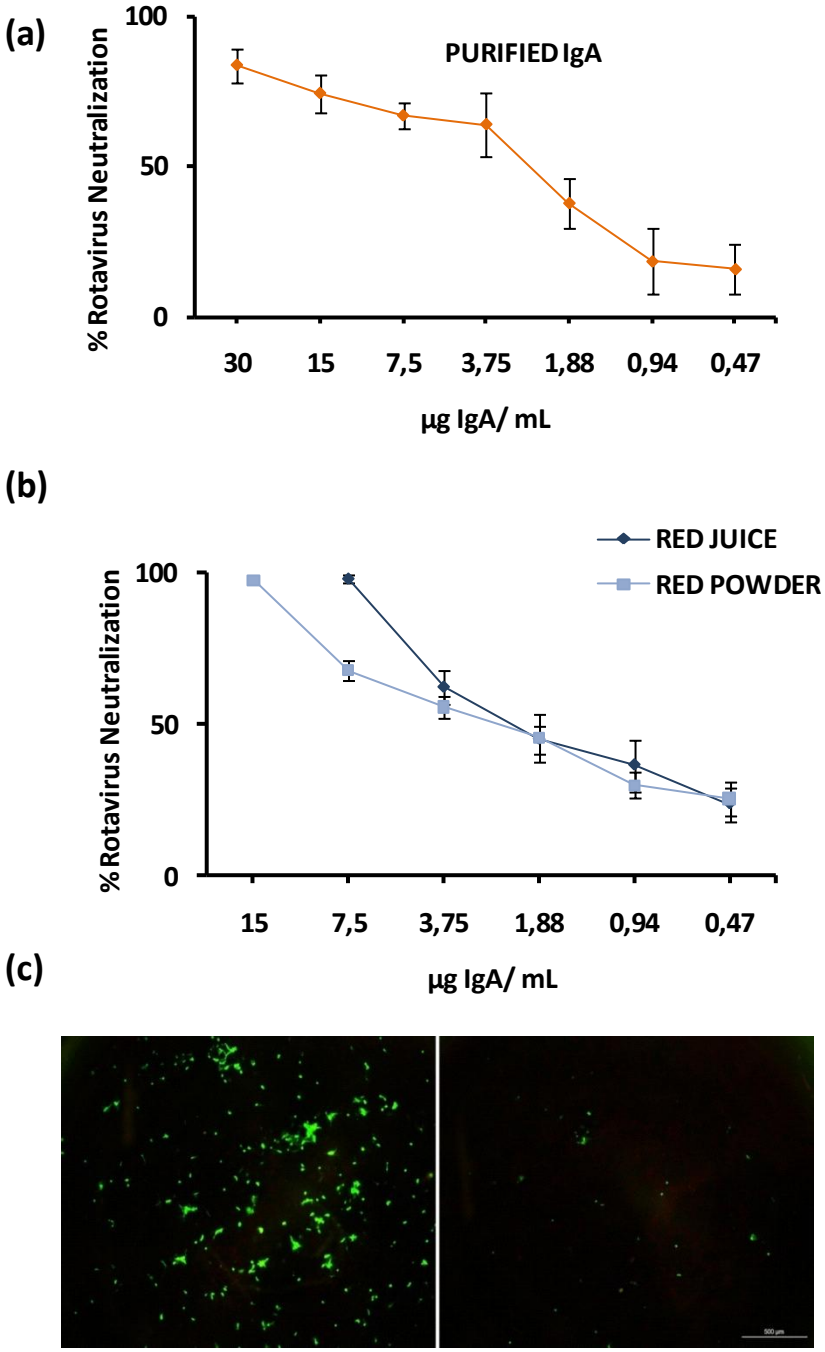
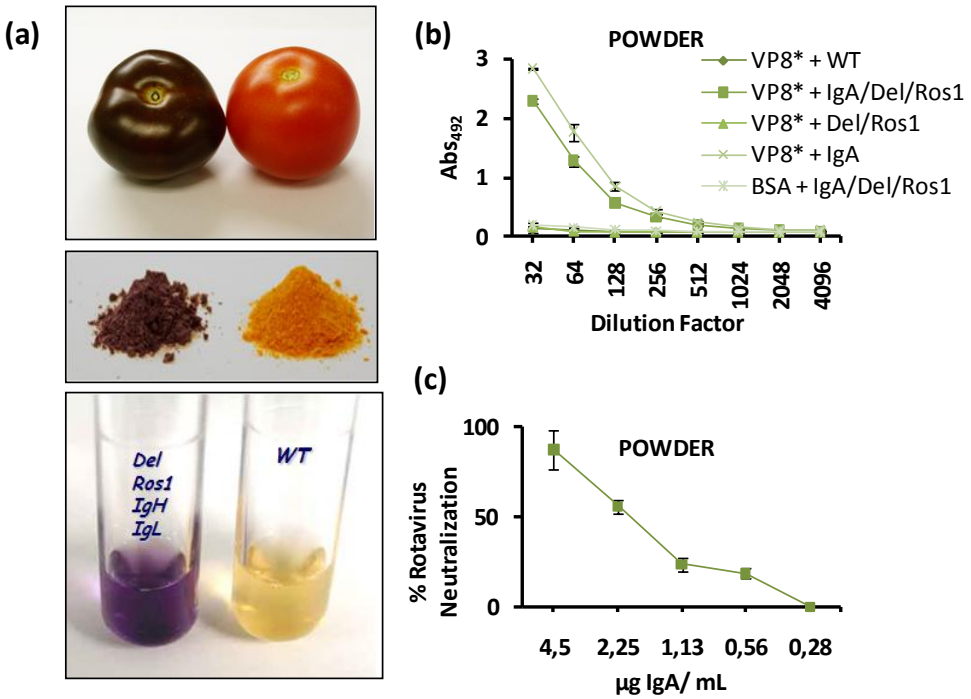

(a)
100
50
0
% Rotavirus Neutralization
PURIFIED IgA
30
15
7,5
3,75
1,88
0,94
0,47
µg IgA/ mL
(b)
RED JUICE
RED POWDER
100
50
0
% Rotavirus Neutralization
15
7,5
3,75
1,88
0,94
0,47
µg IgA/ mL
(c)

FIGURE 6


(a)
Del
Ros1
IgH
IgL
WT
(b)
POWDER
VP8* + WT
VP8* + IgA/Del/Ros1
VP8* + Del/Ros1
VP8* + IgA
BSA + IgA/Del/Ros1
Abs492
3
2
1
0
32
64
128
256
512
1024
2048
4096
Dilution Factor
(c)
POWDER
% Rotavirus Neutralization
100
50
0
4,5
2,25
1,13
0,56
0,28
µg IgA/ mL