UNIVERSITAT POLITÈCNICA DE VALÈNCIA

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Paloma Juárez Ortega
IBMCP
Valencia, January 2014

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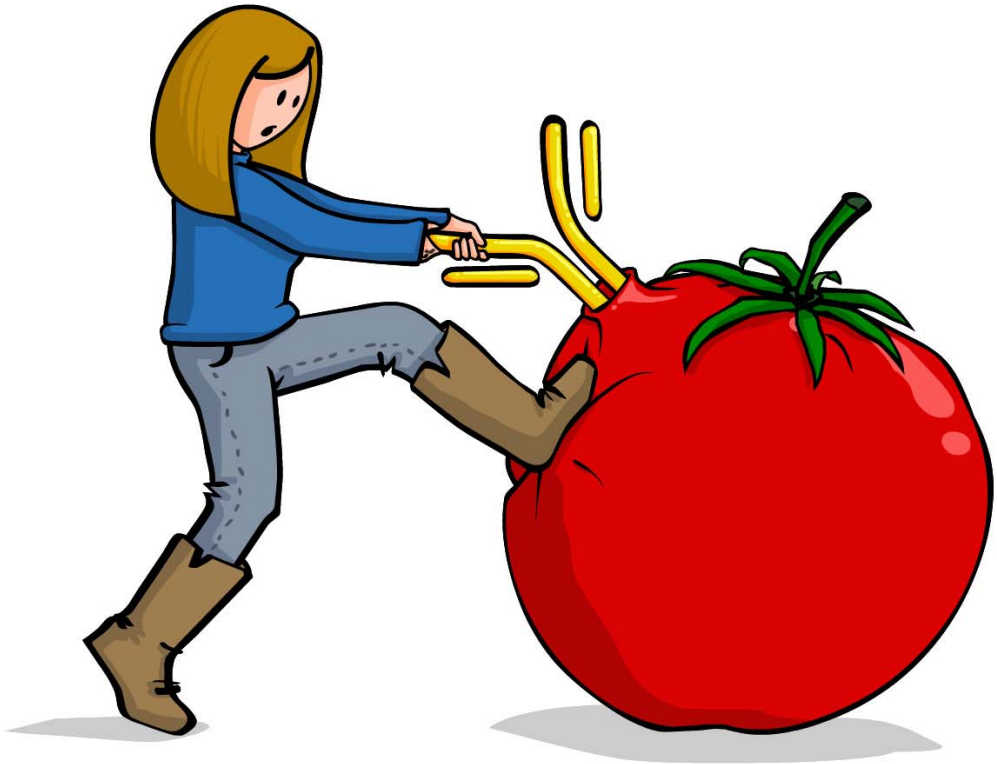

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El **Dr. Diego Orzáez Calatayud**, Científico Titular del Consejo Superior de Investigaciones Científicas y Profesor Asociado de la Universidad Politécnica de Valencia, y el **Dr. Antonio Granell Richart**, Profesor de investigación del Consejo Superior de Investigaciones Científicas, ambos pertenecientes al instituto de Biología Molecular y Celular de Plantas,

CERTIFICAN que la Licenciada en Ciencias Biológicas y Máster en Biotecnología Molecular y Celular de Plantas **Paloma Juárez Ortega,** ha realizado bajo su dirección en el Instituto de Biología Molecular y Celular de Plantas el trabajo que lleva por título ***Production of recombinant Immunoglobulin A in plants for passive immunotherapy***, y autorizan su presentación para optar al grado de Doctor en Biotecnología.

Y para que así conste, expiden y firman el presente certificado en Valencia, a 20 de Enero de 2013

Dr. Diego Orzáez Calatayud **Dr. Antonio Granell Richart**

A mis padres

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Hoy, día 18 de Diciembre vuelvo a casa tras de un día de Jornadas de Navidad y papeleos, dejo las cosas en el sofá y pienso… Cuánto les voy a echar de menos…

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elución y la tiré por la pila sin pensármelo dos veces. Después de un largo rato mirando a la pila y pensando inútilmente como recuperarla, terminamos la tarde maquinando que mentirijilla le diríamos a Diego…(Diego, al final te contamos la verdad). **Estefanía**, cuida bien de las SiPs ehhh! Seguro que lo harás. Ya lo haces! Si no fuera por ti esa parte se habría quedado estancada! Mil gracias también por tu ayuda con el Valenciano, tanto con los intentos fallidos de que aprenda como con las traducciones (como no aprendí me las hicisteis Jose y tú!!). **Jose**, gracias a ti también por tu ayuda con el Valenciano y por haber cuidado bien de mi pipeta de 10 (jijiji). **Jose Luis**, gracias por tu ayuda con toda la parte de volátiles. He aprendido muchas cosas de ti estos últimos meses. **Flor**, tu cuida bien de mis ex pipetas (y ya sabes, escóndelas de las garras de Jose… :P). Gracias también a **Sophie**, **Cris**, **Clara**, **Ale**, **Pathy** y **Leandro** por todas las veces que me habéis echado una mano, y por supuesto a todos los estudiantes que han pasado por el labo y me han ayudado tanto. **Sara Z**, **Sara I**, **Joaquín**, **Ana** e **Irene**, una parte de mi tesis os correponde a cada uno de vosotros. Gracias.

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Summary

Mucosal passive immunization is the transfer of active antibodies from one organism to the mucosal surfaces of another organism for preventing or treating infectious diseases. Mucosal passive immunization has a great potential for the prevention and treatment of enteric infections like Rotavirus, which causes more than 114 million episodes of diarrhoea annually with a death toll of more than 450.000 per year. However, the high cost of recombinant antibodies with the current manufacturing systems based on mammalian cells hampers the production of the high antibody quantities required for passive immunization strategies. Alternative expression platforms such as plants could provide higher scalability and reduced costs. Moreover, the use of edible plant organs, which are *Generally-Regarded-As-Safe* (GRAS), could reduce manufacturing costs even further by easing the requirements for antibody purification. We analyze here the feasibility of utilizing fruits as inexpensive biofactories of human antibodies that can be orally delivered as crude extracts or partially purified formulations in mucosal passive immunization strategies.

In the first section of this thesis, the construction of tomato plants producing a model human Immunoglobulin A (IgA) against rotavirus in their fruits is described. As a result, an elite homozygous line was obtained whose fruits produced on average 41 μg of IgA per gram of fresh weigh, equivalent to 0.69 mg IgA per gram of dry tomato powder. Minimally processed products derived from IgA-expressing tomatoes were shown to strongly inhibit virus infection in an *in vitro* neutralization assay. Moreover, in order to make IgA-expressing tomatoes easily distinguishable from wild-type tomatoes, they 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. The resulting transgenically-labelled purple tomatoes contained not only high levels of recombinant neutralizing human IgA but also increased amounts of anthocyanins.

In the second section of the thesis the composition of IgA-expressing tomatoes was analyzed in search of possible unintended effects that could compromise the GRAS status of the final product. To this end, transgenic IgA-tomatoes were compared with wild type tomatoes and also commercial tomato varieties using proteomic and metabolomic approaches. 2D-DIGE gels coupled with LC-MSMS for protein identification showed that all the uptrend differential proteins detected corresponded only to immunoglobulin chains or antibody fragments. On the other hand, non-targeted metabolite data obtained by UPLC-MS identified variations between transgenic and non-transgenic lines, however such variations could not be associated with the presence of abnormal levels of any particular secondary metabolite in the IgA fruits. Therefore from the analysis conducted here no sign was obtained that could indicate that tomato-IgA formulations are less safe for consumption than their wild type counterparts.

The third section of this thesis focused in optimizing the production of the secretory form of the IgA (sIgA), as this is the most convenient antibody isotype for mucosal passive immunization. SIgA production requires the co-expression of four transcriptional units encoding the light chain (LC), heavy chain (HC), joining chain (JC) and secretory component (SC). In order to optimize its production, sixteen versions of a human sIgA against rotavirus comprising different antibody chain isotypes with or without retention in the endoplasmic reticulum were constructed using the GoldenBraid multigene assembly system. Transient expression in *Nicotiana benthamiana* of all sIgA versions showed that maximum expression levels were achieved by the sIgA version containing alpha1 HC, lambda LC and with a KDEL signal linked to the SC, with an estimated 33% of the total IgA accumulating in the form of a secretory complex.

Resumen

La inmunización pasiva de las mucosas se define como la transferencia de anticuerpos activos de un organismo a las superficies mucosas de otro para la prevención o tratamiento de enfermedades infecciosas. La inmunización pasiva de las mucosas tiene un gran potencial en la prevención y tratamiento de infecciones gastrointestinales como las causadas por rotavirus, con más de 114 millones de episodios de diarrea y más de 450.000 muertes al año. Sin embargo, el elevado coste de los anticuerpos recombinantes que actualmente se producen en células de mamífero, frena la producción de las grandes cantidades de anticuerpos requeridas para estrategias de inmunización pasiva. Las plantas, como plataformas de expresión alternativa, podrían facilitar el escalado y la reducción de costes. Además, el empleo de órganos comestibles, que están catalogados como *Generalmente-Reconocidos-Como-Seguros* (GRAS, de sus siglas en inglés), podría suponer una reducción adicional de los costes al disminuir los requerimientos de purificación de los anticuerpos. En el presente trabajo se analiza la viabilidad de la utilización de los frutos como biofactorías de anticuerpos humanos, de modo que puedan ser administrados oralmente como extractos crudos o bien como formulaciones parcialmente purificadas en estrategias de inmunización pasiva.

En la primera sección de esta tesis se describe la generación de plantas de tomate que producen en sus frutos una inmunoglobulina A (IgA) modelo frente a rotavirus. Como resultado de este trabajo, se obtuvo una línea élite homocigota cuyos frutos producían un promedio de 41 µg de IgA por gramo de peso fresco, equivalente a 0,69 mg de IgA por gramo de polvo de tomate seco. Ciertas formulaciones parcialmente purificadas, derivadas de tomates con IgA, fueron capaces de inhibir fuertemente la infección viral en un ensayo de neutralización *in vitro*. Además, con el propósito de poder distinguir los tomates transgénicos con IgA de los tomates silvestres, los tomates con IgA se cruzaron con una línea de tomate transgénico que expresaba los genes de *Antirrhinum majus* que codifican los factores de transcripción Rosea1 y Delila. Estos factores de transcripción confieren al fruto un intenso color morado. El tomate morado resultante no solo presenta elevados niveles de IgA humana neutralizante sino también altos niveles de antocianinas.

En la segunda sección de esta tesis, la composición de los tomates con IgA fue analizada en busca de posibles efectos no intencionados que pudieran comprometer el estatus GRAS del producto final. Los tomates transgénicos con IgA fueron comparados con tomates silvestres y también con variedades comerciales utilizando técnicas de proteómica y metabolómica. Mediante geles 2D-DIGE y LC-MSMS para la identificación de proteínas se demostró que todas las proteínas diferenciales cuyos niveles aumentaban en las líneas transgénicas correspondían únicamente a cadenas o fragmentos de inmunoglobulinas. Además, un análisis no dirigido mediante UPLC-MS permitió identificar variaciones entre líneas transgénicas y no transgénicas; sin embargo, esas variaciones no se pudieron asociar a la presencia de niveles anormales de

ningún metabolito secundario en particular en los frutos con IgA. Por lo tanto, de este análisis no se pudo deducir que las formulaciones a partir de tomates con IgA fueran menos seguras para el consumo que sus correspondientes formulaciones con tomates silvestres.

La tercera sección de esta tesis se centró en la optimización de la producción de la forma secretora de la IgA (sIgA), ya que es el isotipo de anticuerpo más conveniente para la inmunización pasiva de las mucosas. La producción de sIgA requiere la co-expresión de cuatro unidades transcripcionales que codifican la cadena ligera (LC), la cadena pesada (HC), la cadena J (JC) y el componente secretor (SC). Para optimizar esta producción, se construyeron dieciséis versiones de una sIgA humana frente a rotavirus utilizando el sistema de ensamblaje multigénico GoldenBraid. Estas 16 versiones consistían en diferentes combinaciones de cadenas de anticuerpo, incorporando algunas de ellas una señal de retención en el retículo endoplasmático (KDEL). Mediante expresión transitoria en *Nicotiana benthamiana* de todas las versiones de sIgA se observó que se obtenían niveles máximos de expresión con la versión de sIgA formada por la cadena pesada tipo alfa1, la cadena ligera tipo lambda y una señal KDEL unida al componente secretor. La forma secretora representó unicamente el 33% del total de la IgA acumulada.

Resum

La immunització passiva de les mucoses es defineix com la transferència d'anticossos actius d'un organisme a les superficies de les mucoses d'un altre organisme per a la prevenció o tractament de malalties infeccioses. La immunització passiva de les mucoses té un gran potencial en la prevenció i tractament d'infeccions gatrointestinals com les causades per rotavirus, amb més de 114 milions d'episodis de diarrea y més de 450.000 morts a l'any. Tanmateix, la producció dels anticossos recombinants suposa una forta despesa doncs el sistema de producció actual està basat en cèl·lules de mamífers, fet pel qual relanteix la producció de grans quantitats d'anticossos per a estratègies de immunització passiva.

Les plantes, com plataforma d'expressió alternativa, podrien facilitar l'escalada i la reducció de les despeses. A més a més, la utilització com a plataformes d'expressió dels òrgans comestibles de les plantes, solen estar catalogades com *Generalment-Reconeguts-Com-Segurs* (GRAS), podrien suposar una reducció addicional de les despeses al disminuir els requeriments de purificació dels anticossos. En aquest treball s'analitza la viabilitat de la utilització dels fruits com biofactories econòmiques d'anticossos humans, aquestos anticossos poden ser administrats oralment com extractes crus o bé com formulacions parcialment purificades en estratègies de immunització passiva de mucoses.

En la primera secció d'aquesta tesi es descriu la generació de plantes de tomaca que produeixen en els seus fruits una immunoglobulina A (IgA) model front a rotavirus. El resultat d'aquest treball és l'obtenció d'una línia elit homocigota, de la qual els seus fruits produeixen una promedi de 41µg d'IgA per gram fresc, equivalent a 0.69 mg de IgA per gram de pols de tomaca. Certes formulacions, parcialment purificades, derivades de tomaques amb IgA, foren capaces d'inhibir fortament la infecció viral en un assaig in vitro. A més, amb el propòsit de poder diferenciar les tomaques transgèniques amb IgA de les tomaques silvestres, les tomaques transgèniques amb IgA es creuaren amb una línia de tomaca transgènica que expressava els gens *d'Antirrhinum majus* que codifiquen per als factors de transcripció Rosea1 i Delila. Aquests factors de transcripció donen al fruit un intens color morat. El resultat d'aquest encreuament és l'obtenció d'una tomaca morada que presenta elevats nivells de IgA humana amb, també, alts nivells d'antocians.

En la segona secció d'aquesta tesi, la composició de les tomaques amb IgA sigué analitzada en la busca de possibles efectes no intencionats que pogueren comprometre l'estatus GRAS del producte final. Les tomaques transgèniques amb IgA foren comparades amb tomaques silvestres i també amb varietats comercials utilitzant tècniques de proteòmica i metabolòmica. Mitjançant gels 2D-DIGE i LC-MSMS per a la identificació de proteïnes es demostrà que totes les proteïnes diferencials que augmentaven els seus nivells en les línies transgèniques corresponien únicament a cadenes o fragments de immunoglobulines. A més, un análisis no dirigit a través de UPLC-MS permeté identificar variacions entre línies transgèniques i no

transgèniques. No obstant això, aquestes variacions no es pogueren associar a la presència de nivells anormals de cap metabolit secundari dels fruits amb IgA. Així que, d'aquest anàlisi no es pot concloure que les formulacions a partir de tomaques amb IgA foren menys segures que les seues corresponents formulacions amb tomaques silvestres.

La tercera secció d'aquesta tesi es centrà en l'optimització de la forma secretora de la IgA (sIgA), llavors és l'isòtip d'anticos més rellevant en la imuunització passiva de les mucoses. La producció de sIgA requereix de la co-expressió de quatre unitats transcripcionals que codifiquen per a la cadena lleugera (LC), la cadena pesada (HC), la cadena J (JC) i el component secretor (SC). Per optimitzar aquesta producció, es construïren setze versions de una sIgA humana front a rotavirus utilitzant el sistema d'ensamblatge multigènic GoldenBraid. Aquestes setze versions consisteixen en diferents combinacions de cadenes d'anticos, incorporant alguna d'elles una senyal de retenció al reticle endoplasmàtic. Mitjançant expressió transitòria en *Nicotiana benthamiana* de totes les versions de sIgA s'obtingueren nivells màxims d'expressió amb la versió de sIgA que tenia la cadena pesada de tipus alfa1, la cadena lleugera de tipus lambda i una senyal KDEL unida al component secretor. La forma secretora respresenta un 33% del total de IgA acumulada.

Table of contents

Introduction

1. Plant-made antibodies.

Plants have been proposed as advantageous platforms for large-scale production of antibodies. Plant Biofactories are economically scalable and sustainable production platforms and their phylogenetic distance with humans drastically reduces the chances of contamination with unadverted human pathogens (Ko et al., 2009; Ma et al., 2003). Although mammalian cells (Wurm, 2004) or baculovirus-infected insect cells (Berger et al., 2004) are currently the most used production systems for the majority of applications, the number and types of antibodies expressed in plants have increased incessantly since the first reports in 1989 (Hiatt et al., 1989), illustrating the versatility of plants as production platforms.

In the last years, some plant-made antibodies (PMAbs) have been presented as promising therapeutic solutions. For instance, Large Scale Biology's manufacturing system for Non-Hodgkin's lymphoma antibodies obtained the US Food and Drug Administration (FDA) approval and conducted a Phase I clinical trial that demonstrated their safety and immunogenicity (McCormick et al., 2008). CaroRX®, a mouthwash solution based on antibodies against *Streptococcus mutans* that prevent from dental caries (De Muynck et al., 2010; Larrick et al., 2001; Ma, 1988; Wycoff, 2005), was evaluated in phase I and II clinical trials and has been registered as a medical device in Europe (Larrick et al., 1998).

The use of transgenic plants for the expression of recombinant antibodies is being developed in leaps and bounds. The approach is especially promising when high amounts of antibodies are required. Competitive yields, in comparison to those achieved with CHO cells, have been reported not only at a laboratory scale (Giritch et al., 2006; Petruccelli et al., 2006) but also in prototype industrial setups (Bendandi et al., 2010; Vezina et al., 2009). Up-scaling production can be achieved more easily and economically than with other systems, as for example mammalian cell culture, where scaling up of the fermentation process requires expensive investment. There are no plant-made antibodies yet in commercial production, therefore costs are difficult to estimate. However, it has been reported that, in theory, the costs of an Immunoglobulin A (IgA) expressed in plants are only 1-10% compared to the expression in hybridoma cells (Daniell et al., 2001; Frenzel et al., 2013).

2. Plant-made antibodies in the context of oral passive immunization.

Passive immunity is the transfer of active antibodies from one organism to another that can prevent or treat infectious diseases. Naturally, this occurs for instance with the transfer of antibody from mother to fetus. Maternal antibodies, specifically Immunoglobulin G (IgG), are passed through the placenta to the

foetus around the third month of gestation. Natural oral immunization is also required shortly after birth to prevent infectious diseases. For this, secretory Immunoglobulin A (sIgA) antibodies present in the breast milk are also transferred to the gut of the newborns, protecting them against infections until they can synthesize their own antibodies (Raab, 2011). This maternal oral passive immunity (OPI) can be mimicked when immediate immunity is needed, by orally transferring high levels of ready-made antibodies produced by another organism, to non-immune individuals. OPI is a promising alternative to antibiotics for the prophylaxis and treatment of a wide variety of infectious diseases (Mohan and Haque, 2003; Rahman et al., 2013; Vega et al., 2012). There are several options available to deliver antibodies for OPI. Prophylactic administration of antibody- containing protein preparations from immunized eggs (Xu et al., 2011), or plasma from immunized animals (Marquardt et al., 1999; Niewold et al., 2007) have been successful in preventing infection in different *E. coli* challenge experiments with piglets. However, antibodies from immunized eggs are expensive (65 €/Kg of immunized egg protein) (Chernysheva et al., 2004; Harmsen et al., 2005) and not exent of contamination risks, whereas the use of animal plasma in feed is strongly discouraged as there are serious regulatory concerns about its safety. OPI therapies require large amounts of antibodies to be effective and currently, most recombinant monoclonal antibodies are produced in mammalian cell platforms in which scaling up can result enormously expensive. Therefore, there is a need for alternatives in the production of antibodies for PI which satisfies the conditions of being safe, easy to scale up and inexpensive.

OPI is one of the most promising applications of plant-made antibodies (PMAb), particularly considering that species generally regarded as safe (GRAS) may have lower purification requirements for topic and mucosal applications. Accordingly, several examples in the literature demonstrate the potential of PMAb as anti-microbial agents. Zeitlin et al. (1998) compared a humanized anti-herpes simplex virus 2 (HSV-2) mAb expressed in mammalian cell culture with its counterpart expressed in soybean, proving not only the similarity in their stability in mucosal secretions of the human reproductive tract, but also efficacy for prevention of vaginal HSV-2 infection in mouse. Ko et al. (2003) produced a neutralizing anti-rabies virus IgG in tobacco plants and demonstrated its effectiveness *in vivo*. The virus-neutralizing activity in rabies post-exposure prophylaxis was comparable to that of its commercial counterpart. As more recent examples, Ramessar et al. (2008) developed a PMAb-based vaginal microbicide to prevent HIV transmission and proved that the neutralization capability was equal to or superior to its counterpart produced in CHO cells; more recently Virdi et al. (2013) expressed in *Arabidopsis thaliana* seeds a new format of antibody by fusing VHHs against ETEC to the Fc part (constant region) of a porcine immunoglobulin. These antibodies were shown to efficiently protect ETEC challenged piglets against postweaning diarrhoea.

3. Edible plant organs as a platform for production of antibodies and other mucosal therapeutics.

Edible plant organs present the advantage of being safe and palatable for human consumption, which makes them ideal platforms for production of mucosal therapeutics. Although there are very few examples of antibodies produced in edible plant tissues, during the last years other edible mucosal therapeutics have set a precedent.

The concept of edible plant-made pharmaceuticals was first conceived when in the early 1990s Charles J. Arntzen came up with the idea of making vaccines in edible fruits. While visiting Bangkok, he saw a mother soothe a crying baby by offering pieces of banana. The idea of food being genetically engineered to produce vaccines occurred then to him. The advantages would be enormous: plants grown locally that could regenerate year after year would certainly reduce a huge amount of costs. Not only that, home-grown vaccines would also avoid the transportation over long distances and the need of refrigeration. Last, vaccines would require no medical personnel and no syringes which, apart from their cost, can be contaminated and lead to infections (Corthesy, 2013; Mason et al., 1996; Mason et al., 1998; Mason et al., 2002). Unfortunately, several limitations appeared; among other, the possible development of immunotolerance to target peptides or proteins, dosage requirements and consistency of dosage from fruit to fruit, plant to plant and generation to generation. To deal with the second and third problems, the idea of directly consuming the fruit was substituted by the suggestion of using partially purified formulations, where the dosage could be standardized while costs would still be reduced (Tokuhara et al., 2013).

During the two last decades, a number of vaccines, antibodies and other mucosal pharmaceuticals have been produced in edible plant organs to be used for mucosal treatments. A non-exhaustive list of examples could start with a vaccine candidate based on the heat-labile enterotoxin B subunit of enterotoxigenic *E.coli* (ETEC) produced in transgenic potato, which demonstrated immunogeneicity against ETEC challenge in animal studies (Haq et al., 1995; Mason et al., 1998) and also in humans during the Phase I of the clinical study (Tacket et al., 1998). This ETEC vaccine has also been produced in transgenic corn and also demonstrated immunogeneicity in humans during clinical trials (Tacket, 2007; Tacket et al., 2004). Another relevant example is the oral vaccine against cholera named MucoRice, which consists of the antigen of the cholera toxin B (CTB) accumulated in rice seed storage organelles. Other therapeutic/prophylactic proteins include the human lactoferrin, which was produced by Meristem Therapeutics in maize and later in rice seeds when Ventria Biosciences acquired the technology (Bethell and Huang, 2004; Nandi et al., 2005). This therapeutic protein is currently in clinical development for the prevention of antibiotic-associated diarrhoea (AAD) in adults at high risk for this condition (Ventria-News, 2012). Also, the group of Takeshi Matsumura in Japan expressed canine interferon using strawberry as platform and completed clinical trials.

This work has not been published yet (PBVA, 2013). As a last example, Protalix, in Israel, is developing glucocerebrosidase (GCD) enzyme (PRX-112) for the potential treatment of Gaucher disease aimed at oral delivery. The oral GCD is naturally encapsulated within carrot cells. Protalix has recently initiated a Phase I clinical trial of PRX-112 and initial results were announced in October 2013. Overall, oral GCD was found to be safe and well tolerated in all 12 patients. The presence of the enzyme was detected in patients' blood circulation, and the enzyme demonstrated biological activity. Concluding results are expected during the fourth quarter of 2013 (Protalix, 2013).

Other successful examples of antibodies produced in edible organs are the 2G12 anti-HIV IgG produced in maize seeds (Rademacher et al., 2008; Ramessar et al., 2008), although in this case the product was intended for topical use rather than for oral treatment; more recently a rice-based llama heavy chain antibody fragment against Rotavirus named Mucorice-ARP1 was orally administered to immunocompetent and immunodeficient mice challenged with Rotavirus, notably decreasing the viral shedding in both populations (Tokuhara et al., 2013).

4. Secretory IgA as a target molecule for oral passive immunotherapy.

Although immunoglobulin A (IgA) constitutes only 10%-15% of the total immunoglobulin in serum, it is the predominant immunoglobulin class in external secretions such as breast milk, saliva, tears and mucus of the bronchial, genitourinary and digestive tracts. The daily production of secretory IgA (sIgA) is greater than that of any other immunoglobulin class. Every day, a human secretes from 5 to 15 g of sIgA into mucous secretions (Goldsby et al., 2003).

SIgA is a multiprotein complex comprising two full IgA molecules dimerized by a short Joining chain (JC) and surrounded by the secretory component (SC), a polypeptide resulting from the proteolytic cleavage of the poly-immunoglobulin receptor (pIgR) present in the surface of most mucosal epithelial cells. Each IgA is a tetramer of 2 light chains (LC) and 2 heavy chains (HC). HC may occur in two isotype forms, namely HCα1 and HCα2 which differ structurally in the hinge region between the first and second domains. The hinge region of HCα1 is comprised of 23 residues, while HCα2 has only 10. The greater size of this hinge region provides HCα1 with an elongated structure which confers to the protein more flexibility and a greater antigenic reach. On the other hand HCα2 is more compact, and therefore less susceptible to proteolytic degradation (Furtado et al., 2004). There are also two types of LC designated kappa (κ) and lambda (λ) with neither structural nor functional differences described between them (Foley et al., 1991). Another way to describe the structure of the secretory IgA is attending to the fragments in which it can be pieced when the hinge region is cleaved. These parts are Fab' fragments which comprise the variable regions and first

constant domain of HC and LC, and FC fragments which consist of the second and third constant domains of HC (Figure 1).

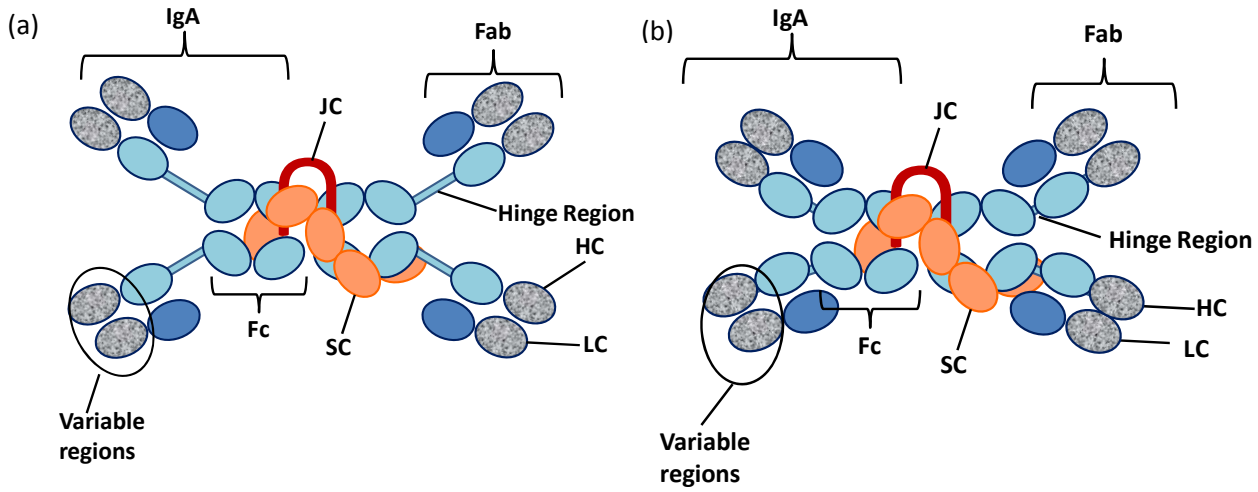

Figure 1. Schematic representation of secretory IgA structure. Each sIgA consists of two IgA molecules joined by a JC and surrounded by a SC. a) sIgAα1 characterised by an elongated hinge region; b) sIgAα2 characterized by a short hinge region. HC, LC, JC, SC correspond to Heavy Chain, Light Chain, J-chain and Secretory Component. Fab and Fc correspond to Antibody-binding Fragment and Fragment crystallisable.

Secretory IgA (sIgA) serves as the first line of defence in protecting the mucosal surfaces by neutralizing pathogenic microorganisms. Because its polymeric form, sIgA can cross-link large antigens with multiple epitopes. Binding of sIgA to bacterial and viral surface antigens prevents attachment of the pathogens to the mucosal cells, thus inhibiting viral infection and bacterial colonization. Complexes of sIgA and antigen are easily entrapped in mucus and then eliminated by the ciliated epithelial cells of the respiratory and genitourinary tract and/or by peristalsis of the gut. For this reason, sIgA is the best suited antibody isotype for OPI (Corthesy, 2003; 2010; 2013).

In spite of all the obvious advantages that sIgA presents as a therapeutic molecule, its recombinant production has been rarely attempted for commercial purposes, probably due to the technical difficulty involving the expression, assembly and recovery of a protein complex made of four different polypeptides and comprising a total of ten individual monomers. In physiological conditions the biosynthesis of a secretory IgA complex requires the cooperation between plasma cells, which produce dimeric IgA (dIgA), and mucosal epithelial cells that express the polymeric Ig receptor (pIgR). After release from the local plasma cells, the dIgA is transported across the mucosal epithelial cells where it binds the pIgR, which is then cleaved in the luminal surface of the epithelium releasing the mature sIgA to the lumen. Some attempts to produce a correctly assembled sIgA by co-expressing all four different genes (HC, LC, JC and SC) in a single

mammalian cell type have been successful (Chintalacharuvu and Morrison, 1997), however, to our knowledge, yields have not been reported and the technology has not been further developed to achieve a commercially viable product.

As stated before, the first attempt to produce a plant-made sIgA for passive immunization was the murine hybrid Guy's 13 (Ma et al., 1995; 1998); this technology was later acquired by Planet Biotechnology (Larrick et al., 2001), evaluated in phase I and II clinical trials and registered as a medical device (Weintraub et al., 2005). Since then, just a few research groups have reported the expression of sIgA in heterologous systems. Wieland et al. (2005) successfully expressed chicken sIgA against *Eimeria acervulina* and more recently Virdi et al. (2013) produced sIgA-like antibodies against enterotoxigenic *E.coli* in seeds of *Arabidopsis thaliana*. All in all, the production of human sIgA antibodies at a commercial viable scale remains an unsolved technological problem. When sIgA production is intended for mucosal passive immunotherapy, plant-based platforms, and most specially those using edible plant organs, appear to be the most promising candidates.

5. Practical considerations for antibody production in plants.

The design of a plant biofactory for antibody manufacturing requires multiple considerations involving not only the expression levels of the antibody but also additional aspects as subcellular targeting, protein degradation, glycosilation patterns and downstream strategies, all of them influencing the yield, quality and cost of the final product (Sarrión-Perdigones et al., 2011). To date, most of these aspects have been addressed separately, mainly on an empirical basis. However, future optimizations will probably require designs that integrate all of them following a global approach.

5.1 Subcellular Localization

Although extracellular secretion is the natural route for antibodies in mammals, targeting antibody chains to specific compartments in the plant cell can result in advantages in terms of stability, yield or downstream processing (De Muynck et al., 2010). Among the different compartments that have been tested as destination for recombinant antibodies (chloroplast, plasma membrane, vacuole, etc.), the secretory pathway seems to be the most convenient route for a correct antibody folding and assembly due to the oxidizing environment, the low abundance of proteases and the presence of molecular chaperones found in the endoplasmic reticulum (Ma et al., 2003). Antibody chains are targeted to the secretory pathway using an appropriate N-terminal signal peptide, either native (Hiatt et al., 1989b; Sainsbury et al., 2008) or replaced by a plant one (Düring et al., 1990). Once there, they can either be efficiently retrieved from the cis-Golgi

back to the ER using a C-terminal H/KDEL retention signal or secreted to the apoplast, following the secretory pathway (De Muynck et al., 2009; Petruccelli et al., 2006). Although several antibodies have been reported as strictly apoplastic (De Muynck et al., 2009; De Wilde et al., 1998; Düring et al., 1990), retention in the ER not only seems a possibility for yield improvement but also for avoiding complex plant Golgi-derived glycosylation patterns that could cause immunogenicity in target organisms (Bencurova et al., 2004; Gomord et al., 2010).

5.2 Glycosylation

One of the main advantages of plants is that, as eukaryotic platforms, they are able to express glycoproteins. However, final glycopatterns differ between plant and animal cells. Whereas ER glycosylation patterns are shared, a number of differences occur at the level of the Golgi apparatus. In the plant Golgi, *N*-linked glycan complexes are decorated with β-1,2 xylose and α-1,3 fucose residues, whereas the human *N*-glycan contain α-1,6 fucose. In addition, β-1,3 galactose and fucose are linked to the terminal N-acetylglucosamine of plant N glycans forming a Lewis structure, whereas in mammals a β-1,4 galactose is often combined with sialic acid (Ko et al., 2009; Saint-Jore-Dupas et al., 2007) (Figure 2).

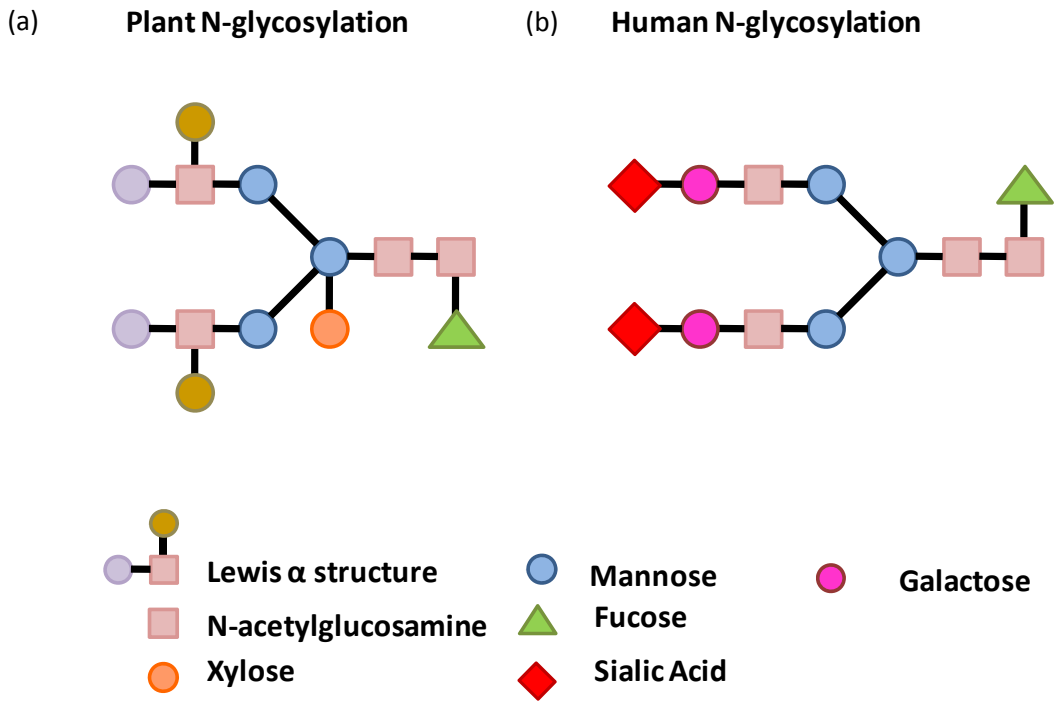

Figure 2. Schematic representation of plant and human N-glycosylation patterns. a) plant N-glycosylation pattern; b) Human N-glycosylation pattern.

Different strategies have been developed to express recombinant proteins with a mammalian-like N-glycosylation pattern. A first approach to humanize plant N-glycosylation is the use of RNAi for the downregulation of endogenous α-1,3-fucosyltransferase (FT) and β-1,2-xylosyltransferase (XT) which leads to complex N-glycosylation lacking immunogenic xylose and fucose epitopes (Schahs et al., 2007; Strasser et al., 2008). The next step consisted on the expression of a chimeric form of the human β1,4-galactosyltransferase (GalT). This was first attempted by Bakker et al. (2001) and later improved by Strasser et al. (2009) who reported the production of mAbs with a homogeneous galactose structure by targeting GalT to a late Golgi compartment where the final steps of *N*-glycan processing occur.

The last approach to achieve the complete human N-glycosylation pattern was the addition of terminal sialic residues, which was achieved by engineering of the full mammalian *N*-acetylneuraminic acid (Neu5Ac) biosynthesis pathway into *Arabidopsis thaliana* (Castilho et al., 2008). With this system, the sialylation of the recombinant monoclonal antibody 2G12 in *Nicotiana benthamiana* retaining full neutralization activity was reported. Moreover, this sialylated antibody was also free of xylose and fucose, as it was produced in mutants that lacked plant-specific *N*-glycan residues (Castilho et al., 2010).

A great effort has been made to adapt N-glycosylation, but it is only recently that attention has been paid to O-glycosylation. As stated by Castilho et al. (2012), plants lack endogenous glycosyltransferases that perform mammalian-type Ser/Thr glycosylation, which could interfere with the production of defined *O*-glycans. For this reason, they are attractive hosts for engineering of *O*-glycosylation steps. Two groups have reported the first approaches to deal with the engineering of sialylated mucine type O-glycans to achieve glycosylation patterns even similar to mammals (Castilho et al., 2012; Yang et al., 2012). This latter step represents an important milestone in the humanization of plant glycosilation patterns.

It is recommendable to mimic mammal glycosylation in therapeutic proteins to avoid unwanted antigeneicity and to gain stability. However, this issue is probably not so determining when the therapeutic proteins are aimed at mucosal treatments. Every day in our diet, we consume plant-specific glycans and for this reason, plant glycans attached to these recombinant proteins are not expected to generate antigenicity. Moreover the concern of regulation agencies on glycosilation patterns has probably decreased significantly after the commercialization of ELELYSO®, the injectable Glucocerebrosidase from Protalix and Pfizer, which showed excellent results in clinical trials in spite of carrying Xylose and Fucose residues (Aviezer et al., 2009; Cox, 2010; Shaaltiel et al., 2007).

5.3 Antibody Degradation

One factor strongly influencing recombinant antibody quality and yield is its stability in a heterologous environment. Proteases may affect the integrity of antibodies during both protein accumulation and protein extraction. Antibodies may undergo complete hydrolysis directly reducing the final

yield or partial degradation which can alter the integrity and activity of the final product (Benchabane et al., 2008; Faye et al., 2005). Moreover, it has been proposed that a balanced coexpression of heavy and light chain is another clue factor for achieving high yield, since unassembled antibody chains, which have been retained by the ER resident chaperone BiP, could be degraded via plant ERAD-like systems, in a similar way as occurs in mammals (Muller et al., 2005; Orzaez et al., 2009).

Gathering together results from various host species and different antibodies, a vast collection of antibody degradation fragments of different sizes have been reported, ranging from partially assembled complexes showing molecular weights between 100 and 150 kDa, to smaller ones as partially degraded un-associated HC (44 kDa), Fab, (Fab)2 and FC fragments around 44 kDa (De Neve et al., 1993), unassembled LCs and eventually, smaller degradation fragments difficult to assign.

To date, several strategies can be used to deal with this quality drawback. Although it has not been fully proved, a balanced expression of antibody chains seems to be the simplest manner to avoid chain degradation. For this, convenient promoters should be used to boost the expression of antibody chains which are limiting the generation of complete antibody complexes. Other approaches are the use of tissue specific promoters to confine transgene expression to compartments with reduced metabolic activity, targeting proteins to specific cellular organelles (Benchabane et al., 2008), or addition of gelatine as competitor substrate for peptidases (Wongsamuth and Doran, 1997). Gene knockout or silencing of plant peptidases is also a tool to take into account if there is a single or only a few target peptidases which are not essential for plant growth (De Muynck et al., 2010). Last, the co-expression of recombinant protease inhibitors interfering with endogenous proteases has also been proposed (Benchabane et al., 2008; Robert et al., 2013).

6. Passive immunization against rotavirus in edible fruits as a proof of concept.

The enteric pathogen Rotavirus is the most common cause of severe diarrhoea among infants and young children. By 5 years of age, almost every child has been infected by rotavirus at least once. It is responsible for more than 114 million episodes of diarrhoea, 25 million clinic visits, 2.4 million hospital admissions and more than 450,000 deaths per year, most of them in developing countries due to the lack of safe drinking water, sanitation and hygiene as well as poor overall health and nutritional status (WHO, 1999).

In countries with temperate climates rotaviruses display a seasonal pattern being usually more frequent in winter, while in the tropics the seasonality of rotavirus diarrhoea is less predictable and infections can occur during all year. Rotaviruses are spread by the faecal-oral route and, although young children are the most susceptible to get infected, it can also occur in adults, especially if they are in contact

with children or in the elderly. Once the infection occurs and the viruses pass the gastric barrier, they get attached to mature enterocytes at the intestinal villi and then they are internalized. The pathophysiology of rotavirus is not completely understood but involves a deficient absorption of water and nutrients due to the damage of intestinal epithelium. After an incubation of 1-3 days, rotavirus gastroenteritis normally begins with fever and vomiting followed by watery diarrhoea. Symptoms usually last 3 to 8 days, with serious complications as dehydration, electrolyte and acid-base disturbance. The most severe symptoms tend to occur in children between six months to two years of age, and those with compromised or absent immune system functions (Parashar et al., 2003).

There are two Jennerian vaccines against rotavirus currently in the market: Rotarix and RotaTeq. However, many times vaccines stimulate the production of IgG antibodies but are poor inducers of mucosal IgA antibodies which are needed to prevent this type of enteric infections (Lycke, 2012). Moreover, general malnutrition and chronic diseases, interference from maternal antibodies and co-infection with other enteric pathogens might also reduce the effectiveness of vaccines. Financial and logistical challenges can also be an obstacle as vaccination campaigns are not always possible in developing countries due to the high prices and transportation of vaccines.

OPI has repeatedly shown to be effective against enteric infections and therefore is a potential alternative or a valuable complement to vaccination (Monger et al., 2006; Virdi et al., 2013; Zimmermann et al., 2009). If competitive yields are achieved, edible fruits could be a convenient and inexpensive platform of production of neutralizing antibodies aimed at OPI. Scaling-up fruit production is simple by following well-established agronomic procedures. Besides, the Generally Regarded As Safe (GRAS) status of palatable plant organs can significantly reduce cost if exhaustive purification is not needed for oral or mucosal use.

Tomato is a world-wide cultivated crop, one of the most important in terms of biomass production. It is edible without thermal treatment, has low allergenicity rates and its allergens are well described (Le et al., 2006). Tomato crops have an optimum growth in differing conditions. It is a high yield edible crop (max 100 Ton/Ha/Year) and it is also well adapted to greenhouse cultivation under the confined conditions required for molecular farming. Tomato and other fleshy fruits are commonly discarded as potential biofactories probably because of their low protein content per fresh weight (Virdi and Depicker, 2013). However, when water is removed by drying, its protein content reaches 14% (while rice seeds contain aprox. 7%). Most interestingly, it is also a model crop, relatively easy to transform and with many biotech tools available. Early naïve misconceptions on plant edible vaccines (often depicted as ready-to-eat raw fruits without dose control), raised scepticism and forced many researchers in the field to move towards non-edible production platforms intended for antigen purification. Despite of this, it is widely recognized that for the production of oral/mucosal therapeutics, the use of GRAS organisms constitutes an important advantage. Although the capacity of tomato fruits to accumulate functional antibodies has not been yet

addressed, they have been reported to successfully produce recombinant oral vaccines (Walmsley et al., 2003; Zhang et al., 2006).

As a proof of this general concept, we decided to engineer tomatoes for the production of anti-rotavirus antibodies. As a first approach, the production of a monomeric human IgA directed against the outer VP8* peptide of the model rotavirus strain SA11 was attempted (chapter 1). To gain further acceptance for the tomato factory concept, the combination of IgA production with a transgenic labelling system based in anthocyanins accumulation was also assayed (also described in chapter 1), as it would serve as an additional colour-based tracing system for increased security .

The proposal of using tomato as production platform for OPI agents is based on the assumption that the expression and accumulation of the recombinant therapeutic product will not alter the edibility status (absence of toxicity) of the wild type fruit. This general assumption has never been contrasted experimentally, and it will be addressed in chapter 2 by comparing the tomatoes developed in chapter 1 with wild type in-house grown tomatoes as well as commercial varieties using proteomic and metabolic approaches.

Once the feasibility and security of fruit-based IgA production is addressed (as described in chapters 1 and 2), it was necessary to proceed to the production of a full sIgA. As described in this introduction chapter, there are many considerations regarding the optimization of the production of an antibody in plants, many of which can only be addressed experimentally. We decided to undertake an exhaustive comparison of different sIgA configurations, comparing heavy and light chain isotypes as well as different subcellular localizations. Such exhaustive comparison requires the combinatorial construction of many multigenic structures, and would not have been possible without the use of a modular cloning system named GoldenBraid (Sarrión-Perdigones et al., 2011b), and the use of *Nicotiana benthamiana* transient expression methodology. Chapter 3 describes the construction and expression analysis of 16 different sIgA configurations, the selection of the best performing configuration and the demonstration of the formation of a sIgA complex in the plant cell.

All together, this work represents an important step forward for the production of a safe and cost-effective production of sIgA as an oral immunotherapy agent in edible plant organs.

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Objectives

Mucosal passive immunization is a strategy with a great potential for fighting against enteric infections. The overall objective of this thesis is to trace a pathway towards the development of fruit-based antibody formulations for mucosal treatments against infectious diseases.

To fulfil this general objective, the following specific objectives were proposed:

1. To generate transgenic tomato fruits expressing a model human immunoglobulin A (IgA) with neutralizing capacity against the enteric pathogen rotavirus.
2. To establish a method to distinguish IgA-expressing transgenic tomato fruits from wild type fruits using anthocyanins as labelling traits.
3. To evaluate possible unintended effects in the composition of IgA-tomato fruits.
4. To select, among different multigenic combinations, the most suitable version of secretory IgA for its recombinant production in plants.

Chapter 1

Neutralizing antibodies against rotavirus produced in transgenically labelled purple tomatoes.

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1. 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 antinutrients 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 owing 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 et al. (Ma et al., 1994; Ma et al., 1995; Ma et al., 1998) who expressed in tobacco a hybrid murine / human secretory immunoglobulin G/ A (Guy's 13 sIgG / A) that is effective against tooth decay. 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 (Hayward, CA). This was the first plant-made antibody that was evaluated in phase I and II clinical trials (Weintraub et al., 2005) as CaroRx^{TM}. In the United States, CaroRx^{TM} 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., 2006a).

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 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 the 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.

2. Results

2.1 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 because of differences in stability conferred by the antibody variable regions, which might be exacerbated by the use of synthetic or semisynthetic 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 that 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 (Figure S1a,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 IgHa1 and Igk, respectively. Convenient restriction sites were engineered at the 5' ends of each constant region to allow for the direct cloning of the variable region of the heavy chain (VH) and variable region of the light chain (VL) derived from the Griffin.1 phage display library (Figure S1a). 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 recombination reaction between attL and attR sites (LR recombination). As a result, plant expression constructs were generated containing either the heavy chain (HC) or the light chain (LC) 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 HC and the LC for each antibody to be analysed were transferred to Agrobacterium and infiltrated into tomato fruits. The high cotransformation 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 coexpression of HC and LC proteins in planta as detected in Western analysis under nonreducing conditions. When IgA_2E4 and IgA_2B3 were transiently cotransformed, only LCs were detected, whereas their respective HCs 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 HC and LC demonstrated that endoplasmic reticulum (ER) retention resulted in higher antibody yields (Figure S2). 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 ERretention signal. For this purpose, an in trans cotransformation 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 cotransformation experiment, an in cis cotransformation approach was also carried out (referred as NH_IgA). In this case, in order to prevent the 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 Figure S1b) carrying 35S:IgH2A1-kdel and NH:IgL2A1-kdel expression cassettes located within the same T-DNA.

2.2 Transgenic fruits accumulate high levels of mAb

Primary transformants from cis and trans cotransformation 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 titres and homozygosity. 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 analysed 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 nonreducing conditions was present in the form of a 45-kDa band, which reacted with both 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.

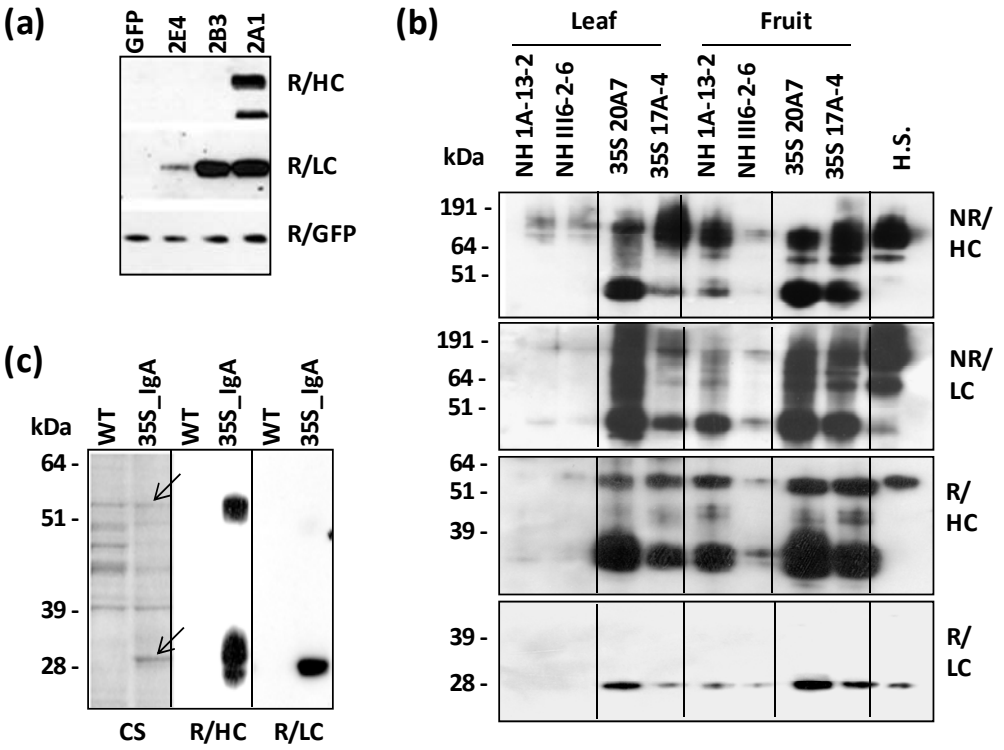


Figure 1. Western blot analysis of IgA expression in tomato. (a) Transient expression analysis of scFV-derived 2E4, 2B3 and 2A1 antibodies using direct fruit-agroinjection. Fruits were co-agroinjeted with ER-retained heavy and light antibody chains, together with GFP as internal control. (b) Western 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.

IgA production levels were quantified by ELISA in the fruits of the NH_1A-13-2 homozygous line, reaching 15.6 ± 1.2 µg IgA equivalents ⁄ g fresh weight (FW) [1.1 ± 0.1% total soluble protein (TSP)] (Table 1). The in trans cotransformation strategy (35S_IgA) resulted in 20% (4 of 20) of the T1 kanamycin-resistant plants showing both HC and LC expression in vegetative tissues. Following the same minibreeding 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 1b). As in the NH_IgA approach, a significant part of the total antibody content in the 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 1b, 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 because of the low total protein content in fruits. By comparison, IgA levels of up to 90 µg IgA / g FW were achieved in the 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 comigrating 55-kDa bands in crude fruit extracts.

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

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

2.3 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 (RR)] were analysed by antigenspecific ELISA to assess the specific VP8* binding activity and by Western blot to check for antibody integrity. In addition, red ripened tomatoes that had been air-dried at room temperature during 1 month were also analysed (dried tomato samples).

As shown in Figure 2a, the anti-VP8* activity, expressed relative to fruit FW, 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 clearthat 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).

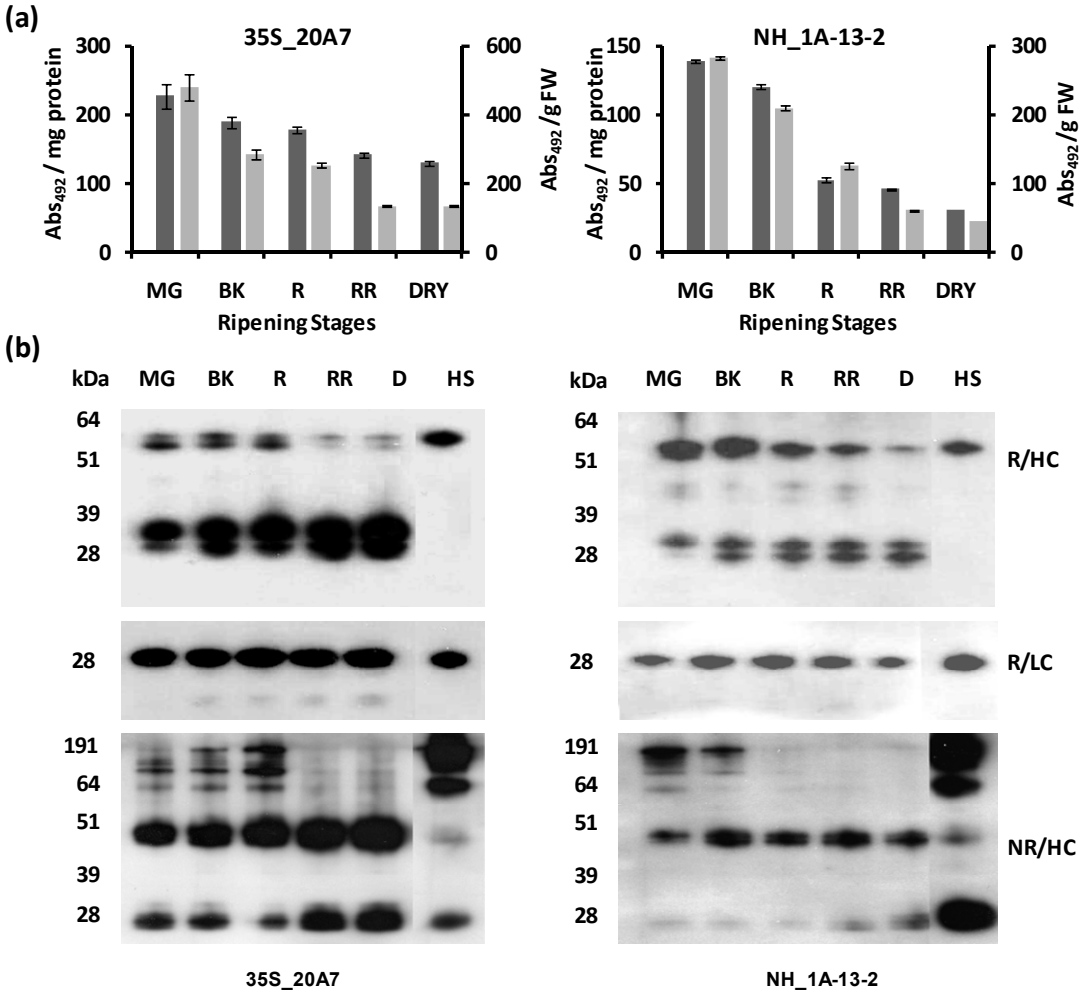
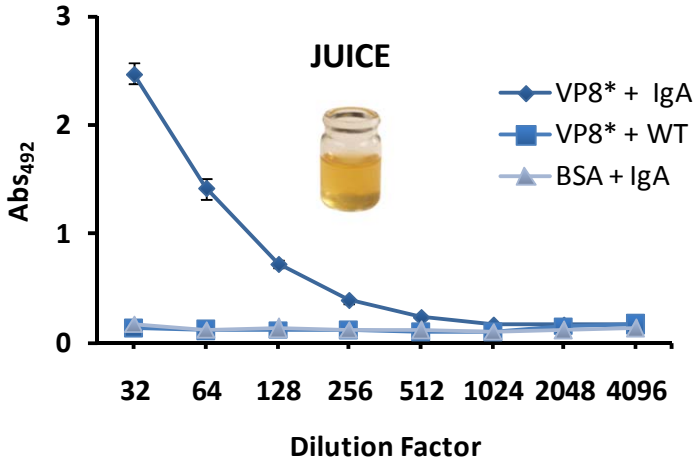


Figure 2. IgA accumulation profile during tomato fruit ripening. (a) Evolution of anti-VP8* activity in fruit extracts of 35S_20A7 and NH_1A-13-2 elite plants referred to fresh weight (light grey) and total protein content (dark grey columns). (b) Western blot analysis of the same fruit samples as in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

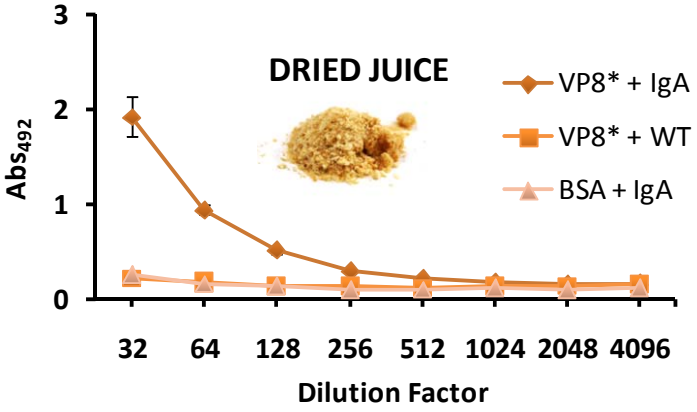
2.4 *Minimally processed tomato-based products show strong anti-VP8* activity*

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 bovine serum albumin (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 phosphatebuffered saline (PBS) (1 : 3) (w:v). The 'powder' format consisted in freeze-dried ground tomato powder, later reconstituted in PBS using three volumes of the original FW. 'Dried juice' consisted in freeze-dried 'juice' sample, later reconstituted in water using three volumes of the original FW. All samples were clarified by centrifugation and incubated in serial dilutions in an antigen-ELISA test. Control 'juice', 'powder' and 'driedjuice' samples from wild-type Moneymaker fruits also were prepared as described earlier. 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 titres showed consistent behaviour, as all three samples reached endpoint titres 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 FW, the recombinant antibody levels in the 'powder' samples reach remarkable levels up to 0.68 mg of IgA equivalents per gram of dry weight (Table 1).

(a)



(b)



(c)

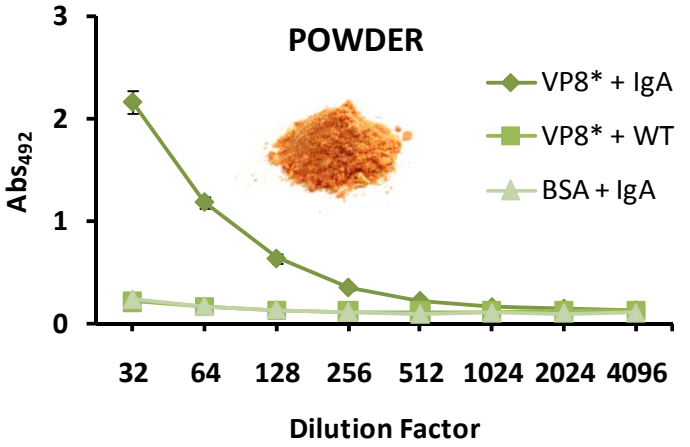


Figure 3. End-point antigen-ELISA titering of minimally-processed fruit-derived products. "Juice" is a clarified fruit extract; "Dried Juice" is a freeze-dried "juice"reconstituted in PBS; "Powder" is freeze-dried tissue reconstituted in PBS. Binding activity refers to the absorbance at 492 nm after 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.

2.5 *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 3.5x 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 because of the lack of Fc domain, as observed in the Western blot analysis in Figure 4b. Silver-stained PAGE of eluted fractions in Figure 4b illustrates the level of purification of the full-size recombinant IgA obtained after a single-step affinity purification with the SSL7 agarose.

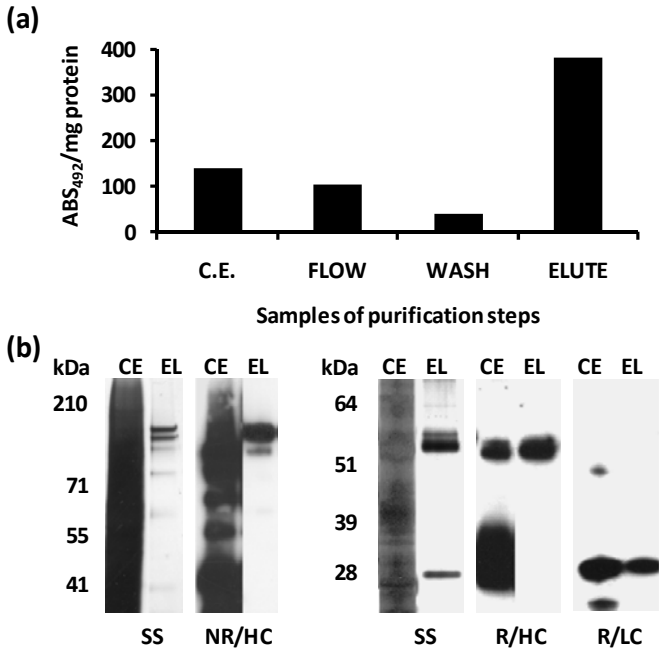


Figure 4. SSL7-based affinity purification of IgA from tomato fruits. (a) Specific Anti-VP8* activity measured in the different fractions of the purification process. (b) SDS-PAGE and western analysis of the starting tomato crude extract (CE) and the final SSL7-agarose eluted fraction (EL). SDS-PAGE 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.

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 titres (>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 carried out 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 titres >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 IC50 values of purified and unpurified 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.

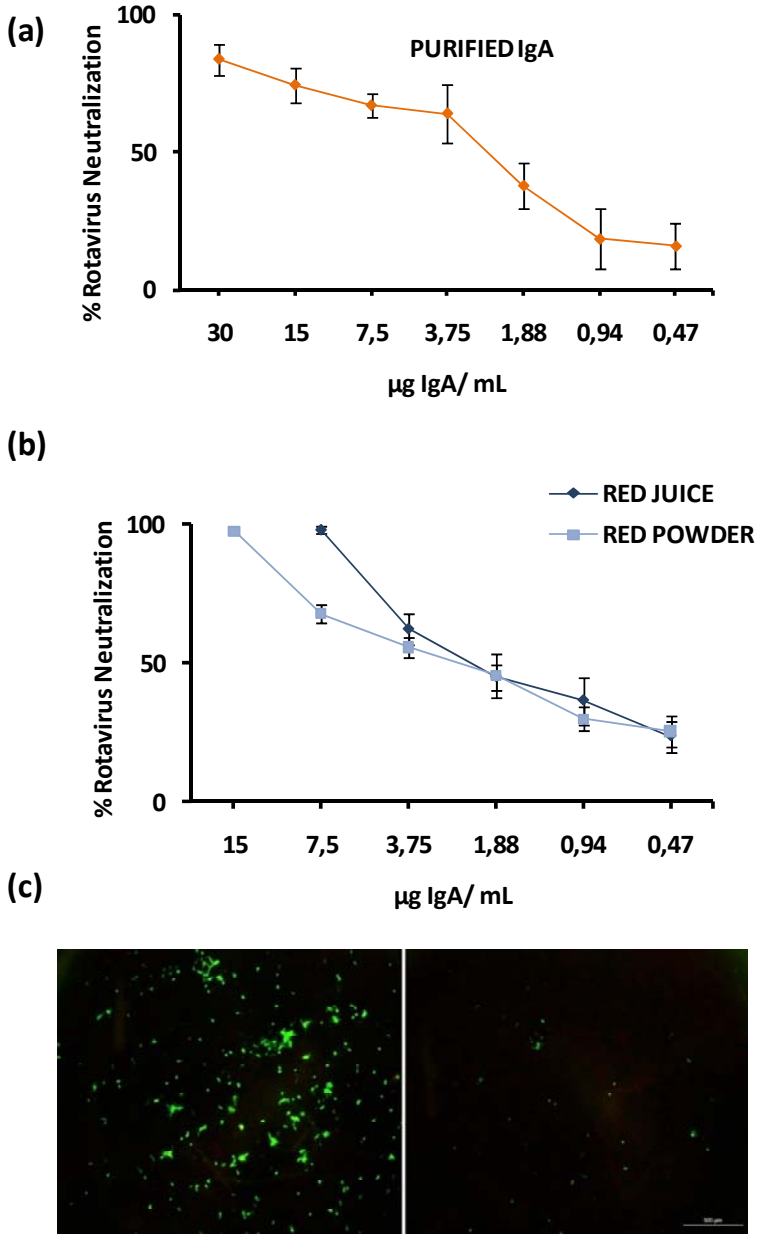


Figure 5. ***In vitro*** **rotavirus neutralization assays.** Trypsin-activated rotavirus of SA11 strain (10.000 ffu/mL) were pre-incubated for 1h with serial dilutions of IgA-containing samples or equivalent control samples and used to infect MA104 cell monolayers (2 x 10^4 cells/well). Infective foci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

2.6 *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 owing to the 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, purplefruited 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 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 lg µgA / 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 titres of tomato powder were also conducted, showing titration 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 virus neutralization (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 titres 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 (Figure 6c), a value similar to that observed previously in the red tomato 'powder'.

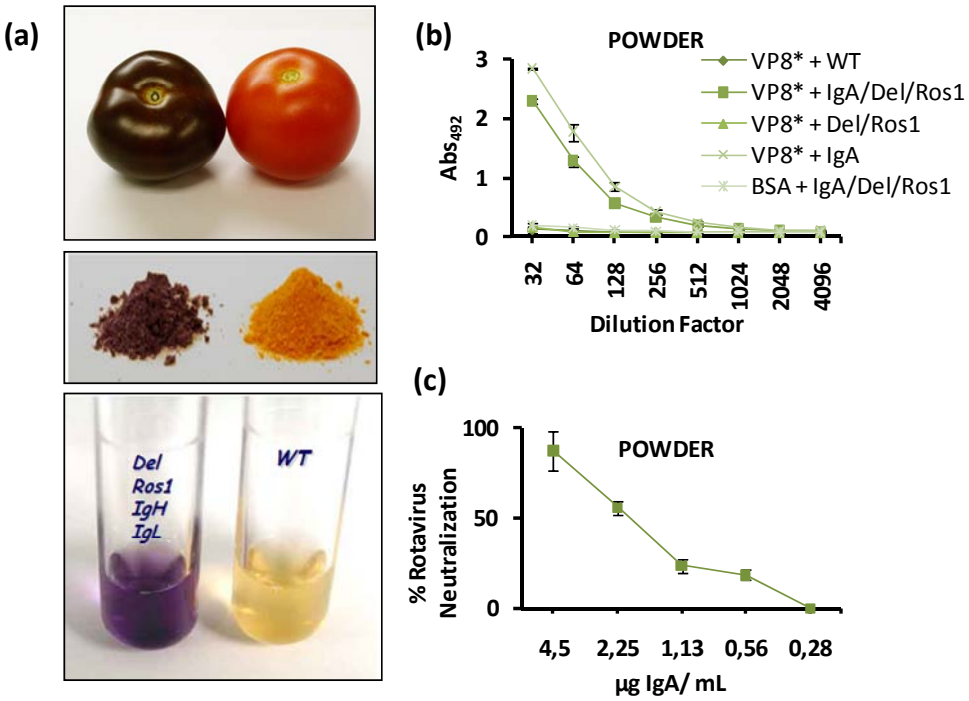


Figure 6. Purple tomatoes anti-VP8*. (a) Tomato fruit, "powder" and "juice" samples from WT (right) and Del/Ros1/IgH/IgL tomatoes (left); (b) Endpoint anti-VP8* ELISA titering of purple and red tomato "powder". Samples were obtained by pooling three fruits from each plant, namely a wild type plant (WT), 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.

3. Discussion

Diarrhoeas 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 in both 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 virus-like particles were used for oral delivery (Zhang et al., 2006b). 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 subcutanous 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 transmissible 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 VN. 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 contribution of additional components of the extract acting cooperatively with the antibody in neutralizing the infection. The effect of the edible plant extracts to enhance VN 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 greenhouse 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 g of antibody (IgA plus Fab's) per tomato plant, which can be administered as semicrude 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 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 the activity levels of nuclear-encoded antibody in ripened fruit. Moreover, the stability of Fab' fragments during ripening and postharvest 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 in antinutrients 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 by 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 laboratory, 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 HC accumulation.

In the final design of the recombinant antibody, a number of additional decisions were made. The endogenous signal peptide (SP) was substituted with the SP 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 cotransformation, 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 are 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 in IgA content was observed, reaching levels of 33.5 μg IgA / g FW, close to those reported for red IgA plants (41.2 μg / g FW). 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., 2011a).

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 into 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.

4. Experimental procedures

4.1 DNA constructs and vectors

The DNA sequences corresponding to the constant regions of human alpha heavy (HCa) and lambda light (LCk) antibody chains were obtained from the human library clones EHS1001- 9024643 and EHS1001-9024145, respectively (Open Biosystems, Huntsville, AL). LCk 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 LCk a number of elements, namely an attB1 recombination site, a Kozak sequence, the 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 LCk coding region (Table S1). The resulting LCk expression cassette still lacking the sequence corresponding to the VL was BP-cloned in pDNOR221 vector, generating an ENTRY vector named as pENTR_LCk. In a similar fashion, a pENTR_HCa vector was created for the convenient cloning of variable regions of the HC into an IgCa 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 preamplified 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 reamplified 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 x 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_LCk 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_HCa, generating pENTR_IgHa 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 Karimi et al. (2002). pBINJGW was engineered by introducing a Gateway cassette (Invitrogen, Carlsbad, CA) in the SmaI site of pBINJIT plasmid, following manufacturer's procedures. Binary expression vectors for heavy and light

antibody chains were obtained by performing LR reactions between ENTRY vectors (e.g. pENTR_IgL_2A1 and pENTR_IgHα_2A1) and binary destination vectors, generating plant antibody expression clones (e.g. pEXP_ 35SIgL_2A1 and pEXP_ 35SIgHα_2A1).

Heavy and LCs expression cassettes were also combined in a single binary vector under the constitutive 35S and the fruitoperating 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.

4.2 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 PBS pH 7.4. After mixing, the suspension was centrifuged twice at 4°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 4ºC at maximum speed, and the supernatant was used for the analysis.

4.3 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, Spain). VP8* expression was performed using the QIAexpressionist protocol

(http://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 s) 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 25.000 g spin, cells were sonicated (6 x 25 s) one more time and washed again with the same buffer. A last wash with ureafree buffer A was made.

Inclusion bodies were solubilized with 20 mM Tris–HCl, 0.5 M NaCl, 6 M guanidine HCl, 5 mM imidazole and 1 mM 2-b-mercaptoethanol at pH 8.0. The sample was stirred at room temperature for 1 h and then centrifuged for 15 min at high speed. The remaining particles were removed by passing the sample through a 0.22-lm filter. Purification was performed using HisTrap HP affinity columns (GE Healthcare, Buckinghamshire, UK) following manufacturer's procedures. Protein refolding in column was performed with 30 mL of a lineal 6–0 M urea gradient at a flow rate of 1 mL ⁄ min, starting with buffer B (20 mM Tris–HCl, 0.5 M NaCl, 6 M urea and 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.

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

Plates (Corning, New York, NY) were coated overnight with 10 lg ⁄ mL of recombinant VP8* for the detection of VP8* binding activity or 2 µg ⁄ mL of anti-human IgA a specific (Sigma Aldrich, St. Louis, MO) for IgA determination, in coating buffer (50 mM carbonate buffer pH 9.8) at 4ºC. Plates were then washed four times in PBS and blocked with a 2% (w⁄ v) solution of ECL AdvanceTM 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 h at room temperature. After incubation, plates were washed four times in PBS and the anti-human IgA a-specific horse radish peroxidase (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 four PBS washes, the substrate (*o*-phenilenediamine from Sigma Aldrich) was added and the reactions were stopped with 3 M HCl. Absorbance was determined at 492 nm. As a control for specificity, plates were coated with 10 µg ⁄ mL of BSA. A sample was considered positive when its absorbance was at least three 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 to 0.1 µg ⁄ mL of commercial IgA from human colostrum (Sigma Aldrich) was obtained 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 analysed and the mean ± SD for each line was calculated.

4.5 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 with silver staining following standard procedures. For Western blot analysis, blots were incubated with 1 : 20.000 anti-human IgA a-specific HRP (Sigma Aldrich) for the detection of the HC and 1 : 10.000 anti-human k LC (Sigma Aldrich) followed by 1 : 10.000 μg ⁄ mL HRP-conjugated anti-rabbit IgG (GE Healthcare) for the detection of the LC. Blots were developed with ECL plus Western blotting Detection System (GE Healthcare).

4.6 Protein SSL7 affinity purification

Protein extracts prepared as explained previously were further clarified using a 0.22-μm Stericup (Millipore, Billerica, MA) on ice. The clarified extract was directly used for purification with staphylococcal superantigen-like protein 7 (SSL7) agarose columns from InvivoGen (San Diego, CA) following manufacturer ¢ s protocol with minor modifications. Wash buffer was substituted by PBS (0.1 M phosphate and 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).

4.7 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 minimum essential medium (MEM from Gibco- Invitrogen, Carlsbad, CA) supplemented with 100 U⁄ mL penicillin, 100 lg ⁄ mL streptomycin, 2 mM glutamine, 0.7% CO3H and 10% foetal bovine serum (FBS) and incubated for 24 h at 37 ^{o}C 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 37 ^{o}C). After incubation, the monolayers were washed with MEM and incubated for 18 h in MEM containing 1 μg ⁄ mL trypsin at 37^{o}C 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 Moneymaker 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 37^{o}C, followed by three washes with PBS. The secondary antibody was an antimouse IgG FITC-labelled (Sigma, Sigma Aldrich, St. Louis, MO) (1 : 400). The monolayers were washed three more times with PBS and stained with Evans blue for 5 min, washed again with PBS and 20 µL of mounting fluid (Millipore) was applied to each well. Images were obtained by excitation with 480-nm light and acquired at 6.3x magnification factor using a Leica MZ16F binocular equipped with a Leica DFC300FX digital camera and Leica application suite software. The 50% inhibiting concentrations (IC50) were calculated by the method of Reed and Muench (Reed and Muench, 1938), using the concentrations present during the antibody-virus pre-incubation step.

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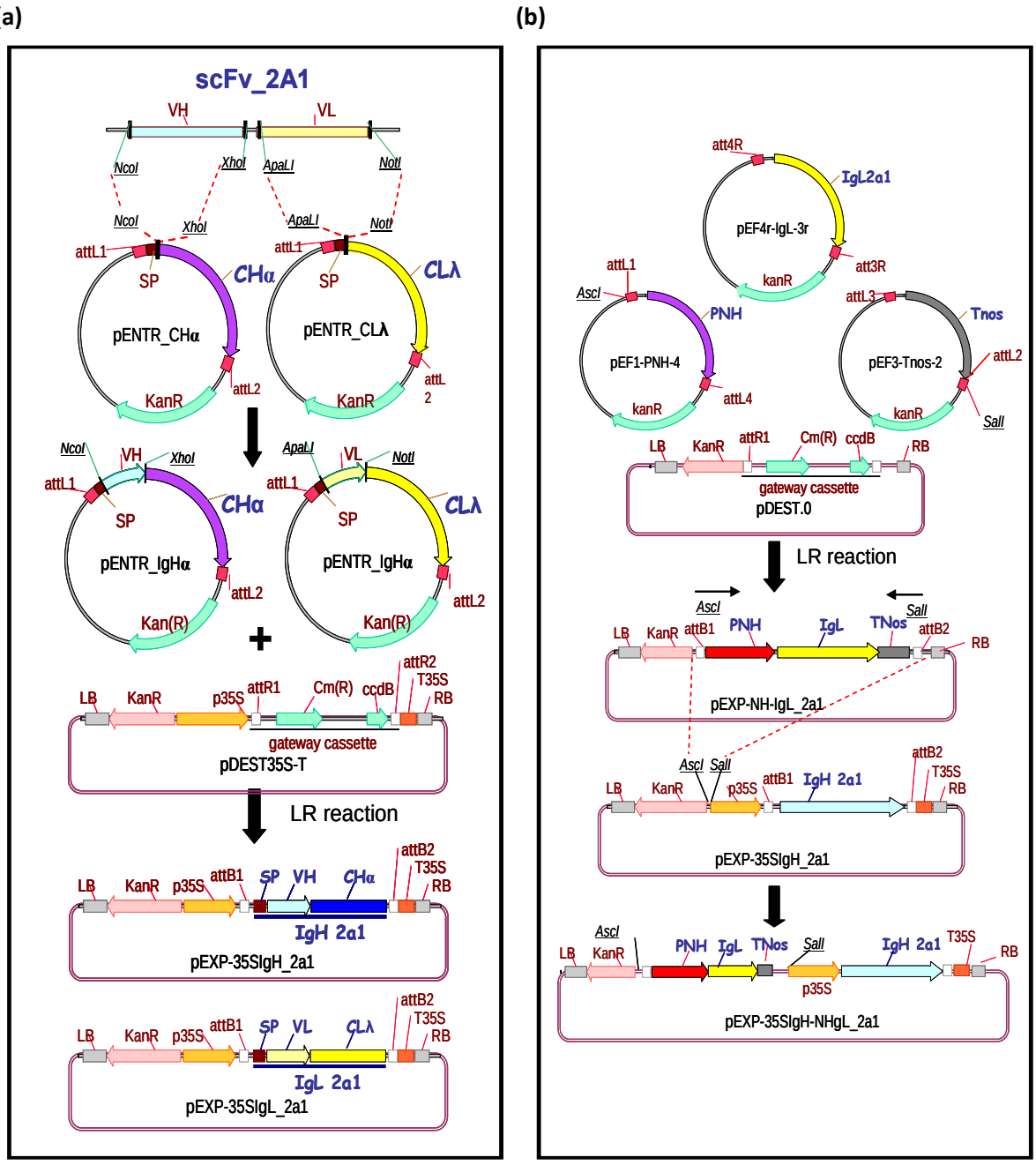
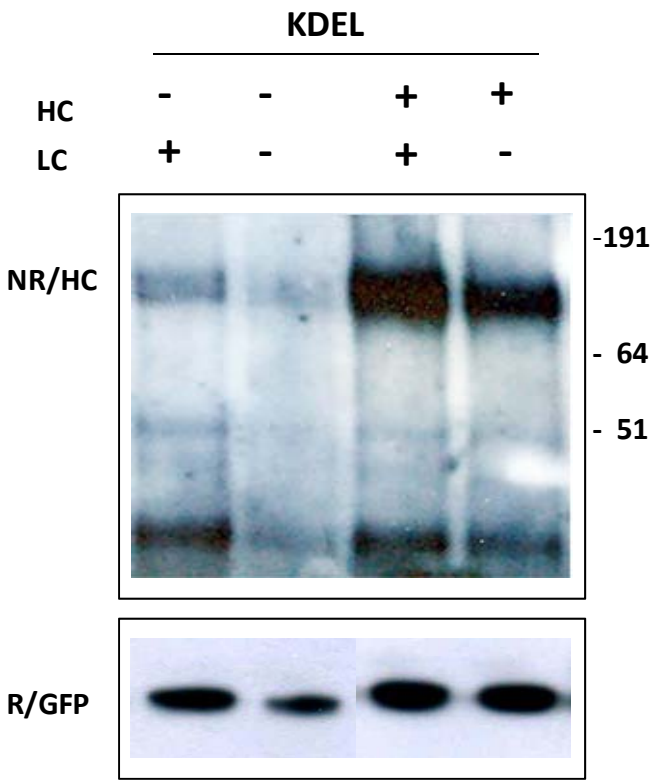
7. Supplementary material

Supplementary Table 1. Primer Sequences for DNA constructs and Vector.

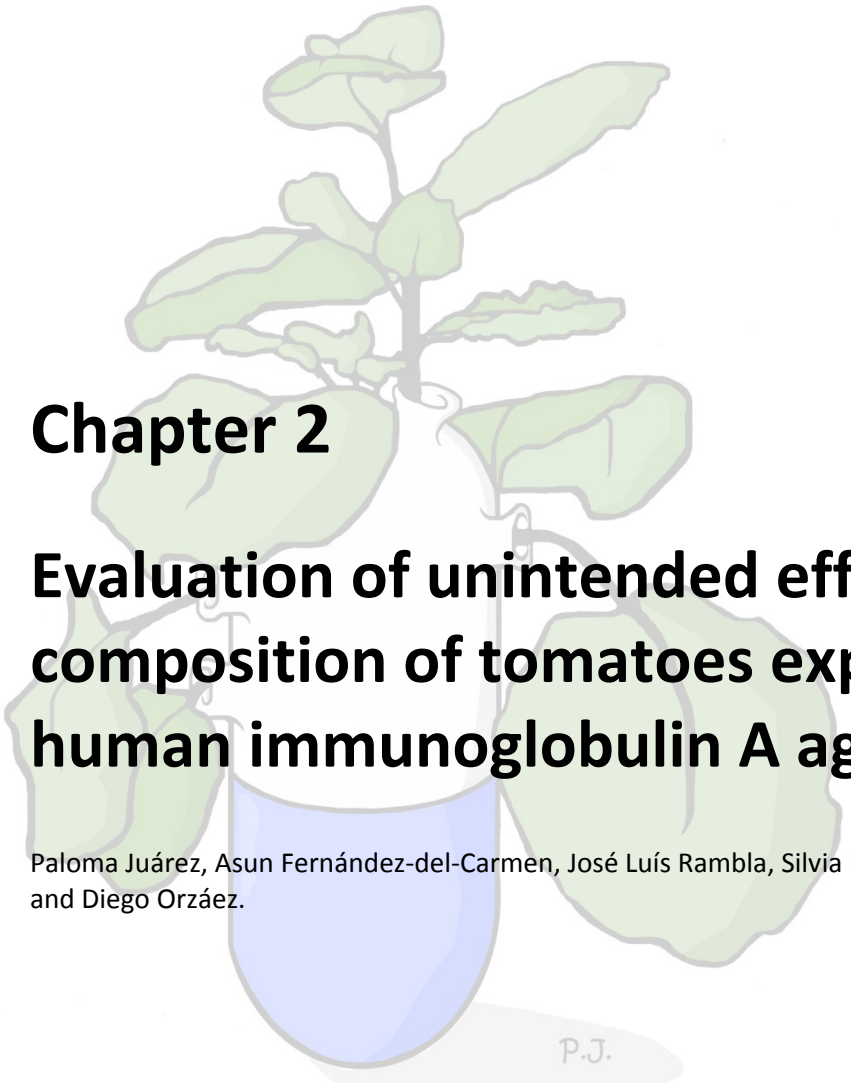
Primer	Sequence
D06Mar01	ggggacaagtttgtacaaaaaagcaggcttccacaatgggcacttcctctgtttttctactattccttctttctttttcttctccttctcccg
D06Mar07	tttcttttcttctccttctcccgagtgcacagtcgacagcggccgccgcccccactgtcactctgttcc
D06Mar08	ggggaccactttgtacaagaaagctgggttctagactatgaacattctgtaggggccactg
D06Mar00	cgagcgcatccccgaccagccccaaggtct
D06Mar19	tgaacctaagagcaggtcctcaagggccgg
D06Mar03	tgcaccgaccggcccttgaggac
D06Mar04	tgaaggtcttcccatgattccaaggc
D06Mar20	ccgggctgtgccgagccttggaatcatggga
D06Mar02	ttcttctccttctcccgtccctccttgccatggccgtcaccgtctcgagcgcatccccgaccagccccaa
D06Mar06	ggggaccactttgtacaagaaagctgggttcaaagttcatctttgtagcaggtgccgtccacct
L07Nov07	ggggacaacttttctatacaaagttgcaatgggcacttcctctgtttttc
L07Nov08	ggggacaactttattatacaaagttgtctaaagttcatcttatgaacat
L07Jun05	cgacaatctgatccaagctcaa
L08Jan01	ccgcgtcatcggcgcgccgggtcataacgtgactcccctt



Supplementary Figure 1. **Grafting of scFV 2a1 derived variable fragments into plant expression cassettes carrying constant frames of human IgA.** (a) Schematic overview for the cloning of HC and LC in plant expression cassettes. VH and VL were inserted into pENTRY-CHα and pENTRY-CLλ using restriction-ligation reactions. The combination of variable and constant regions were incorporated into destination vector PDEST35S-T by an LR gateway reaction to construct the final expression vectors pEXP-35SIgL_2α1 and pEXP-35SIgH_2α1. (b) Incorporation of NH promoter to LC. NH promoter, LC and Tnos terminator were grafted into PDest.0 by triple gateway LR reaction obtaining the pEXP-NH-IgL_2a1. The NH-IgL-Tnos construct was incorporated into the pExp-35SIgH-2α1 using restriction-ligation reactions to construct the pExp-35SIgH-NHIgL_2α1. VH and VL are variable regions of heavy and light chain respectively; Nco1, Xho1, ApaL1, Not1, Asc1, Sal1 are restriction enzymes; attL1, attL2, attR1, attR2, attB1, attB2 are gateway restriction sites. CHα and CLλ the constant regions of the heavy and light chain respectively; sp is the pectate lyase signal peptide. KanR is kanamycin resistant; p35S and pNH are respectively constitutive and fruit-operating promoters.



Supplementary Figure 2. Transient expression of apoplastic and ER-retained versions of IgA2A1. Heavy (HC) and ligh (LC) chains with (+) or without (-) KDEL extensions were co-agroinjected in fruits and analyzed by western blot under non-reducing conditions (upper panel). A 35S:GFP construct was added to the agroinfiltration mix to serve as a control of transformation efficiency (lower panel).



Chapter 2

Evaluation of unintended effects in the composition of tomatoes expressing a human immunoglobulin A against Rotavirus.

Paloma Juárez, Asun Fernández-del-Carmen, José Luís Rambla, Silvia Presa, Amparo Micó, Antonio Granell and Diego Orzáez.

1. Introduction

Antibody-based treatments aimed at mucosal passive immunisation (MPI) have a great potential in medicine and veterinary for the prevention of infection diseases. MPI is particularly advantageous for the treatment of enteric diseases (Monger et al., 2006; Virdi et al., 2013; Zimmermann et al., 2009). For most applications, passive antibody treatments require large amounts of antibodies to be delivered in the target mucosa in order to ensure protection (Virdi and Depicker, 2013). Therefore, MPI requires antibody production platforms which are cost-effective at a large scale. Recombinant monoclonal antibodies are excellent candidates for MPI due to their high specificity; however, as most recombinant antibodies are currently produced in mammalian cell platforms in which up-scaling leads to increasing production costs, their application in MPI is seriously hampered.

Low-cost neutralizing recombinant antibodies aimed at MPI can be inexpensively produced in edible plant organs. Many seeds, fruits, leaves, tubers and roots are considered safe and palatable for human consumption in unheated, minimally processed formulations (Tokuhara et al., 2013); therefore, it has been proposed that antibodies produced in plant organs with *Generally-Regarded-As-Safe* (GRAS) status could be delivered as dose-controlled ingredients in partially processed formulations without the need exhaustive purification (Virdi and Depicker, 2013). This would certainly reduce the manufacturing costs and would facilitate scaling-up, as plant platforms are reportedly easier to scale up than other platforms as e.g. those based on fermentation (Tokuhara et al., 2013).

The lack of exhaustive purification can be, on the other hand, a double-edged sword, as the composition of the final product ultimately results from an event of genetic modification. It could be argued that, besides inducing the intended production of a recombinant antibody, transgenesis could eventually lead to unintended effects in the final fruit composition (Gong and Wang, 2013; Rischer and Oksman-Caldentey, 2006). Such unintended effects could be derived from the integration of the transgene and/or from biological interactions caused by transgene-encoding proteins. With commercialization of GM crops, unintended effects have become one of the most controversial issues when debating their biological safety (Herman and Price, 2013). Although during the last 20 years many studies made to assess the compositional safety of genetically modified crops pointed out the evidence that transgenesis is even less disruptive of crop composition than traditional breeding, (Di Carli et al., 2009; Herman and Price, 2013; Kusano et al., 2011), to our knowledge the absence of unintended, deleterious effects in the composition of genetically modified edible plants organs expressing recombinant antibodies has never been assessed. Therefore, it is important to perform a systematic evaluation of the possible unintended effects by comparing the genetically modified plant organs with wild type organs and also with traditional varieties.

Recently, our group produced a model human Immunoglobulin A (IgA) for passive protection against the enteric pathogen rotavirus in transgenic tomato fruits. Dry formulations compatible with oral intake and long term storage presented a high concentration of active antibodies (≈0,7 mg/g DW), which were shown to strongly inhibit virus infections in an *in vitro* virus neutralization assay. Minimally-processed fruit-derived formulations containing recombinant IgA were proposed as potential vehicles for low cost MPI treatments. The availability of IgA-producing fruits provides an excellent opportunity to test the possible unintended effects that the expression of a human antibody could have in the final composition of the fruit, and to infer the possible deleterious effects to human health that such unintended effects could have.

Safety concerns in tomato fruit composition arise fundamentally from two sources: proteins (allergens) and secondary metabolites (toxicants). So far, only three tomato protein allergens, Lyc e 1 (profilin), Lyc e 2 (invertase) and Lyc e 3 (nsLTP) are listed in the official IUIS allergen database, although additional potential allergens have been reported (Welter et al., 2013); Westphal et al. (2004; 2003) concluded that tomato profilin, Lyc e 1, is a minor human allergen whereas profilin Lyc e 2, beta-fructofuranosidase, is an important human allergen. Additionally, lipid transfer protein (Lyc e 3), is a potentially severe food allergen due mainly to its extreme resistance to pepsin digestion and is, therefore, considered a pan-allergen (Asero et al., 2000; OECD, 2008). To investigate the changes the in fruit protein profile associated with the over-expression of recombinant proteins, a proteomic analysis is required. Proteomics is a powerful non-targeted tool used to detect unintended differences derived from genetic manipulation. Proteomic based approaches have been used a number of times to compare protein profiles of transgenic maize (Coll et al., 2011), tomato (Corpillo et al., 2004) or potato (Khalf et al., 2010; Lehesranta et al., 2005) with their non transgenic counterparts (Herman and Price, 2013).

A second possible source of concern is the presence of toxic metabolites. Fruits are rich in chemically diverse compounds which can be present in a wide range of different concentrations. The most important toxicans in tomato are the steroidal glycoalkaloids α-tomatine and dehydrotomatine. Tomato glycoalkaloids are synthesized in tomato fruits during early development and then degraded during fruit maturation (Eltayeb and Roddick, 1984; Eltayeb and Roddick, 1985; Friedman and Levin, 1995; Kozuke et al., 1994). There is no analytical method capable to extract and detect all metabolites, however, in the last two decades, several methods have been established for large-scale analysis and comparison of metabolites in plant extracts (De Vos et al., 2007; Herman and Price, 2013). Among them, UPLC-MS specifically allows the detection and quantification of the kind of semipolar secondary metabolites which are the main concern in tomato fruit composition.

The goal of this work is to examine a broad set of components in the fruit in order to search for unintended effects produced by the expression of a human immunoglobulin A, which could eventually affect the safety of minimally-processed formulations derived from IgA tomatoes. In this chapter we will perform a

proteomic analysis to detect possible changes in the fruit proteome associated with the production of human IgA. In addition, a metabolomic profile by UPLC-MS comparing IgA and non-transgenic tomatoes will be carried out to detect possible unintended effects in the metabolome.

2. Results

2.1 Evaluation of IgA content in the fruit.

In order to evaluate possible unintended effects in fruit composition, two types of IgA-producing plants were selected for analysis. The first group was generated in co-transformation experiments (referred as 35S_IgA) in which tomato explants were simultaneously co-inoculated with two *Agrobacterium* cultures carrying heavy (HC) and light (LC) antibody chains respectively, both of them expressed under the control of constitutive 35S promoter. In the second group (referred as NH_IgA), both HC and LC were linked in a same T-DNA, with LC placed under the control of the tomato NH promoter (Estornell et al., 2009). In the second generation (T2), different segregating lines were obtained. Some NH_IgA segregant lines lacking the transgene (nullizygotes) were selected to serve as appropriate negative controls in subsequent assays. On the contrary, all 35s_IgA segregants analyzed, coming from a *co*- transformation approach, showed at least one of the two antibody chains. Consequently no 35S_IgA nullizygote could be included in the analysis. In total seven independent transgenic events from both groups (NH_IgA and 35s_IgA) showing a broad range of IgA levels were used in the different comparative analysis (see Table 1). This includes an "elite" T3 homozygous IgA line (line I) previously selected from the 35S_IgA group for its high antibody levels. A number of IgA-free control lines were also included in the analysis, which comprised (i) the above mentioned nullizygote plants from the segregating NH_IgA lines (ii) two wild type Money Maker plants from different seed batches (iii) three commercial tomato varieties from a local supermarket (Cherry, Plum and Round).

Tomato plants of all non-commercial lines were grown in the same greenhouse conditions. Fruits were collected 15 days after breaker stage and frozen tomato powder of each fruit was prepared for further analysis. A first step in the analysis consisted in assessing the antibody content of the different lines. For this, tomato powder samples coming from individual fruits were extracted in PBS buffer and the IgA content in the resulting crude extracts was quantified by means of ELISA analysis. As expected, the "elite" homozygous 35S_IgA line (line I) showed the highest IgA levels, with an average content of 37,5 µg/g FW. The IgA content of the remaining transgenic lines ranged from 25,5 µg to 1,9 µg IgA/g FW (see Table 1). To check the integrity of the IgA in the powder samples, a representative number of transgenic fruits was analyzed by Western blot. As expected, blots revealed specific bands corresponding to the HC (55 kDa) and the LC (28 kDa) in all the transgenic samples analyzed. A major degradation band earlier characterized as a 28 kDa HC fragment was also observed in blots developed with anti-HC antibody. In all cases the relative expression levels observed in Western blots were consistent with the ELISA quantification. No HC or LC bands were detected in non-transgenic control samples (Figure 1).

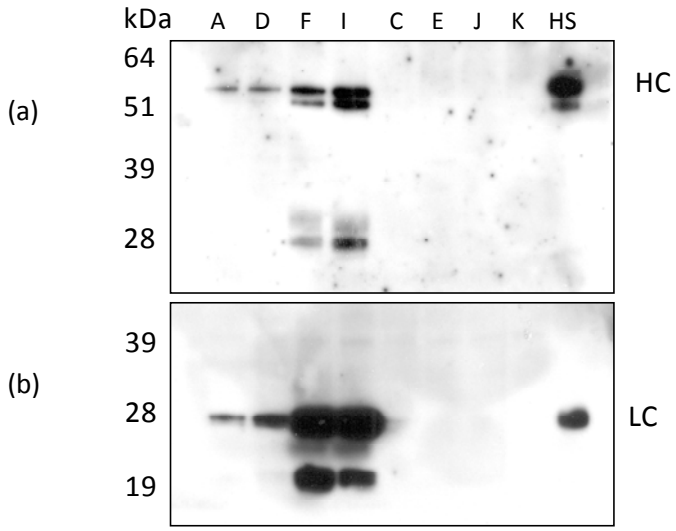


Figure 1. Western blot analysis of IgA expression in tomato fruit. (a) Western blot under reducing conditions, developed with anti Heavy chain antibody (HC) (b) Western blot under reducing conditions developed with anti Light chain antibody (LC). A and D are NH_IgA lines; F and I are 35S_IgA lines; C and E are Nullizygote; J and K are Wild Type Money Maker samples from two different seed batches and HS is a Human Serum sample.

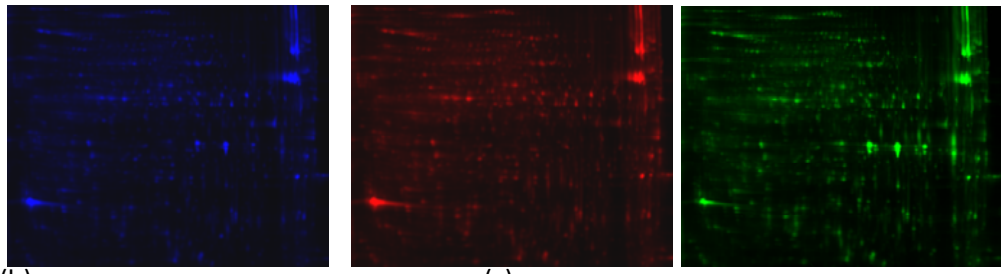
Table 1. IgA levels referred to fresh weight (FW) and to total soluble protein (TSP) in all lines.

ID	Line	Total IgA (µg/gFW)	Total IgA (% TPS)
A	NH-IgA	2,5 ± 0,3	0.2 ± 0.1
B	NH-IgA	5,7 ± 1,6	0.4 ± 0.1
C	NH-IgA Nullizygote	0	0
D	NH-IgA	1,9 ± 0,3	0.2 ± 0.1
E	NH-IgA Nullizygote	0	0
F	35s_IgA	13,0 ± 2,2	1.0 ± 0.2
G	35s_IgA	25,5 ± 6,5	2.0 ± 0.5
H	35s_IgA	20,9 ± 5,2	1.6 ± 0.4
I	35s_IgA Homozygous	37,5 ± 5,2	2.9 ± 0.4
J	Money Maker Seed batch 1	0	0
K	Money Maker Seed batch 2	0	0
L	Plum	0	0
M	Round	0	0
N	Cherry	0	0

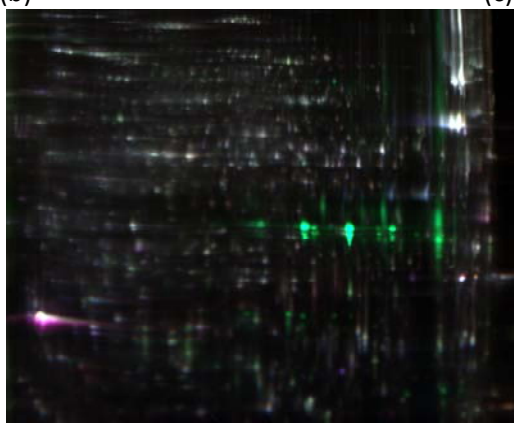
2.2 *Identification of differentially expressed proteins in IgA-tomatoes by 2D-DIGE and LC-MSMS.*

The first step in the assessment of the fruit content was directed to the analysis of the protein composition by 2D-DIGE. To maintain DIGE experiments within operative limits, a total of eight tomato lines were included: four selected lines comprising the whole IgA expression range were paired with four non transgenic lines, which included two in-house grown Money Maker plants as well as two NH_IgA nullizygote plants. To identify differentially regulated proteins, each gel was loaded with an internal standard (Figure 2a, blue), a selected transgenic line (Figure 2a, green) and a selected non transgenic line (Figure 2a, red), summing a total of four gels in the whole experiment. The internal standard was composed of a pool of all the samples used in the analysis. Each of the three samples in a gel was labelled with a different CyDye (Cy2, Cy3 and Cy5). The NH_IgA tomato samples (A and D from table 1) were paired with their corresponding nullizygote sample (C and E). The other two gels were loaded with 35s_IgA samples (F and I), each one in combination with one wild type (J and K). The three different images created for each gel were overlapped and the spots from the different gels were matched (Figure 2b). A total of 1001 spots were located in the four gels. Differential proteins are shown as green spots (with higher levels in transgenic line) or red spots (with higher levels in the control line) in Figure 2b. All spots were quantified, and the resulting data was used for the comparative analysis between the compositions of the different samples.

(a)



(b)



(c)

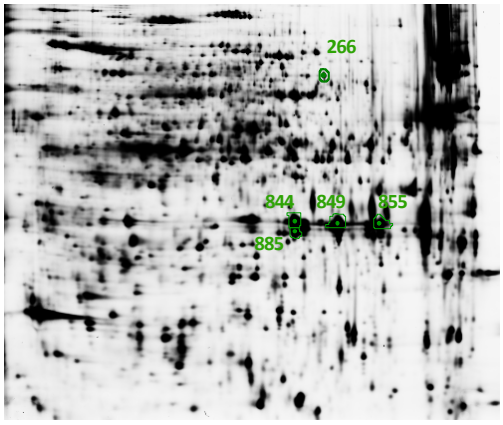


Figure 2. Representative 2D DIGE gel of tomato fruit proteins. (a) Fluorescence images of gel loaded with equal amounts (50 µg) of the reference sample (Cy2-labelled, blue), non transgenic samples (Cy5-labelled, red) and transgenic samples (Cy3-labelled, green) were loaded in the same gel. (b) Overlay of the three fluorescence images. Differential proteins appear in green or red, unaffected proteins appear in white. (c) Silver stained 2D DIGE gel. Spots showing significant differences between transgenic and non-transgenic samples picked for sequencing are outlined in green.

In order to find possible differences between transgenic and non transgenic tomato fruits a Principal Component Analysis (PCA) was performed with the quantification data extracted from the gels (Figure 3). The first two principal components of the PCA model explained 42% and 18% of the total variance, respectively. As depicted in the score scatter plot shown in Fig. 3a, PC1 mainly separated one of the wild type samples (line J) from the rest of the group. This aberrant separation of line J was most probably due to sample preparation and therefore it was decided to remove this line from the PCA analysis. Figure 3b shows the score scatter plot after removing line J. The first two principal components of this alternative PCA model show 28% and 23% of the total variance, respectively. Here, PC1 slightly separates 35s_IgA lines (F and I) from the rest of the group which remain together with exception of line K which is not completely grouped with the rest of the lines. PC2 further discriminates between both 35s_IgA lines (F and I). Samples from NH_IgA transformation events (both transgenic and Nullizygote) are grouped together in the scores space, thus not being separated by any of the components of the PCA model.

(a) (b)

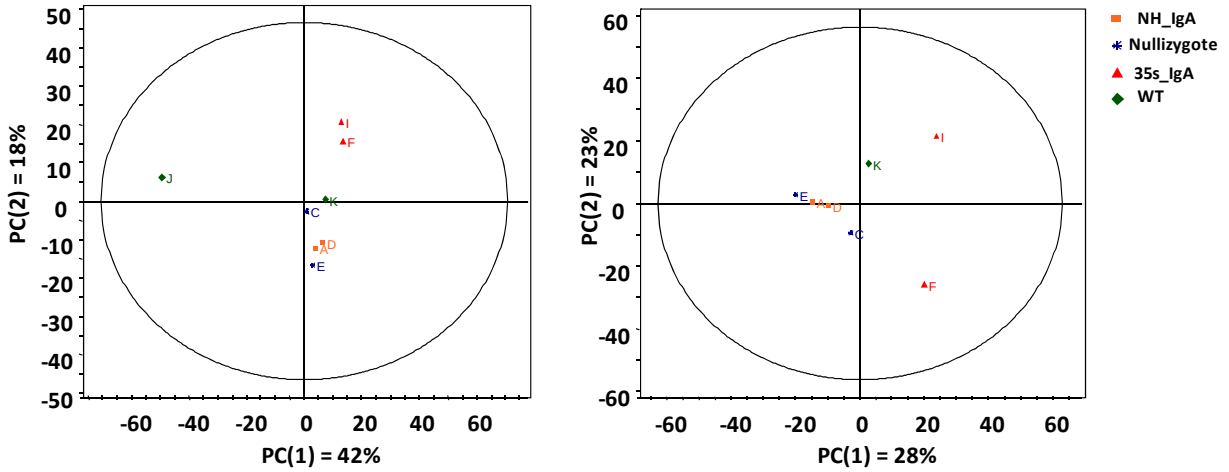


Figure 3. PCA of proteomic data of transgenic and non transgenic tomato lines. (a) all samples are included in the plot. (b) sample J is removed from the analysis.

In order to identify those spots which are the main contributors for class separation, a quantitative comparison between transgenic and non transgenic lines was performed. A total of 37 spots were found whose average ratio was higher than 2 (|ratio| > 2). Among them, only 20 showed an increase in both 35s_IgA and NH_IgA lines when considered separately, and only 5 of those were found statistically significant ($p \leq 0,05$) (Figure 2c, outlined in green). All 20 IgA-associated spots were picked and analyzed by LC-MSMS. Table 2 shows the successful identification of the 5 significant spots: spot 266 was identified as

Human HC, and spots 844, 855, 885 and 849 were identified as Human LC. Additionally Table 3 shows the remaining non-significant spots which were also successfully identified. Only one of the 15 non-significant, IgA-associated spots was identified as mainly human keratin and therefore was discarded. The rest were identified as HC or LC with exception of spot 783 in which an ACC oxidase from tomato was identified together with Human HC.

Table 2. List of identified significant proteins with ratio > 2

Spot	Paragon score	%Cov	Accession	Name	Species	Peptides (95%)
266	17,6	29,8	Sp|P01876|IGHA1_HUMAN	Ig alpha-1 chain C region	HUMAN	9
844	22,5	41,1	Sp|B9A064|IGLL5_HUMAN	Immunoglobulin lambda-like polypeptide 5	HUMAN	30
885	6,1	19,2	Sp|B9A064|IGLL5_HUMAN	Immunoglobulin lambda-like polypeptide 5	HUMAN	3
849	12,6	42	LV301_HUMAN	Ig lambda chain V-III region SH	HUMAN	4
855	14,7	39,7	Sp|B9A064|IGLL5_HUMAN	Immunoglobulin lambda-like polypeptide 5	HUMAN	18

Table 3. List of identified proteins with ratio > 2

Spot	Paragon score	%Cov	Accession	Name	Species	Peptides (95%)
843	6,3	34,3	sp|P01714|LV301_HUMAN	Ig lambda chain V-III region SH	Human	4
896	6,2	22,4	sp|P01876|IGHA1_HUMAN	Ig alpha-1 chain C region OS=Homo sapiens	Human	6
783	2	52,3	sp|P10967|ACCH3_SOLLC	1-aminocyclopropane-1-carboxylate oxidase	Solanum lycopersicum	1
	1,7	10,5	sp|P01876|IGHA1_HUMAN	Ig alpha-1 chain C region	Human	1
792	9,0	17,9	gi|46561796	rpL23-ScFv fusion protein	---	4
797	2,4	10,5	sp|P01876|IGHA1_HUMAN	Ig alpha-1 chain C region	Human	1
852	8,1	45,3	sp|P0CG04|LAC1_HUMAN	Ig lambda-1 chain C regions	Human	4
868	9,3	45,3	sp|P0CG04|LAC1_HUMAN	Ig lambda-1 chain C regions	Human	4
869	3,6	23,6	sp|P0CG04|LAC1_HUMAN	Ig lambda-1 chain C regions	Human	2
875	2,0	17,0	sp|P0CG04|LAC1_HUMAN	Ig lambda-1 chain C regions	Human	1
886	10,1	38,0	gi|21669631	immunoglobulin lambda light chain VLJ region	Human	5
899	2	10,5	sp|P01876|IGHA1_HUMAN	Ig alpha-1 chain C region	Human	1
956	2,37	10,5	sp|P01876|IGHA1_HUMAN	Ig alpha-1 chain C region	Human	1
1186	2	15,5	gi|7438718	Ig lambda chain NIG250 precursor	Human	1
1169	1,82	23,6	sp|P0CG04|LAC1_HUMAN	Ig lambda-1 chain C regions	Human	1

*** Paragon Score:** A measure of the protein confidence for a detected protein, calculated from the peptide confidence for peptides from spectra that are not already completely "used" by higher scoring winning proteins.

**** % Cov:** The percentage of matching amino acids from identified peptides having confidence greater than 0 divided by the total number of amino acids in the sequence.

2.3 *Metabolic profiling for the evaluation of unintended effects by UPLC-MS.*

Unintended effects on the metabolome of transgenic tomato fruits generated by the expression of human IgA were also evaluated by non-targeted metabolomics. Tomato methanol extracts were used for the analysis, which was carried out by means of reverse-phase UPLC coupled to mass spectrometry (UPLC-QTOF). This technology is specially suited for the detection of semipolar secondary metabolites, primarily tomato polyphenols and alkaloids (Moco et al., 2006). Between four and five different fruits from seven transgenic lines containing different levels of IgA, two nullizygote plants, two wild type moneymaker lines and three commercial varieties (plum, round, and cherry) were analyzed.

The mass spectra obtained from the analysis of tomato methanol extracts by means of UPLC-QTOF was first processed with XCMS, an integrated metabolomics analysis platform for the determination of metabolic profile differences and metabolite identification (Smith et al., 2006). The pre-processing of the data generated two discrete data matrices, one for each ionization mode: ESI- (electrospray ionization in negative mode) and ESI+ (electrospray ionization in positive mode). The ESI- data set contained 386 mass variables while 835 peaks were initially identified in the ESI+ mass spectra. In order to remove low quality data, a first filtering step was done by imposing two different conditions: (i) a mass feature should be detectable in a minimum of three biological replicates in at least one of the tomato lines, and (ii) its average day-coefficient of variation for the reference sample (computed on normalised data) should not be greater than 40%. Mass peaks that did not fulfil these requirements were considered unreliable and were thus, excluded from the study. A second filtering step was accomplished by removing the redundant isotopic features which were identified by the package CAMERA (a collection of algorithms for metabolite profile annotation with the primary purpose of annotation and evaluation of isotope peaks, adducts and fragments in peak lists) (Kuhl et al., 2012).The final ESI- and ESI+ data sets, with 107 and 249 peaks, respectively, were joined together in a single matrix for further analysis.

PCA was performed as first step in data exploration to examine the relationship between the different sample classes. The first two principal components of the PCA model explained 30% and 17% of the total variance, respectively. As can be seen in the scores scatter plot depicted in Figure 4a, PC1 clearly separated the commercial varieties from the remaining samples, while PC2 allowed further discrimination within the commercial group between cherry (N) and plum and round tomatoes (L and M lines). The samples from IgA-producing lines and control nullizygote and Moneymaker WT lines, however, grouped together in the scores space not being separated by any of the components of the PCA model. As the largest variance in the data set was between commercial and Moneymaker lines, a new PCA was performed in which commercial lines were excluded from the analysis to thus better explore the differences associated to IgA production. The obtained PCA model (Figure 4b) did not reveal any clear group structure in the dataset; only

35S_IgA lines F, G, and H, slightly separated from the remaining samples whereas significant confusion occurred between the homozygous 35S_IgA line I, NH_IgA lines, and the control lines. This does not exclude, however, changes associated to the expression of the foreign protein, it rather points out that any possible variation introduced in the metabolome by the expression of IgA in the fruit is smaller than the existing within-group variation.

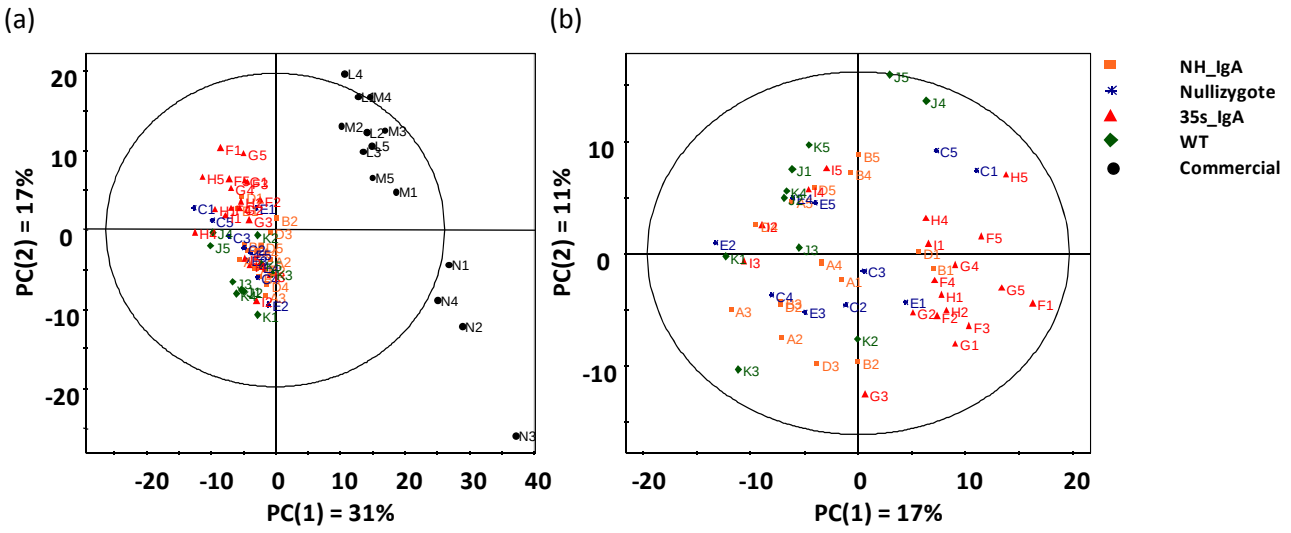


Figure 4. Principal component analysis score plots of LC-MS spectra. (a) Complete dataset set of tomato cultivars. (b) Moneymaker lines.

To directly search for specific metabolites that discriminate between IgA-producing and control lines, an orthogonal partial least squares discriminate analysis (OPLS-DA) was set. OPLS-DA makes use of *a priori* information on class membership (e.g. IgA content) to extract class-related variance in a predictive component and class-uncorrelated variation in one or more orthogonal components. Thus, even in the presence of uncontrolled co-variates, OPLS-DA allows a direct identification of class-discriminant variables. As 35s_IgA lines and NH_IgA lines contain very different IgA levels, they were treated separately so that each transgenic group could be compared with its closest control (non-IgA) group. Thus, three separate comparisons were made involving 35s_IgA vs. WT, NH_IgA vs. WT, and NH_IgA vs. nullizygote lines (Figure 5). The fitted models comprised one predictive (IgA content) plus 1, 2, and 1 orthogonal components, respectively. The variance related to class separation explained by the models was 82%, 98%, and 84% with a cross-validated predictive ability $Q^2(Y)$=0.31, 0.84, and 0.40, indicating a higher uncertainty in the 35s_IgA vs. WT and NH_IgA vs. Nullizygote models regarding class separation. The homozygous line I formed a separate subgroup within the 35s lines as revealed by the first orthogonal component of the model.

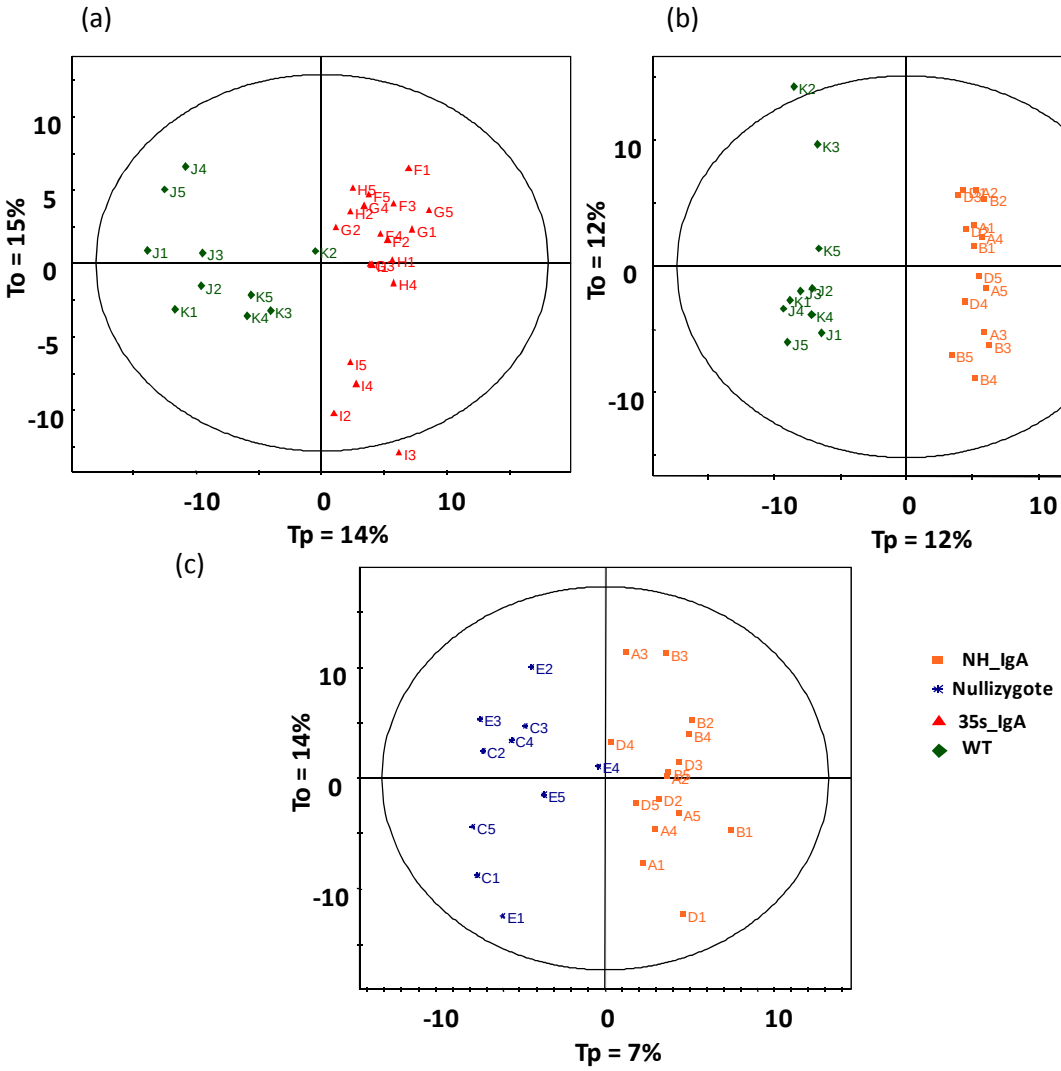


Figure 5. OPLS-DA score plots of the (a) 35S_IgA vs. WT (b) NH_IgA vs. WT, and (c) NH_IgA vs. Nullizygote.

The most relevant metabolite ions for class discrimination were selected, according to the following rules: the selected ions should present a high variable influence on projection (VIP ≥ 1), a high correlation with IgA content (| p (corr) | ≥ 0.5), and narrow jack-knifed 95% confidence intervals of the variable coefficient (confidence interval not crossing zero). A total of 41, 39, and 27 discriminating mass peaks (counting 91 different masses) were chosen according to these criteria from the 35s_IgA vs. WT, NH_IgA vs. WT, and NH_IgA vs. Nullizygote models, respectively. A large proportion of these peaks, however, showed a very modest variation between the transgenic lines and their counterpart controls with less than half of them, 42 masses, reaching fold ratios ≥ 2. The complete list of 91 masses is provided in Table S1 as supplementary material. The three models were then compared by means of SUS-plots (Shared and Unique Structures plot, an analysis that combines the p(corr) profiles from two models) constructed with all the selected variables (Figure 6). The first SUS-plot showed the correlation between models (35s_IgA vs. WT) and (NH_IgA vs. WT) (Figure 6a). The second SUS-plot correlated models (35s_IgA vs. WT) and (NH_IgA vs. Nullizygote) (Figure 6b). The last SUS-plot showed the correlation between models (NH_IgA vs. WT) and

(NH_IgA vs. Nullizygote) (Figure 6c). Only two discriminating ion masses were shared by the three models, namely masses Mp232 and Mn44. In general, there was little overlap between the (35s_IgA vs. WT) and the (NH_IgA vs. Nullizygote) models, with only the two mentioned masses, Mp232 and Mn44, sharing high correlation loadings and covariance. Comparison of the (35s_IgA vs. WT) / (NH_IgA vs. WT) and (NH_IgA vs. WT) / (NH_IgA vs. Nullizygote) demonstrated greater correspondence, with 9 and 6 common discriminating features, respectively (Figure 6d).

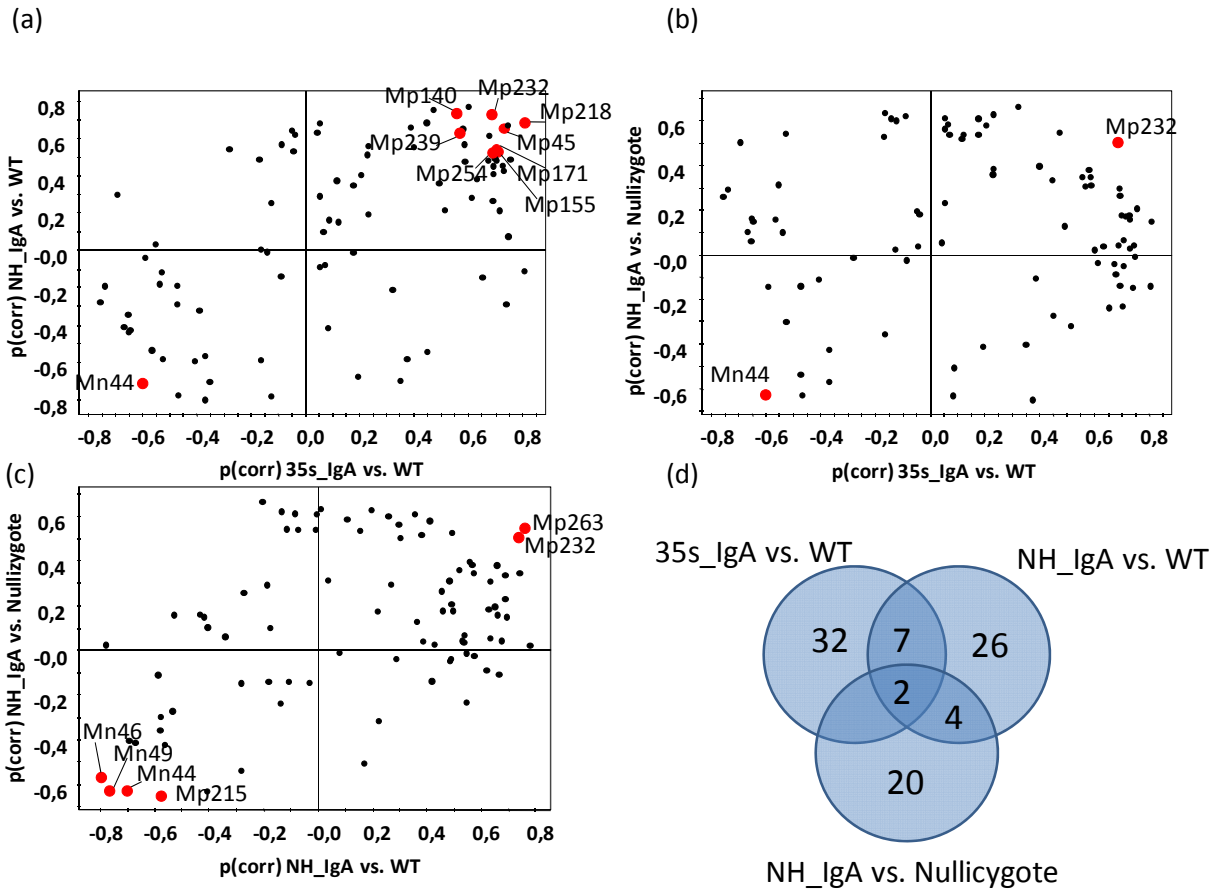


Figure 6. SUS plots between (a) (35S_IgA vs. WT) / (NH_IgA vs. WT), (b) (35S_IgA vs. WT) / NH_IgA vs. Nullizygote), and (c) (NH_IgA vs. WT) / (NH_IgA vs. Nullizygote) models. Common features are marked in red. Only masses selected as potentially important for class separation in any of the three models were plotted. (d) Venn diagram showing the number of unique and overlapping discriminating mass features.

In order to determinate the statistical significance of the observed differences, a univariate analysis was also carried out. A t-test was performed for the comparison between (NH_IgA vs. WT), (NH_IgA vs. Nullizygote), and (35s_IgA vs. WT). Seventeen masses, 4.8% of the total number of variables, resulted significant (FDR < 0.05) in at least one of the comparisons (Table 4). All these metabolic features, which included the ion masses Mp232 and Mn44 above mentioned, were among the 91 masses selected as major contributors to class separation in the OPLS-DA model. Among them, nine peaks showed an uptrend in the transgenic lines when compared to the WT, which could represent a potential concern regarding safety of

the final product. These mass features, with an average fold variation ranging from 1.6 to 3.4, showed similar tendency in both 35s and NH lines, with the only exception of Mp173, which remained unaltered in NH lines. However, the same uptrend was observed for the Nullizygote lines when compared to the WT suggesting a lack of real association between these changes and IgA production. It is also worth noticing that no significant differences between the NH_IgA lines and their Nullizygote controls were found.

Table 4. List of significantly different mass features (T-test, FDR ≤ 0.05)

			Fold Change			
Mass ID[(a)]	m/z	Rt	35S/WT	NH/WT	NH/Nullizygote	Nullizygote/WT
Mp30	385.0445	0.85	0.97	0.38*	0.91	0.42
Mp46	218.1005	0.87	0.78	0.22*	0.45	0.50
Mp140	284.1009	1.32	**1.48**	**1.61***	**1.18**	**1.37**
Mp148	278.1484	1.34	**2.04***	**1.60**	**0.94**	**1.70**
Mp157	316.1386	1.44	**2.13***	**1.80**	**0.91**	**1.97**
Mp171	303.1081	1.74	**1.86***	**1.54**	**0.79**	**1.95**
Mp173	538.1281	1.75	**1.89***	**1.18**	**0.65**	**1.83**
Mp232	287.1153	7.36	**1.96**	**3.38***	**1.57**	**2.16**
Mp241	497.1543	9.26	**2.26**	**3.21***	**1.67**	**1.92**
Mp245	425.1431	10.58	0.85	0.40*	0.76	0.52
Mp263	359.1337	21.05	**2.68**	**3.19***	**2.07**	**1.54**
Mp335	666.3875	30.90	0.34*	0.78	1.51	0.51
Mp350	415.3230	33.79	0.56*	1.18	1.44	0.82
Mn44	386.9989	4.06	0.52	0.37*	0.53	0.70
Mn49	285.05766	4.54	0.66	0.23*	0.48	0.48
Mn84	1430.6555	27.99	**1.79**	**2.23***	**0.99**	**2.24**
Mn97	1120.5295	30.87	0.38*	0.89	1.44	0.62

Significant differences (FDR < 0.05) are indicated with an asterisk. Mass features showing an uptrend in the transgenic lines are in bold. Contribution to class separation in an OPLS-DA model is indicated by an underline. (a) Mp: mass detected in ESI+ mode; Mn: mass detected in ESI- mode. Rt: retention time

In addition to the assessment of the statistical significance of the differences between the transgenic lines and their counterpart control lines (proof of difference), the safety of the metabolic changes in IgA-producing fruits was also evaluated following a proof-of-safety approach. According to this test, differences will be regarded as safe if the metabolite levels fall within an established safety range (Hothorn and Oberdoerfer, 2006; Kusano et al., 2011). When *a priori* defined safety thresholds are not available, as it is necessarily the case for untargeted metabolomic studies, these can be derived from the metabolite levels

found in commercial varieties. Thus, following the approach of Kusano et al. (2011) symmetric safety limits were defined as the 90% confidence interval for the ratio to control (WT) of the commercial variety furthest away from the WT. When, for any given metabolite, the confidence interval for the ratio of the transgenic fruits to the WT control is within this safety range, the levels of the metabolite can be regarded as safe. When this analysis was performed, only one mass, Mn52, showed ratios to WT outside the acceptable limits in the 35s and NH line groups with an average fold variation of 2.4 and 1.7, respectively. When analyzing lines individually, 42 additional features were identified with ratios outside the established safety range in at least one of the transgenic tomato lines, but only 22 out of these fell outside the upper boundary and could therefore represent a potential safety concern (Cases 2 and 3 in Figure 7). This is becouse IgA tomato fruits are not intended to be used as food product, and therefore safety concerns are constrained to the presence of new or increased levels of toxicologically relevant compounds, the loss of for example key nutrients being unimportant. The majority of the 22 remaining masses (86%), showed modest average fold variation which was in all the cases lower to the established threshold (Case 2 in Figure 7) while the remaining 14% (3 masses, showed in red in table 5) showed an average fold variation above, although sometimes close to the established threshold (Case 3 in Figure 7) (Table 5). This indicates that strong variance rather than consistent higher abundance is the reason that makes those masses to fall slightly outside the established safety range. It should be noticed that in one case safety cannot be concluded for Nullizygote line C. Most importantly, it should be noticed that for the homozygous elite 35s line I all measured variables were within the acceptable limits.

Table 5. List of potentially unsafe masses of the upper boundary. Nullizygote lines underlined in blue. Ratios in red showean average fold variation above but close to the established threshold.

Mass ID[(a)]	m/z	Rt (min)	Line	Ratio Line/WT	Safety threshold	Mass ID[(a)]	m/z	Rt (min)	Line	Ratio Line/WT	Safety threshold
Mp31	385.0	0.85	B	2.38	4.08	**Mn2**	386.9	0.74	B	1.71	1.85
Mp36	176.0	0.86	A	1.57	2.22	**Mn30**	496.0	1.34	B	3.57	5.80
			D	1.98					F	4.28	
			E	1.44		**Mn50**	281.0	5.64	F	3.27	9.18
Mp53	322.0	0.90	B	1.75	2.09				G	3.43	
			D	1.90		**Mn51**	179.0	6.57	B	1.58	2.17
			F	1.94					F	2.26	
Mp102	348.0	1.18	D	1.89	2.32				G	1.65	
			E	2.26		**Mn52**	341.0	6.57	A	2.07	2.15
Mp140	284.1	1.32	B	1.89	2.23				C	1.96	
			F	2.02					E	1.67	
Mp153	307.0	1.35	B	3.14	3.13				F	3.90	
			F	4.11					G	2.14	
Mp173	538.1	1.75	C	2.38	2.33				H	2.36	
			F	2.79		**Mn55**	281.0	6.96	F	2.26	11.12
			H	1.92					G	1.65	
Mp175	385.0	1.89	B	2.54	3.04	**Mn57**	181.0	7.14	F	3.69	4.68
			F	4.54		**Mn90**	1336.	28.79	B	1.40	1.67
Mp208	267.1	4.44	G	3.23	17.61				C	1.20	
Mp216	365.0	5.57	F	3.71	7.21				H	1.20	
Mp225	365.0	6.94	G	3.49	7.03	**Mn94**	917.2	29.22	H	1.66	2.76
Mp231	265.1	7.30	G	6.65	14.39						
Mp237	381.1	8.59	F	1.82	2.22						

(a) Mp: mass detected in ESI+ mode; Mn: mass detected in ESI- mode. Rt: retention time

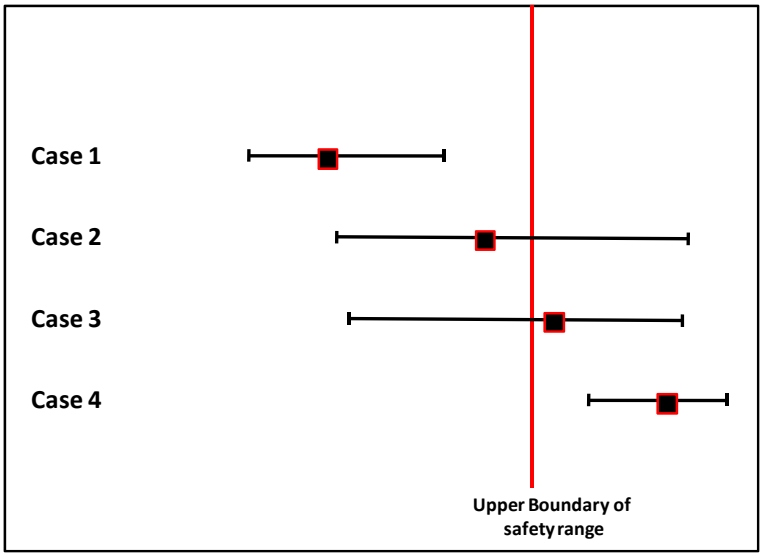


Figure 7. Simplified schematic representation of the possible outcomes of the proof of safety assessment. Only the upper boundary limit is considered. Case 1 represents a situation where the metabolite can be regarded as safe. Case 2-4 represent situations where safety cannot be concluded. Squares show the mean ratios between the transgenic line and WT. Bars represent the confidence intervals.

3. Discussion

Plant organs are considered GRAS when at their mature stage they contain no toxicants, allergens or anti-nutrients that could result harmful for the consumer. It is expected that recombinant production of IgA in edible tomatoes does not compromise fruit composition and consequently its edibility. However, this hypothesis had never been corroborated experimentally before; the most important compounds concerning edibility in tomato are proteins (allergens) and secondary metabolites (alkaloids) (OECD, 2008). Here, we analyze proteomic and metabolomic profiles of IgA tomatoes and compare them with non-transgenic profiles, as a way to evaluate the changes in tomato composition introduced by IgA production. Whole proteome was analyzed by means of 2D-DIGE gels and UPLC-qTOF was used to analyze secondary metabolites. The changes observed, or the absence of them, are taken as an indication of the safety of minimally processed IgA-tomato products.

Four representative transgenic lines paired with four non-transgenic lines were chosen for the proteomic analysis by means of 2D-DIGE. These included NH_IgA compared with their corresponding nullizygote fruits as well as 35s_IgA paired with WT samples. Principal component analysis (PCA) from the quantification data highlighted the presence of an aberrant WT sample. The tomato powder which was used to prepare this sample was also used for subsequent metabolomic analysis showing no abnormal performance in this case. This suggests that the aberrant behaviour in proteomic analysis was due to protein precipitation procedures. Therefore, this sample was removed from PCA analysis.

To quantify the magnitude of the changes between transgenic and non-transgenic groups, mean ratios were calculated for each spot between IgA and non IgA samples. In a first arbitrary cut only spots with ratio >2.0 were selected, as only a higher abundance of a protein can be considered as a potential thread. As a result, a total of 20 spots were selected for identification by LC-MSMS, with only 5 of them showing significant increase in a T-test analysis (p-value < 0,05). All the sequenced spots matched human IgA chains except for spot 783, which showed an overlap between human IgA HC and tomato ACC oxidase, an ethylene-forming enzyme involved in climacteric fruit ripening. ACC oxidase is a common protein in climacteric fruits with no allergenic properties reported, thus, a slight increase in this enzyme probably does not represent a threat for human consumption. Therefore, it can be deduced from this analysis that no other protein apart from IgA is substantially changing the composition of the transgenic tomato. However, further analysis of allergenic proteins could be made in order to further assure the absence of novel allergens in the fruit composition (Gubesch et al., 2007).

Regarding the analysis of secondary metabolites, PCA including commercial varieties Cherry, Plum and Round showed a clear separation between these and the remaining Moneymaker samples. This observation could be due to several reasons. It is possible that inherent variety differences are sufficient to

separate the samples, but other possible explanations are the cultivation conditions of each tomato batch, the post-harvesting conditions to which they have been subdued, e.g. refrigeration, etc. In any case, those differences are much more evident and by far, sufficient to mask any differences generated by the production of an immunoglobulin. For this reason, another PCA was made after removing the commercial varieties from the data set. This second PCA showed no separation between IgA lines and IgA-free lines. Both groups showed a similar distribution, meaning that any environmental component that is affecting the metabolite composition is more significant than any difference generated by the production of IgA.

Subsequently, OPLS-DA models were set in order to search for specific unintended effects between transgenic and non transgenic lines directly related with IgA production. As NH_IgA lines and 35s_IgA lines presented a drastic difference in IgA levels, they were treated separately. All three OPLS-DA models were able to separate transgenic and non-transgenic lines, however, with exception of the model set for NH_IgA vs. WT, they presented a low predictive power with values of $Q^2 < 0,5$.

In principle, those masses correlating with IgA levels which are common within the three models are expected to be truly related with the IgA production. Only two masses, namely Mn44 and Mp232, were found to fulfil this criterion. Of these, only Mp232 showed an uptrend in transgenic lines, although this uptrend was only significant when comparing NH_IgA samples with WT but not in the two remaining comparisons. The fact that Mp232 uptrend is only significant in one of the group comparisons indicates that it has only minor effects in fruit composition. Moreover the average increase ration is 3.38 fold, and increase that would only be a concern if high daily doses of tomato-derived products were required. Nevertheless, targeted identification of Mp232 could be advisable to better evaluate its possible safety implications.

An additional proof-of-safety approach of the metabolic changes in IgA-producing fruits was performed following the approach of Kusano et al. (2011). In this analysis, all the measured variables in the elite line homozygous I line fell within the acceptable limits. However safety could not be concluded for 42 masses in the remaining lines, 22 of which showing an uptrend in transgenic lines. Among those 22 masses, just three of them showed an average fold variation above the established threshold (case 3 in Figure 7), none of them being Mp232. This indicates that in most cases a high variability is the reason of these confidence intervals lying beyond the safety range. It is also important to point out that for 5 of the 22 different masses, nullizygote lines also appeared to be outside the established safety range. Taking together, these observations suggest a lack of a real association between these changes and IgA production.

It is a common mistake to expect that the composition of a transgenic crop is identical to its non-transgenic comparator. As shown here in PCA and OPLS-DA models, IgA expression leaves an associated footprint in the fruit composition, which can be detected and partially described using proteomic and metabolomic profiling. Similarly, a metabolic signature introduced by the recombinant over-expression of the taste-modifying protein miraculin was found before in tomato fruits using a multi- platform approach, in

the context of a substantial equivalence assessment (Kusano et al., 2011). However, to our knowledge, the metabolic signature of a therapeutic protein produced in an edible plant organ was not analyzed before. The over-expression of xenoproteins in the plant cell can induce sequence-specific responses in the host, which range from the suppression of the expression of the foreign gene via post-transcriptional gene silencing, to the triggering of a general stress response or the induction of a program of cell death (see Orzaez et al. (2009) and references therein). Therefore, the accumulation of harmful compounds in response to IgA expression in fruits could not be initially discarded. By incorporating several independent transformation events leading to different IgA expression levels, this work tries to evaluate unintended effects arising from the general strategy of IgA-based oral passive immunotherapy in tomato. However this work alone cannot rule out the possibility of harmful unintended effects associated with any strategy involving IgA production in fruits. At least three potential sources of uncertainty still remain. First, the unexpected accumulation of harmful compounds other than the "usual suspects", namely proteins and semi-polar secondary metabolites, cannot be discarded with the methodology used here. A single method enabling complete metabolome analysis does not exist, and therefore additional comprehensive profiling methods should be considered (Rischer and Oksman-Caldentey, 2006). Secondly, any new transgenic event involving IgA production in fruits should evaluate possible unintended effects associated specifically with the site of insertion, which cannot be evaluated here. Finally, as observed in the proteomic profile, the antibody itself accumulates in the form of different polypeptides with distinct electrophoretic mobilities, probably arising from partial proteolytic degradation and/or different glycosylation patterns. Certainly, the pool of IgA-related polypeptides present in the fruit is different to those occurring in a natural OPI and therefore the safety of the plant-made IgA itself and in the context of the fruit matrix needs to be thoroughly evaluated. All in all, the data shown here constitutes a first "clearway" for the fruit-based IgA strategy, by establishing that not unsafe compositional changes associated with IgA production are detected when un-targeted analysis profiling the unsafe "usual suspects" in tomato are performed.

4. Experimental Procedures

4.1 Plant material

Seven transgenic lines (A, B, D, F, G, H, I) of tomato (*Solanum lycopersicum*, cv. Moneymaker) expressing human immunoglobulin A (IgA) were used in this study along with the parental line – K – used for genetic transformation. Lines A, B and D were obtained by *Agrobacterium* mediated transformation of a plasmid carrying the heavy (HC) and light chains (LC) of human IgA under the cauliflower mosaic virus 35s constitutive promoter and the tomato NH promoter, respectively. Lines F, G, H, and I were obtained by co-transformation with two *Agrobacterium* cultures, one carrying a plasmid with the 35s:IgA_HC and the second carrying a plasmid with the 35s:IgA_LC construct. A detailed description of the generation of these transgenic lines can be found in Juarez et al. (2012). Additionally, two nullizygot segregant lines – C and E – from the 35S:IgA_HC / NH:IgA_LC transformation, and one Moneymaker line – J – obtained from a different laboratory were also included as additional controls. Finally, fruits from three widely consumed tomato types – plum (L), round (M), and cherry (N) – purchased from a local grocery completed the experimental dataset. Tomato seeds (excluding commercial lines L, M, and N) were grown in pots in a greenhouse with a light/dark cycle of 16/8 hours. Fruits were harvested 15 days post-breaker. Five independent samples from each plant line were collected. After removing gel and seeds, tomato pericarp (with the peel) was ground to a fine powder in liquid nitrogen and stored at -80ºC for further analysis.

4.2 Protein extraction, SDS-PAGE, Western blot and ELISA tests

For protein analysis, frozen tomato powder samples from each group were pooled together and the total soluble protein (TSP) was extracted in three volumes (w/v) of ice-cold PBS (100 mM sodium phosphate, 150 mM NaCl, pH 7.4), followed by 2 x 15 min centrifugation steps in a tabletop centrifuge at 16,000 g and 4ºC.

Protein separation was carried out by SDS-PAGE on NuPAGE® 10% Bis-Tris polyacrylamide gels (Invitrogen, Life Technologies, Paisley, UK). Gels were stained with Coomassie blue. For western blot analysis, proteins were transferred to PVDF membranes (Amersham Hybond™-P, GE Healthcare, Buckinghamshire, UK) by semi-wet blotting (XCell II™ Blot Module, Invitrogen, Life Technologies) following the manufacturer's instructions. Membranes were incubated with 1:20.000 Anti-Human IgA α-chain specific peroxidase conjugated antibody (SIGMA, St. Louis, USA) for the detection of the HC, and with 1: 10.000 anti-human λ LC (SIGMA) followed by 1:10.000 µg/mL HRP-conjugated anti-rabbit IgG (GE Healthcare) for the detection of the LC. Blots were developed with the Amersham™ ECL Plus Western Blotting Detection System

(GE Healthcare) following the manufacturer's instructions and were visualized by exposure to X-ray film (Fujifilm Coorporation, Tokyo, Japan).

IgA quantification was carried out by sandwich ELISA as previously described (Juarez et al., 2012). Briefly, 96-well ELISA plates (Corning, NY, USA) were coated overnight at 4ºC with 2 µg/mL of Anti-Human IgA α-chain specific antibody (SIGMA). Plates were blocked with a 2% (w/v) solution of ECL Advance™ Blocking agent (GE Healthcare), 0.1% Tween-20 (v/v) in PBS (blocking solution) for 2h at RT. Tomato extracts, serial two-fold dilutions in PBS, were applied in triplicate and incubated for 1h at RT. A human colostrum IgA standard (SIGMA) was included for the construction of a standard curve (0.8 to 0.1 $\mu g/ml^{-1}$ concentration range). A 1:5000 dilution of Anti-Human IgA α-chain specific peroxidase conjugated antibody (SIGMA) in blocking solution was used as detection antibody; SIGMAFAST™ OPD (*o*-phenilenediamine) tablets (SIGMA) was used as the enzymatic substrate. Absorbance was determined at 492 nm. IgA concentration in each sample was obtained by interpolation with the IgA standard curve.

4.3 2D DIGE analysis

TSP extracts were incubated with an equal volume of ice-cold 20% TCA for 2 h at 4ºC. Proteins were recovered by centrifugation (16,000 g, 15 min at 4ºC) and the obtained pellets washed three times with cold acetone before being solubilised in lysis buffer (7M urea, 2M thiourea, 4% CHAPS). Samples were then cleaned up with the 2D Clean-up kit (GE Healthcare) following the manufacturer's instructions. Cleaned protein precipitates were solubilised in Tris lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 20 mM Tris pH 8.5). Protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad, California, USA) using bovine serum albumin as standard.

Protein samples were labelled with CyDye DIGE fluor minimal dyes (GE Healthcare) according to manufacturer's instructions. IgA producing and control lines were alternatively labelled with Cy3 or Cy5 in dye-swap approach. A reference sample (consisting of pooled aliquots of all biological samples within the experiment) labelled with Cy2 was run on every gel together with the Cy3 and Cy5 experimental samples.

2D electrophoresis was developed as described by (Munoz-Fambuena et al., 2013) and 2D gels were scanned using a Typhoon Trio (GE Healthcare). Gel image analysis was performed with DeCyder 2D software v6.5.

4.4 Protein identification

For picking spots of interest, gels were first Silver stained with Silver Staining Kit, Protein (GE Healthcare). Spots were manually excised from analytical gels and distained with 2x 5-min washes with

acetonitrile (CAN)/water (1:1, v/v). Subsequently, samples were rehydrated with 50 mM ammonium bicarbonate for 5 min and 25 mM ammonium bicarbonate in 50% (v:v) CAN for 15 min. Proteins were then digested with sequencing grade trypsin (Promega) as described elsewhere (Shevchenko et al., 1996). The digestion was stopped with TFA (1% final concentration), 1 µL of the digestion mixture was spotted onto the MALDI target plate. A BSA plug was analyzed in the same way to control the digestion process.

Liquid chromatography and tandem mass spectrometry (LC–MS/MS): 5 µl of every sample were loaded onto a trap column (NanoLC Column, 3µ C18-CL, 75umx15cm; Eksigent, Radio Rd Redwood City CA) and desalted with 0.1% TFA at 3µl/min during 5 min. The peptides were then loaded onto an analytical column (LC Column, 3 µ C18-CL,75umx25cm, Eksigent) equilibrated in 5% acetonitrile 0.1% FA (formic acid). Elution was carried out with a linear gradient of 5a35% B in A for 30min. (A: 0.1% FA; B: ACN, 0.1% FA) at a flow rate of 300 nl/min. Peptides were analysed in a mass spectrometer nanoESI qQTOF (5600 TripleTOF, ABSCIEX, Framingham, MA). The tripleTOF was operated in information-dependent acquisition mode, in which a 0.25-s TOF MS scan from 350–1250 m/z, was performed, followed by 0.05-s product ion scans from 100–1500 m/z on the 50 most intense 2-5 charged ions.

ProteinPilot default parameters were used to generate peak list directly from 5600 TripleTof wiff files. The Paragon algorithm of ProteinPilot was used to search SwissProt protein database (22470027 proteins searched) with the following parameters: trypsin specificity, cys-alkylation, no taxonomy restriction, and the search effort set to rapid. To avoid using the same spectral evidence in more than one protein, the identified proteins are grouped based on MS/MS spectra by the Protein-Pilot Pro group algorithm. Thus, proteins sharing MS/MS spectra are grouped, regardless of the peptide sequence assigned. The protein within each group that can explain more spectral data with confidence is shown as the primary protein of the group. Only the proteins of the group for which there is individual evidence (unique peptides with enough confidence) are also listed, usually toward the end of the protein list.

4.5 UPLC-QTOF

Frozen tomato powder (500 mg) was extracted by vigorous mixing in 3 volumes cold (-20ºC) methanol acidified with 0.125% formic acid. Following centrifugation (10 min, 16,000 x g), the supernatant was dried (in two separate 750 µl aliquots) in a speed-vac and stored at -80ºC. Prior to analysis, dry extracts were solubilised in 150 µl methanol-0.1% formic acid and filtered through a 0.2 µm Anotop 10 membrane filter (Whatman, Kent, UK). Samples were analysed by UPLC-MS using an ACQUITY®UPLC-PDA system coupled to a Q-TOF Micro mass spectrometer (Waters Corporation, Milford, MA, USA). Separation was performed on an ACQUITY BEH C18 column (150 x 2.1 mm i.d., 1.7 µm). The mobile phase consisted of formic acid:ultrapure water (1:1000 v/v, phase A) and formic acid:acetonitrile (1:1000 v/v, phase B). Gradient conditions were as follows: from 95 to 90% of A in 14 min, from 90 to 80 % of A in 15 min, from 80

to 65% of A in 10 min, from 65 to 57% of A in 1 min, from 57 to 0% of A in 1 min, then held at 100% of B for 3 min, returned to 95% of A in 1 min, and equilibrated for 4 min before next injection. The flow rate was 0.4 ml min^{-1}; the column and sample temperature were kept at 40 and 10ºC respectively; the sample injection volume was 5 µl. UV spectra were acquired between 220 and 800 nm with a 1.2 nm resolution and 20 points sec^{-1} sampling rate. Mass spectrometry analysis was performed by electrospray ionization (ESI) in positive and negative modes (using one of the extract aliquots for each ionization mode). Q-Tof parameters were as follows: capillary voltage 3.0 kv, cone voltage 30 eV (ESI+) or 45 eV (ESI-), desolvation temperature 300ºC, source temperature 120ºC; cone gas flow 50 l h^{-1}, desolvation gas flow 600 l h^{-1}, collision energy 5 eV. MS data was acquired in centroid mode in the m/z scan range 100-1500 with a scan time of 0.5 s and an interscan time of 0.1 s. Leucine enkephalin was used as the lock mass using a LockSpray exact mass ionization source. A reference sample (made by pooling equal amounts of all biological sample) was injected four times (at regular intervals) in each injection series.

MassLynx raw files converted to netCDF format with DataBridge (Waters Corporation, Milford, MA, USA) were processed with the XCMS package version 1.34.0 (http://bioc.ism.ac.jp/2.11/bioc/html/xcms.html) run under R version 2.15.2 (http://www.r-project.org/). The centWave algorithm (Tautenhahn et al., 2008) was used for feature detection; peak grouping and retention time correction were performed with the density and peakgroups algorithms (Smith et al., 2006), respectively. Mass features were grouped using the CAMERA algorithm (Kuhl et al., 2012). The area of each extracted mass feature was first normalised to the total chromatogram area; the reference sample was then used to normalise the data between different runs (for each mass feature, the test sample area is divided by the mean area of the reference samples included in that run).

4.6 Statistical data analysis

PCA and OPLS-DA of the protein and metabolite profiles of tomato fruits were performed with the software package SIMCA-P 11 (Umetrics, Sweden). Variables were log-transformed, mean centred, and scaled to unit variance prior to analysis.

Two-sample t-tests on log transform data were performed using MeV v4.9 (http://www.tm4.org/mev.html), an application of the TM4 Microarray Software Suite (Saeed et al., 2003). Safety thresholds in the proof-of-safety analysis were defined by the symmetric maximum boundaries of the non parametric 90% confidence intervals for the ratio to WT of the commercial variety furthest away from the WT. Safety was concluded when the confidence intervals for the ratio to control of the transgenic lines were within the safety limits. For the calculations of the confidence intervals the R package pairwiseCI was used.

5. Acknowledgements

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7. Supplementary material

Table S1. List of peaks contributing to class separation in OPLS-DA. Underlining is used to indicate contribution to a model. Contribution to a model is indicated by an underline. Significant differences (T-test, FDR ≤ 0.05) are marked with an asterisks.

			Fold Change			
Mass ID[(a)]	m/z	Rt	35S/WT	NH/WT	NH/Nullizygote	Nullizygote/WT
Mp30	385.0445	0.85	0.97	0.38*	0.91	0.42
Mp38	249.0409	0.86	1.06	1.67	1.61	1.04
Mp41	348.0744	0.86	1.06	1.11	0.69	1.60
Mp43	138.5901	0.87	0.52	0.70	0.46	1.51
Mp45	381.0807	0.87	1.85	1.72	0.99	1.74
Mp46	218.1005	0.87	0.78	0.22*	0.45	0.50
Mp49	138.0563	0.88	0.64	0.69	0.65	1.07
Mp53	322.0595	0.90	1.24	1.63	1.25	1.30
Mp60	248.1139	0.90	1.24	1.63	1.25	1.30
Mp70	498.9294	0.92	1.26	1.40	1.51	0.93
Mp71	230.1051	0.92	1.24	0.63	0.64	0.98
Mp73	268.9478	0.92	1.11	1.32	1.37	1.92
Mp75	536.8865	0.93	1.18	1.27	1.45	0.87
Mp96	365.1066	1.05	1.69	1.69	0.98	1.72
Mp104	470.1442	1.18	1.30	1.21	1.16	1.05
Mp120	268.1054	1.24	1.28	1.75	1.26	1.39
Mp140	284.1009	1.32	1.48	1.61*	1.18	1.37
Mp148	278.1484	1.34	2.04*	1.60	0.94	1.71
Mp151	294.1572	1.34	1.77	1.68	1.17	1.44
Mp153	307.0690	1.35	2.17	2.01	1.63	1.24
Mp155	332.1122	1.43	1.90	1.76	1.06	1.66
Mp157	316.1386	1.44	2.13*	1.80	0.91	1.97
Mp159	258.1354	1.44	2.03	1.76	1.10	1.60
Mp167	315.0515	1.65	1.58	1.01	0.95	1.06
Mp168	299.0768	1.65	1.59	0.90	0.87	1.03
Mp171	303.1081	1.74	1.86*	1.54	0.79	1.95

Mp173	538.1281	1.75	1.89*	1.18	0.65	1.83
Mp174	160.0764	1.83	1.21	0.65	0.75	0.86
Mp175	385.0469	1.89	2.83	1.64	1.09	1.51
Mp176	342.1213	2.12	0.64	0.55	0.67	0.82
Mp209	298.1003	4.44	1.76	1.31	1.21	1.08
Mp214	146.0620	4.48	0.93	0.62	0.71	0.87
Mp215	265.1569	4.94	1.62	0.43	0.40	1.09
Mp216	365.0867	5.57	2.51	1.72	1.20	1.42
Mp218	449.1637	6.10	3.04	4.17	1.42	2.93
Mp219	367.0882	6.16	1.91	0.92	0.94	0.98
Mp221	144.0820	6.26	1.08	2.00	0.97	2.07
Mp224	377.0868	6.66	1.10	1.11	1.57	0.71
Mp225	365.0877	6.94	2.49	1.60	1.11	1.44
Mp227	377.0872	6.99	1.21	1.10	1.65	0.67
Mp231	265.1570	7.30	3.48	0.43	0.75	0.58
Mp232	287.1153	7.36	1.96	3.38*	1.57	2.16
Mp233	349.0860	7.37	2.11	1.86	1.37	1.36
Mp236	375.1286	7.99	1.05	2.02	1.13	1.78
Mp239	435.1845	9.23	2.30	3.33	1.77	1.88
Mp241	497.1543	9.26	2.26	3.21*	1.67	1.92
Mp242	321.1954	9.46	1.48	0.50	0.45	1.11
Mp245	425.1431	10.58	0.85	0.40*	0.76	0.52
Mp246	375.1280	11.06	1.05	1.63	1.08	1.51
Mp250	405.1744	13.14	1.77	1.56	1.34	1.16
Mp252	469.1330	15.19	1.18	0.76	2.47	0.31
Mp254	373.1122	17.73	2.80	2.82	1.15	2.46
Mp255	355.1758	18.15	0.60	0.95	1.71	0.56
Mp263	359.1337	21.05	2.68	3.19*	2.07	1.54
Mp270	773.4557	21.94	0.65	0.82	1.20	0.68
Mp294	976.5158	28.01	1.30	1.69	0.95	1.78
Mp297	670.4179	28.54	0.56	0.97	1.19	0.81
Mp335	666.3875	30.90	0.34*	0.78	1.51	0.51
Mp339	652.4069	31.26	0.41	0.59	1.33	0.44
Mp343	1108.5583	31.28	0.43	0.61	1.31	0.47

Mp348	636.4118	31.29	0.91	2.52	1.12	2.25
Mp350	415.3230	33.79	0.56*	1.18	1.44	0.82
Mp362	416.3537	34.52	0.48	0.34	1.69	0.20
Mp371	987.4454	38.20	0.70	0.60	0.94	0.64
Mn8	183.9948	0.83	1.04	1.23	1.46	0.85
Mn15	341.1073	0.87	2.59	1.48	0.90	1.64
Mn17	111.0013	0.94	1.08	0.95	1.16	0.82
Mn19	458.9570	0.94	0.96	1.01	1.39	0.72
Mn20	496.9136	0.95	0.97	1.20	1.55	0.77
Mn24	111.0017	1.24	1.07	0.99	1.19	0.83
Mn25	427.0073	1.24	1.07	0.95	1.35	0.70
Mn27	448.9894	1.25	0.95	0.99	1.39	0.71
Mn28	678.9744	1.25	0.95	0.88	1.46	0.60
Mn30	496.0915	1.34	2.37	2.58	1.95	1.32
Mn44	386.9989	4.06	0.52	0.37*	0.53	0.70
Mn49	285.0576	4.54	0.66	0.23*	0.48	0.48
Mn50	281.0629	5.64	2.58	1.48	0.96	1.54
Mn55	281.0635	6.96	3.27	1.70	1.25	1.36
Mn56	375.0682	7.05	1.02	1.04	1.62	0.64
Mn59	351.1276	8.08	1.15	2.28	1.00	2.29
Mn62	351.1272	11.10	1.11	2.03	1.08	1.89
Mn67	609.1456	21.64	1.17	1.50	1.32	1.13
Mn73	387.1073	24.01	1.85	2.52	1.01	2.49
Mn75	357.0958	24.01	1.68	2.24	1.06	2.12
Mn78	1336.5886	24.64	0.68	0.90	0.97	0.93
Mn81	1244.6025	24.88	0.78	1.00	0.92	0.93
Mn84	1430.6555	27.99	1.79	2.23*	1.00	2.24
Mn97	1120.5295	30.87	0.38*	0.89	1.44	0.62
Mn98	1174.5372	31.25	0.40	0.67	1.27	0.52
Mn99	1106.5492	31.25	0.43	0.62	1.17	0.53
Mn106	1108.5653	32.25	0.90	2.47	1.08	2.29
Mn118	963.4523	38.21	0.78	0.69	0.80	0.86

Chapter 3

Combinatorial analysis of secretory Immunoglobulin A (sIgA) expression in plants

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1. Introduction

Monoclonal antibodies (mAbs) have been used in research and diagnosis for many years, and their application in health is increasing rapidly. They play an essential role in cancer therapy (Reichert and Valge-Archer, 2007), however topical and oral immunotherapy of mucosal surfaces with mAbs is also of great interest, as it can block the entry and transmission of human pathogens (Corthesy, 2003; Corthesy, 2009; Corthesy, 2010).

There are different expression systems available for the production of recombinant antibodies, each with its advantages and shortcomings. Plants are one of the most interesting platforms for recombinant antibody production, because they are cost-effective, highly scalable, have a low risk of contamination with mammalian pathogens (Fischer et al., 2004; Paul et al., 2011; Sarrion-Perdigones et al., 2011b; Twyman et al., 2003) and also can perform post-translational modifications similar to mammals, as, for example, *N*- and *O*-glycosylation (Saint-Jore-Dupas et al., 2007). IgA is the most abundant antibody in mucus, and it forms part of the first line of defense against infectious agents. IgA can be present in the body fluids in its monomeric form (mIgA), containing only heavy chain (HC) and light chain (LC) or forming a secretory IgA (sIgA), a multiprotein structure comprising two full IgA molecules dimerized by a short joining chain (JC) and surrounded by the secretory component (SC), a polypeptide resulting from the proteolytic cleavage of the poly-immunoglobulin receptor (pIgR). IgA and, particularly, sIgA are good candidates for mucosal passive immunotherapy, having a number of advantages over IgG (e.g., the presence of four antigen binding sites, increased resistance against proteolysis in the gastrointestinal tract and the blocking of some bacterial pathogens mediated by carbohydrates, both in the HC and the SC) (Corthesy, 2003; Corthesy, 2009; Corthesy, 2010). Despite this, most research effort on plant-made recombinant antibodies has been made on monoclonal IgG antibodies.

The first attempt to produce a plant-made sIgA for passive immunization was the murine hybrid Guy's 13 sIgG/A (Ma et al., 1995a; Ma et al., 1998), which was evaluated in phase I and II clinical trials (Weintraub et al., 2005) as CaroxR™. Since then, various groups have expressed sIgA in plants (Larrick et al., 2001; Nicholson et al., 2005; Wieland et al., 2006; Wycoff, 2004). However, there is a lack of information about what structural requirements are the best to produce functional, fully human sIgA in plants with maximum activity.

There are a number of options available when designing a new antibody in sIgA format, which could lead to completely different products for the same purpose. Therefore, it is important to study how modifications in the engineered parts will affect antibody function. In this work, we focus on the study of the *in vitro* binding activity of the final product, although other functional considerations, such as *in vivo* activity or stability, could also be tested with an adequate setup. In the first place, there are several options concerning structural design: LC may occur in two isotype forms, designated kappa (κ) and lambda (λ), with

no functional differences described between them (Foley et al., 1991), and two types of HC, namely the α1 and the α2. In particular, the hinge region differs significantly between the two HC isoforms. The hinge region of HCα1 is comprised of 23 residues, while HCα2 is made of only 10 residues. The greater number of amino-acids in the IgA α1 provides an extended structure and a greater antigenic reach, while IgA α2 is more compact and, therefore, less susceptible to proteolitic cleavage (Furtado et al., 2004).

Secondly, subcellular localization may also affect the overall efficacy of the antibody. Targeting antibody chains to specific compartments in the plant cell can improve the stability, yield and/or downstream processing (De Muynck et al., 2010). The secretory pathway appears to be the most convenient route for a correct antibody folding and assembly, due to the oxidizing environment of the endoplasmic reticulum (ER), the low abundance of proteases and the presence of molecular chaperones. Moreover, protein glycosylation occurs only in the endomembrane system (Ma et al., 2003).

Once in the secretory pathway, there are several possible options; for example, the antibody can be efficiently retrieved from the *cis*-Golgi back to the ER using a *C*-terminal H/KDEL retention signal or deposited in the vacuole (Petruccelli et al., 2006). Several antibodies have also been reported as apoplastic (De Muynck et al., 2009; Petruccelli et al., 2006); however, in some cases, retention in the ER leads to a yield improvement (Schouten et al., 1996) and avoids plant complex glycosylation patterns that could cause an unwanted immune response (Bencurova et al., 2004; Gomord et al., 2010).

An appropriate way to produce the most efficient antibody design is to perform a combinatorial analysis of several versions of sIgA and select those which accumulate at higher levels and/or present an improvement on stability and activity. With traditional DNA assembly systems, this can become tedious work. However, with new standard modular cloning tools, like GoldenBraid (GB) (Sarrion-Perdigones et al., 2011a) or MoClo (Weber et al., 2011), to achieve all the combinations should be facilitated. These technologies open a new way for optimization of antibody production in plants by experimentally testing the best subcellular targeting and isotype combinations for the expression of a target antibody.

As a proof of this concept, we have followed a combinatorial approach to optimize the plant production of a sIgA version of an anti-rotavirus monoclonal antibody (2A1 sIgA). The variable regions of 2A1 antibody were initially selected by phage display against the VP8* peptide of the VP4 protein of the rotavirus SA11 strain capsid (Monedero et al., 2004). This antibody was previously described in its monomeric format, showing a strong rotavirus neutralization activity (Juarez et al., 2012).

2. Results

2.1 GoldenBraid-assisted multigene assembly of 16 versions of secretory IgA

A number of standard basic DNA pieces, named GBparts, were used as a starting point in the building of sIgA multigene structures. Basic GBparts comprised non-coding DNA regions, as the CaMV 35S constitutive promoter (35s) and the strong nopaline synthase transcription terminator (Tnos), together with a number of GB-adapted coding sequences required for the assembly of a functional sIgA, namely a signal peptide derived from the tomato pectate lyase gene (SP), the constant regions of the human HCα1 and HCα2, the constant regions of human LCλ and LCκ, the extracellular region of the human SC and the complete coding sequence of the human JC. In addition, the heavy and light variable regions of the anti-rotavirus VP8* peptide scFv antibody fragment 2A1 were also GB-adapted and incorporated to the GBpart collection (Figure 1a). GB-adaptation, also called GB-domestication, consisted of: (i) removal of internal BsaI and BsmBI restriction sites by PCR-directed mutagenesis; (ii) PCR-mediated addition of flanking GB standard bar-codes, which consist of standard labels of 11 nucleotides that serve to facilitate cloning and, ultimately, to specify the relative position of each GBpart in the assembly (see Figure 1b and Table S1); and (iii) TA-cloning of the resulting domesticated PCR products into pGEM-T vectors.

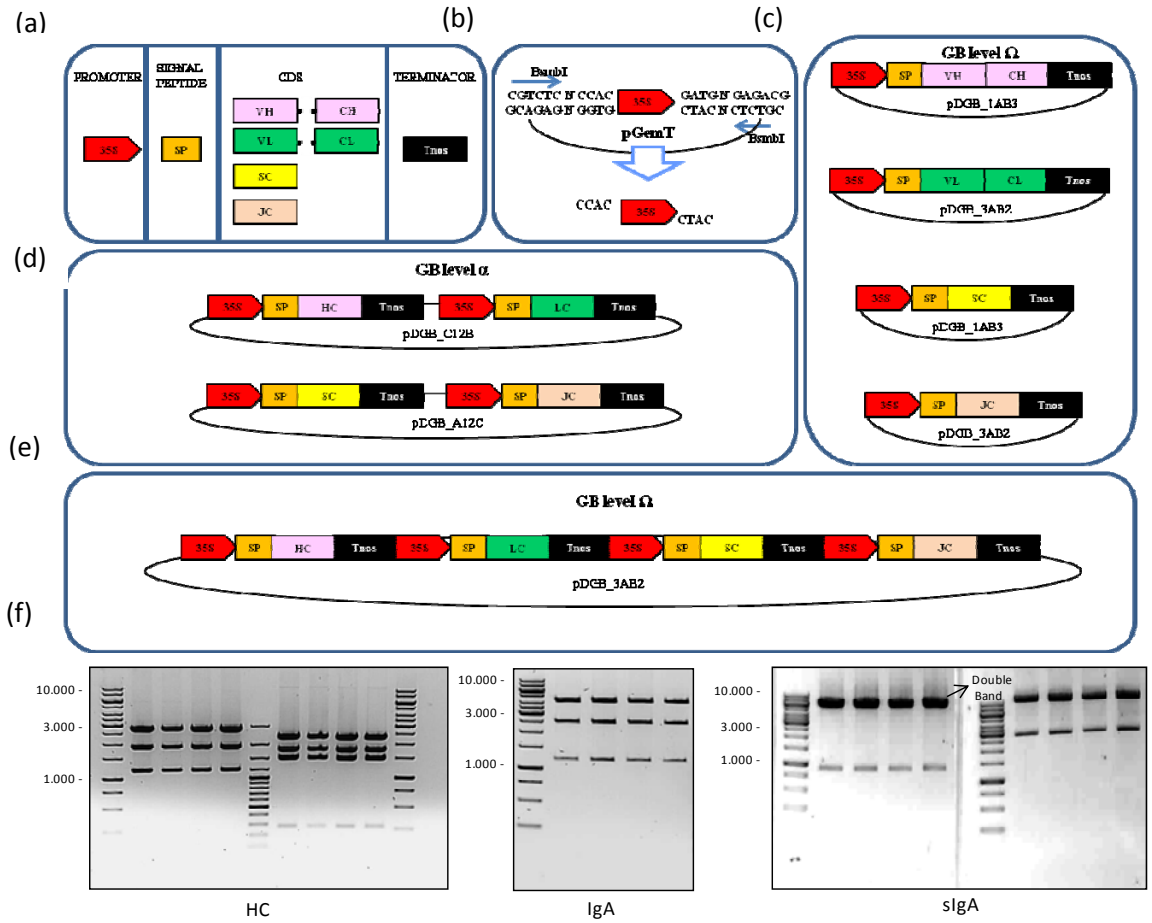

Figure 1. Assembly process of the secretory immunoglobulin A (sIgA). (**a**) Collection of basic parts necessary to construct a secretory IgA. Each basic part is cloned in a pGem-T vector. 35S, SP, VH-CH, VL-CL, SC, JC, Tnos, correspond, respectively, to the 35s CMV promoter, pectate lyase signal peptide, variable and constant regions of the heavy chain, variable and constant regions of the light chain, secretory component, J-chain and nopaline synthase terminator; (**b**) Example of domestication of a basic part. The 35s promoter is flanked by fixed BsmbI recognition-cleavage sites. The overhangs left by the BsmbI restriction enzyme converge with GB pDGB vectors on 5' and on 3', with the next basic part to assemble; (**c**) Multipartite assembly of the basic parts to form the four different transcriptional units: heavy chain (HC), light chain (LC), secretory component (SC) and J-chain (JC), into level Ω-GB destiny vectors (pDGB_1AB3 and pDGB_3AB2); (**d**) Binary assembly of transcriptional units in level α-GB destination vectors (pDGB_C12B and pDGB_A12C), in order to construct two different composite parts—IgA and JC-SC; (**e**) Last construct of sIgA by binary assembly of two composite parts in a final pDGB; (**f**) Example of restriction analysis of four colonies of each construct: left, BglII (expected bands of 2825, 1886 and 1197) and BglI (expected bands of 2345, 1790, 1498 and 275) restriction analysis of the HC transcriptional unit; middle, BglII (expected bands of 4183, 2495 and 1228 kDa) restriction analysis of IgA; right, BamHI (expected bands of 6815, 5857 and 913 kDa) and BsaI (expected bands of 10,664 + 2921 kDa) restriction analysis of sIgA.

Standard modular cloning allows the combinatorial seamless assembly of synonymous GBparts. Thus, 16 versions of sIgA were constructed by combining the two types of HC (HCα1 and HCα2) with the two versions of LC (LCκ and LCλ) and retaining or not the complex in the endoplasmic reticulum by adding (or not) a *C*-terminal KDEL signal to the HC and/or SC (see Table 1). For the assembly process of the four genes in a single T-DNA, the GoldenBraid cloning system was used.

Table 1. Screening of 16 versions of sIgA by ELISA test. Anti VP8* binding activity is expressed in terms of Abs492 (Abs, absorbance). +++ refers to Abs > 2.0; ++ refers to Abs > 1.0; − refers to Abs < 0.3.

	LCλ/SC	LCλ/SCkdel	LCκ/SC	LCκ/SCkdel
HCα1	++	++	−	−
HCα1kdel	+++	++	−	−
HCα2	++	++	−	−
HCα2kdel	++	++	−	−

A number of steps were followed to construct each sIgA version. First, the so-called multipartite assembly reactions were performed. In multipartite reactions, GBparts are assembled together into binary destination vectors (Ω-GB vectors) to produce individual transcriptional units (TUs). To this end, GBparts are simply mixed together in a single tube with Ω-GB destination vector and subjected to a highly efficient restriction/ligation reaction, which will orderly assemble all the elements of the transcriptional unit together (Figure 1c). In GB multipartite assembly, properly assembled structures are distinguished from the initial destination plasmids using positive blue/white β-galactosidase selection. Multipartite assemblies comprising four individual GBparts (e.g., SC TUs) resulted in 95% white colonies on average, whereas 5-part GBpart assemblies (e.g., HC TUs) resulted in 85% white colonies on average. In all cases, four individual white colonies were selected and tested by restriction analysis and a minimum of three out of four colonies were found correct (see HC panel in Figure 1f as an example).

Once individual transcriptional units were constructed, they were assembled together into 2-part TUs structures using level α-GB destination vectors in a so-called binary GB reaction. Binary reactions combining LC + HC produced genetic modules for the expression of full monomeric IgA (mIgA), whereas SC and JC TUs were also combined in a single genetic module named SC-JC (Figure 1d). Last, mIgA and SC-JC composite parts were assembled together into a final level Ω-GB destination vector in order to construct the complete sIgA (Figure 1e). The final size of the sIgA constructs was of 13.6 kb. Binary assemblies ranged between 5% and 20% white colonies. For each construct, four colonies were selected for digestion, resulting in 100% correct colonies (see examples in Figure 1f).

2.2 Transient expression in Nicotiana benthamiana of 16 versions of sIgA against Rotavirus

All 16 binary plasmids containing sIgA versions against VP8* (see Table 1) were transferred to *Agrobacterium tumefaciens* and transiently transformed in *Nicotiana benthamiana* by means of agroinfiltration. For the resulting 16 plant samples, an initial screening was performed by antigen ELISA to detect anti-VP8* IgA activity in the clarified crude extracts of agroinfiltrated leaves using an anti-HC antibody

for detection. To avoid potential proteolysis, protease inhibitor, PMSF, was added to every extract. In order to ensure the accuracy of the comparison among all the combinations, all samples were equalized on the basis of the luciferase activity of a cotransformed plasmid in which the nopaline synthase promoter drives the luciferase gene. Antigen ELISA tests showed high anti-VP8* binding activity in half of the samples (Table 1). Surprisingly, a very low activity was observed in all sIgA versions containing the LCκ. The integrity of kappa-sIgA constructs was confirmed by retro-transformation of *Agrobacterium* plasmids into *E. coli* and subsequent restriction analysis and sequencing. In addition, the low anti-VP8* activity was confirmed in a second ELISA experiment that yielded similar results (not shown). Consequently, work with LCκ versions was discontinued and all further analyses were done with the LCλ-containing sIgA versions.

A detailed examination of the remaining eight combinations was subsequently performed. The analysis was completed with TUs expressing monomeric IgA and free SC. Leaf age is a known factor influencing recombinant protein expression levels. When leaves of three different ages were transiently transformed and assayed for luciferase expression, a coefficient of variation of 22% was observed. Therefore, in order to increase the accuracy of the comparison, three independent leaves taken from different plants (leaves number 4, 5 and 6, counting from the base of the plant) were infiltrated per each construct. Each leaf was used as an individual biological replicate, and all results were subsequently normalized using a luciferase reporter system as an internal standard. The anti-VP8* activity of each combination was analyzed in detail by antigen-ELISA using three different detection tools, namely anti-HC, anti-LC and anti-SC antibodies. Antigen-ELISA tests against the HC were first carried out in order to give a first view of total IgA content (including mIgA and sIgA). Considerably high binding activity values were observed in all eight sIgA combinations, while the SC control remained negative. Figure 2a shows that ER retention had a positive effect, resulting in significantly higher anti-VP8* activity (p-value < 0.01). This effect is more noticeable when the KDEL signal is added to the SC than to the HC, and the comparison between these two types of samples yielded also significant differences (p-value <0.01). The increase in anti-VP8* activity observed when the SC is targeted to the ER was the first evidence of the correct formation of a secretory complex. Interestingly, the ER-associated activity was shown to increase in a non-additive fashion, as the simultaneous targeting of both HC and SC to the ER did not yield significantly higher activity than individual targeting. A second interesting observation extracted from Figure 2a is that all the combinations containing HCα1 appear to be significantly more efficient than their HCα2 counterparts (p-value <0.01). The above-described results were also confirmed using anti-LC as the detecting antibody, confirming the presence of LC in the functional complexes detected in the previous ELISA test. (Figure 2b).

Finally, antigen-ELISA tests using anti-SC as the detecting antibody were developed in order to specifically detect the anti-VP8* activity of sIgA complexes, as only SC-containing, VP8*-binding antibody complexes can be detected using this ELISA set up. Interestingly, the activity pattern observed for sIgA

combinations was very similar to the one observed for total IgA using anti-HC (compare Figure 2a,c). In particular, the positive effect provided by the presence of a KDEL peptide in the SC was also observed using anti-SC detecting antibody, further confirming that the retention of the SC in the ER increases the overall anti-VP8* IgA activity by specifically stabilizing the sIgA subfraction.

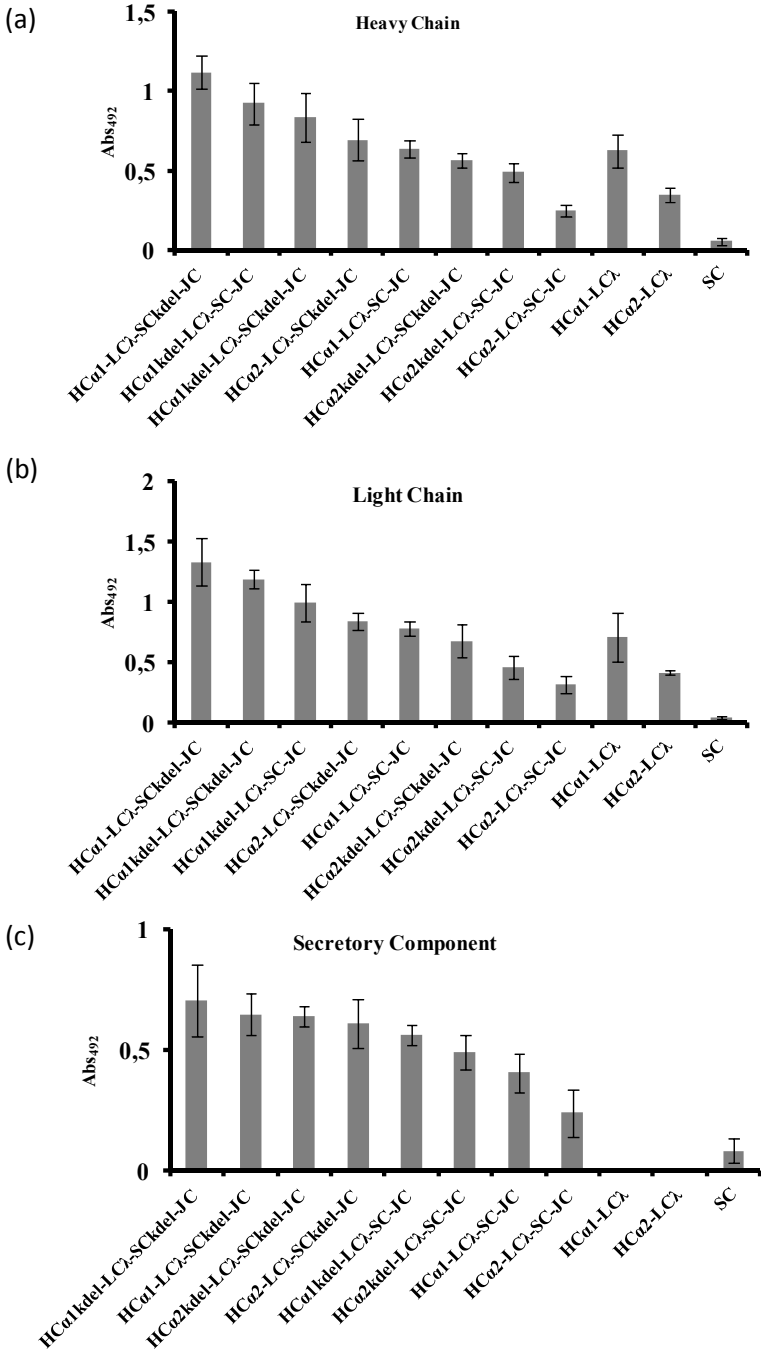

Figure 2. Combinatorial analysis of eight λ versions of sIgA by ELISA tests. (**a**) ELISA assay developed with anti-HC; (**b**) ELISA assay developed with anti-LCλ; (**c**) ELISA assay developed with anti-SC. All plates were coated with VP8* antigen. Three different leaves were infiltrated and tested for each sample. Means of the three biological replicates are represented, with error bars representing the standard deviation. All samples were equalized, with the luciferase reporter system as an internal standard.

Taking into account the whole analysis, the maximum anti-VP8* activity, detected both as total IgA and sIgA, was achieved with the HCα1-LCλ-JC-SCkdel combination. Among non-ER-retained forms, the maximum anti-VP8* activity was achieved with the HCα1-LCλ-JC-SC combination (Figure 2).

2.3 Detailed characterization and purification of the HCα1-LCλ-JC-SCkdel combination

Once the best performing combination was identified, a detailed characterization of the resulting product was undertaken. *N. benthamiana* leaves were agroinfiltrated with the best sIgA-encoding multigenic construct (HCα1-LCλ-JC-SCkdel). A mIgA construct (HCα1kdel-LCλ) and a free secretory component construct (SC) were also agroinfiltrated to be used as controls. At 5 dpi, leaves were harvested, and crude extracts were clarified and used for analysis. The antibody content was quantified by sandwich ELISA using plates coated with anti-HC antibody. mIgA control was also analyzed by Western blot to assess the integrity of the HC and LC when transiently produced in plants, as shown in Figure 3b. It was anticipated that the agroinfiltration of HCα1-LCλ-JC-SCkdel would result in a mix of mIgA and sIgA. The total IgA content (calculated as HC equivalents) in clarified crude extracts was estimated by sandwich ELISA with an anti-HC detecting antibody, whereas an anti-SC detecting antibody was employed to estimate the sIgA content, using a standard curve made with sIgA from human colostrum. As shown in Table 2, both IgA and sIgA constructs yielded similar amounts of total IgA, estimated as HC equivalents, namely 31.6 ± 3.7 and 32.5 ± 1.1 µg/g fresh weight (FW) and representing 1.1% and 1.5% of the total soluble protein (TSP) in leaves, respectively. Expectedly, only background sIgA values were detected for the mIgA and SC-alone constructs, whereas the HCα1-LCλ-JC-SCkdel construct yielded 11.0 ± 0.2 µg/g FW of sIgA (expressed as equivalents of HC), corresponding to a 0.5 ± 0.1% TSP. According to these calculations, it was estimated that at least 33% of the total HC in HCα1-LCλ-JC-SCkdel-infiltrated leaves is present in the form of a sIgA complex (Table 2).

Table 2. IgA/sIgA levels, calculated as HC equivalents, from clarified crude extracts, referred to total soluble protein (TSP) and fresh weight (FW) of the two IgA and sIgA best performing combinations. Means plus or minus the standard deviation of three biological replicates are indicated for each section.

Construct	Best Performing Combination	Total IgA (%TSP)	Total IgA (µg/g FW)	sIgA (%TSP)	sIgA (µg/g FW)
IgA	HCα1kdel-LCλ	1.1 ± 0.1	31.6 ± 3.7	0	0
sIgA	HCα1-LCλ-JC-SCkdel	1.5 ± 0.1	32.5 ± 1.1	0.5 ± 0.1	11.0 ± 0.2

In principle, both mIgA and sIgA can be purified using SSL7-affinity chromatography, which binds the Cα2/Cα3 domain junction of the Fc (Fragment crystallizable) portion of the HC (Langley et al., 2005). For affinity purification, clarified crude extracts were passed through SSL7-agarose columns. The purification steps were monitored by antigen-ELISA, coomassie stained SDS-PAGE and Western blot. Upon PAGE separation of the elution fractions, a 25 kDa band corresponding to the full size LC and a 55 kDa band corresponding to the full size HC were observable in coomassie stained gels from IgA and sIgA samples, after a single step of affinity chromatography (Figure 3a). The 64 kDa coomassie band that would correspond to the free SC was not detected by coomassie staining, but was readily detectable in Western Blot analysis from sIgA samples (Figure 3d). In contrast, SSL7 control purifications from SC-alone and mIgA agroinfiltrations did not yield any detectable SC band (Figure 3d).

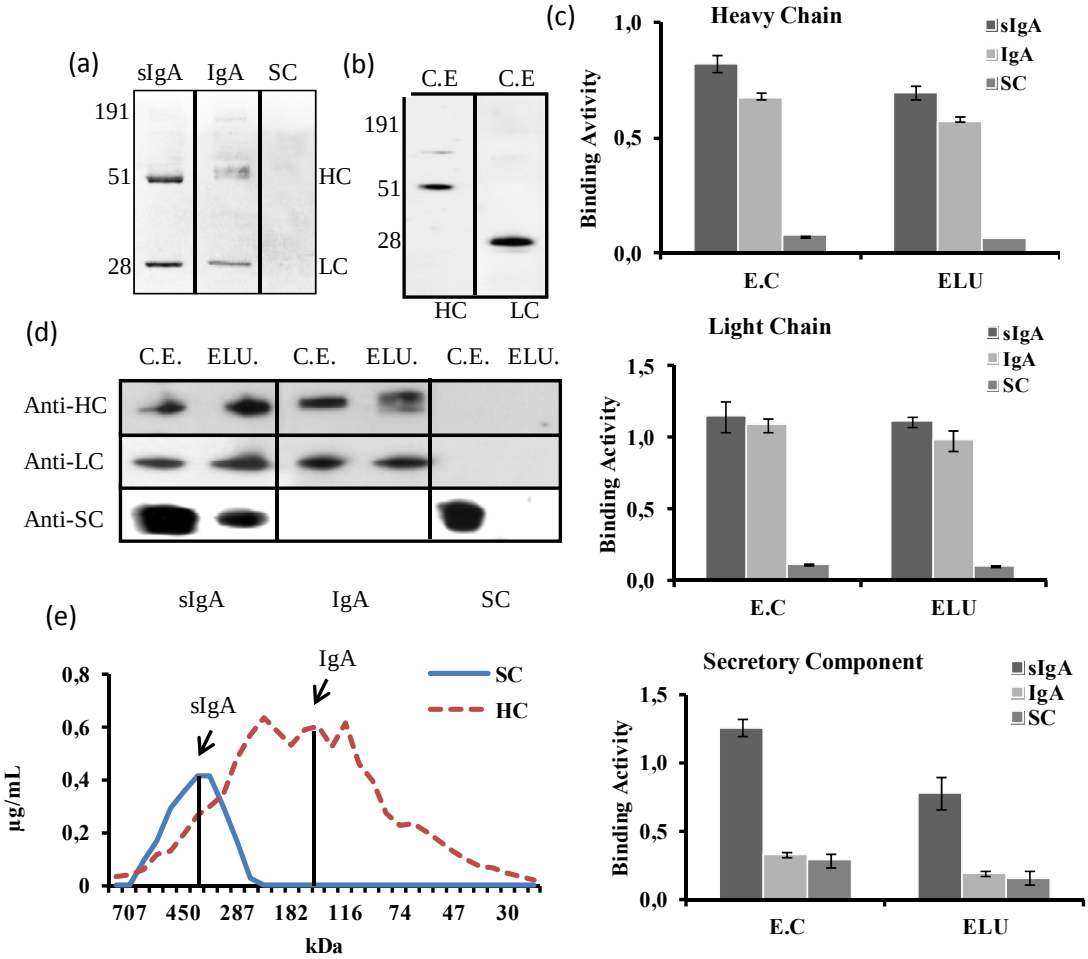

Figure 3. Characterization of SSL7-purified sIgA. (**a**) Coomassie stained SDS-PAGE under reducing conditions of elution fractions corresponding to HCα1-LCλ-JC-SCkdel (sIgA), HCα1kdel-LCλ (IgA) and free secretory component (SC) purification. Bands correspond to the heavy chain (HC) and light chain (LC); (**b**) Western blot analysis under reducing conditions of a clarified crude extract (CE) sample of HCα1kdel-LCλ (IgA) developed with anti-HC (left) and with anti-LC (right); (**c**) ELISA analysis, coated with VP8*, of starting clarified crude extract (CE) and elution fraction (ELU) of three samples: sIgA, IgA and free SC, performed as described in Figure 2. Upper panel developed with anti-HC, medium panel

developed with anti-LC and lower panel developed with anti-SC. Means of three technical replicates are represented with error bars, indicating the standard deviation; (**d**) Western blot analysis under reducing conditions of the starting clarified crude extract (CE) and elution fractions (ELU) of three samples: sIgA, IgA and free SC. Upper lane developed with anti-HC, medium lane developed with anti-LC and lower lane developed with anti-SC; (**e**) ELISA analysis of fractions proceeding from gel filtration chromatography of a sample of SSL7-purified HCα1-LCλ-JC-SCkdel construct. Plates were coated with anti-HC and developed with HRP conjugated anti-HC (red) and anti-SC (blue). A standard curve of commercial IgA from human colostrum was obtained to calculate the concentration of IgA and sIgA (expressed as HC equivalents for proper comparison) for every fraction.

The anti-VP8* binding activity of the three samples was also followed by antigen ELISA. ELISA plates were coated with VP8*, incubated with crude and SSL7 purified extracts and developed with anti-HC, anti-LC and anti-SC antibodies. As expected, anti-VP8* antibody formats containing HC and LC peptides were detected in crude and purified samples from IgA and sIgA agroinfiltrated constructs (Figure 3c), whereas anti-VP8* antibody formats containing SC were only observed in crude and purified samples derived from the sIgA construct. No SC was detected from SC-alone constructs, indicating that free-SC does not bind to VP8* nor SSL7. Interestingly, the relative content of SC-containing antibody seems to decline after SSL7 purification (compare EC and ELU fractions of sIgA sample in Figure 3c, lower panel). This decline is not observed when the same samples are tested for their HC and LC content. Indeed, quantification of purified samples confirmed these results. Of a total of 0.7 µg/mL of HC, only 0.1 µg/mL were in the form of sIgA, which accounts for 21%. This could indicate that at least part of the secretory complex present in the clarified crude extract is disassembled during the purification process, losing its SC peptide and yielding monomeric IgA structures.

With these results and in order to learn more about the proportion of sIgA in the total IgA, a gel filtration assay was performed. For this, sIgA from the best performing combination was purified and 500 µL of the elution loaded into a prepacked high-resolution gel filtration column with a separation range between 10 and 600 kDa. Fractions of 250 µL were collected and monitored by sandwich ELISA assays developed both with anti-HC and anti-SC. Figure 3d shows two partially overlapped peaks, the larger one corresponding to the total content of HC equivalents (including sIgA (371 kDa), mIgA (146 kDa) and possibly unfolded chains) and the smaller one developed with anti-SC, corresponding to 371 kDa sIgA (an estimated 20% of the total IgA content) (Figure 3e).

3. Discussion

The selection of the most appropriate isotype for recombinant antibody production in plants is rarely addressed using experimental approaches, as practical hurdles often override technical or functional considerations. Moreover, sIgA has been only occasionally considered as a feasible option for mucosal passive immunotherapy, despite its demonstrated appropriateness (Ma et al., 1995b). Even more, certain specific decisions concerning antibody design, as the choice for LC and HC isotypes or for subcellular localization, are rarely made on the basis of an exhaustive experimental analysis. However, it has been repeatedly observed that antibody expression levels diverge dramatically from case to case and that, e.g., the experimental selection of the most stable idiotypes, can bring considerable advantages in terms of yield (Juarez et al., 2012; Wieland et al., 2006). A technical hurdle that compromises the selection among different antibody formats is the difficulty to produce multigenic structures that can be assayed in a combinatorial way. In the case of recombinant production of protein complexes in plants, the assembly of multiple transcriptional units in a single T-DNA is an inefficient and tedious task, particularly in the case of sIgA, which requires the co-expression of four TUs. As an alternative approach, some labs, including our own, have relied on *trans*- co-transformation, even though it implies reduced reproducibility and unwanted heterogeneity in the expression levels of the different proteins, often impeding the reach of solid conclusions (Sarrion-Perdigones et al., 2011a). Here, we demonstrate that the recently developed GB assembly system facilitates the combinatorial assembly of the four transcriptional units necessary for sIgA expression, ensuring the coordinated expression of the four genes in transient expression experiments.

In a first screening, it was determined that the use of LCκ severely reduces the activity levels of IgA 2A1. The LCκ used in this assay was codon-optimized for *Nicotiana benthamiana* expression, and therefore, inefficient codon-usage can be discarded as a possible cause for the observed expression. This observation was particularly surprising and, although the integrity of the constructs containing LCκ were exhaustively examined, we could not find any element in the genetic design that could account for the low activity observed in this set of samples. Provided that LCκ have been successfully employed before in the production of other plant-made antibodies (Ramessar et al., 2008; Sack et al., 2007), it is quite possible that this is a specific feature of the 2A1 variable region. If so, this would highlight the importance of combinatorial screenings for successful plant-made antibody expression. Further experiments using variable regions other than 2A1 will serve to discern if the low activity of kappa light chain is an isotype or idiotype-specific feature.

Detailed examination of the remaining combinations led to a number of conclusions. First, these experiments served as a demonstration that a fully human sIgA complex is formed in the plant cell that retains activity against VP8* antigen. In particular, the detection of anti-VP8* activity using an anti-SC detecting antibody is, to our knowledge, the strongest evidence provided so far in support for the formation

of a fully human sIgA complex in the plant cell, as previous reports made use of hybrid murine IgG/A (Ma et al., 1995a). The presence of JC was not assayed, as it is technically challenging, due to the poor accessibility of the molecule and the lack of appropriate detecting antibodies. Therefore, the presence of incomplete complexes within the plant sIgA pool containing only SC bound to IgA in the absence of J-chain cannot be formally discarded. However, this is very unlikely, as it is well established that SC can only bind IgA when this is dimerized by the J-chain (Braathen et al., 2007; Brandtzaeg and Prydz, 1984; Johansen et al., 2000). The small background signal observed when developing antigen-ELISA plates with anti-SC (Figure 2c) is probably due to un-specific binding of glycan structures within the SC, which are known to un-specifically bind to certain pathogens (Hu et al., 2012; Mathias and Corthesy, 2011; Murthy et al., 2011). Also, VP8* has been previously described to bind glycans of the family of sialic acid (Haselhorst et al., 2009; Yu et al., 2012). However, plants lack this type of glycosylation, and therefore, it is unlikely that the observed reactivity is due to VP8* binding activity.

In addition, it was found that IgAα1-based designs presented better results than IgAα2 in all the combinations tested. This is somehow surprising, because the extended hinge of the IgAα1 isotype was expected to result in lower stability against protease degradation, therefore leading to lower activity levels (Bonner et al., 2009). However, it seems that a long hinge does not suppose a handicap for this specific antibody. The IgAα1 hinge is *O*-glycosylated in animal cells with mucin-like sugars, which is thought to confer additional proteolytic defense (Yoo and Morrison, 2005). Mucin-like *O*-glycosylation cannot be achieved natively in plant cells. Instead, arabinose residues linked to hydroxyproline have been reported in the hinge of plant-made IgAα1 (Karnoup et al., 2005). Whether plant specific *O*-glycosylation patterns confer additional stability to IgAα1 remains to be elucidated (Saint-Jore-Dupas et al., 2007).

Finally, a positive effect of ER retention in the overall anti-VP8* activity was clearly observed. It is well established that antibodies retained in plant ER by the addition of a KDEL signal accumulate at higher levels than antibodies that are secreted to the apoplast (De Muynck et al., 2009; Petruccelli et al., 2006; Schouten et al., 1996). Interestingly, we observed that this effect is more noticeable when the KDEL signal is added to the SC. It is worthwhile to notice that the addition of KDEL to the SC increases both the anti VP8* sIgA activity (as measured using anti-SC antibody), as well as the overall anti-VP8* IgA activity, as detected with anti-HC and anti-LC antibodies. This served as an additional indication that a significant portion of the anti-VP8* IgA pool is present in the form of a sIgA.

Further confirmation of the integrity of sIgA complex was obtained with the affinity purification of the resulting recombinant product. As shown, SC co-purifies with IgA when SSL7 is used in affinity binding experiments. SSL7 is reported to bind HCα between domains Cα2 and Cα3 (Langley et al., 2005; Ramsland et al., 2007), therefore discriminating between full-size IgA and Fab fragments. In the same conditions, recombinant SC alone was not recovered from SSL7 columns, indicating that the SC is unable to bind SSL7 on

its own. Interestingly, the recovery rate of sIgA (as detected using an anti-SC antibody prior and after affinity purification) was lower than the recovery rate measured for total IgA (compare panels 1 and 2 with panel 3 in Figure 3c). A possible explanation is that only a fraction of the sIgAα1 complexes are covalently stabilized by disulfide bonds, whereas the remaining complexes are weakly kept together by non-covalent bonds, which can be broken apart during the purification process. Non-covalent complexes were previously described for native sIgAα2 (Almogren et al., 2007), and therefore, it is plausible that non-covalent sIgAα1 complexes could occur in plants due to, e.g., partial assembly and/or inappropriate redox conditions in the plant ER. Although the molar ratio between SC and HC (1:4) could partially explain the low abundance of the SC band in purified IgA samples, the low recovery rate of sIgA compared to mIgA in SSL7 purification is probably contributing to these results.

Overall, it was established that maximum anti-VP8* activity was achieved by transient transformation of a multigene design comprising HCα1, LCλ, KDEL-tagged SC and JC. Using this combination, up to 32.5 µg of HC in its different IgA assembly forms was obtained per gram of fresh weight, with at least one third of this amount being present in the form of sIgA. The remaining two thirds of measurable IgA activity do not form secretory complexes. Free SC in relative large amounts has been detected in all sIgA combinations (data not shown), which would suggest that SC is not a limiting factor in sIgA formation. Thus, it is likely that the low level of complex formation is due to limiting JC expression. This could be resolved by placing the JC under the control of a stronger promoter and/or a stronger expression system.

Further improvements in terms of both the total IgA yield and the sIgA/mIgA ratio may be necessary for those applications involving high antibody doses. Moreover, additional considerations, such as the *N*- and *O*-glycosylation patterns of each antibody form in the context of passive mucosal immunotherapy, should be also considered (Saint-Jore-Dupas et al., 2007). Nevertheless, we think that the current production levels, combined with the high speed and combinatorial versatility of the described platform may provide sufficient competitive advantages for the production of monoclonal sIgAs in low-dose applications and/or in minimally processed formulations.

4. Experimental Procedures

4.1 Cloning and assembly of modular parts

The DNA sequences corresponding to the constant regions of human alpha1 heavy chain (HCα1), alpha2 heavy chain (HCα2), lambda light chain (LCλ) and secretory component (SC) were obtained from Open Biosystems, Huntsville, AL, USA. Kappa light chain (LCκ) and J-chain (JC) were codon optimized for *Solanum lycopersicum* and synthesized by GeneScript, NJ, USA (NCBI Accession numbers KC515402 and KC515401, respectively). The variable regions against VP8* were obtained from a scFv phage display, selected as described earlier (Juarez et al., 2012). The DNA module encoding the signal peptide for secretion was obtained by PCR from tomato SGN-U212775 unigene. Taking advantage of the property of seamless assembly, the junctions between the signal peptide and the coding sequences, e.g., HC (PSLLA-QVQLL), were tested with the signal P algorithm to ensure a correct processing into mature protein. PCR amplification was performed by using the Advantage-2 DNA Polymerase Mix (Clontech, Mountain View, CA, USA). The primers used for amplification of each basic part were synthesized by IDTdna, Coralville, IA, USA (Table S1). PCR was analyzed by agarose 1% gel electrophoresis and purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Amplified parts were TA cloned using the pGEM-T Easy Vector System (Promega, Madison, WI, USA), and 1 µL of the ligation was transformed into DH5α electrocompetent cells. Plasmid DNA preparations were obtained by using the E.Z.N.A. Plasmid Mini Kit I (Omega Bio-Tek, Norcross, GA, USA). Plasmid DNA concentration was measured using a Nano Drop Spectrophotometer 2000 (Thermo Scientific, Rockford, IL, USA). Positive clones were selected in ampicillin-containing plates and confirmed by plasmid restriction analysis (EcoRI, NotI from Thermo Fisher Scientific, Waltham, MA, USA) and by sequencing. Assembly reactions were performed basically as described by (Sarrion-Perdigones et al., 2011a) using BsaI and BsmBI (New England Biolabs, Ipswich, MA, USA) as restriction enzymes in 25-cycle digestion/ligation reactions. T4 DNA ligase was purchased from Promega. One microliter of the reaction was transformed into DH5α electrocompetent cells. Positive clones were selected in kanamycin or spectinomycin-containing plates. Plasmid DNA preparations were made by using the E.Z.N.A. Plasmid Mini Kit I (Omega Bio-Tek). Plasmid DNA concentration was measured using a Nano Drop Spectrophotometer 2000 (Thermo Scientific). Constructs were confirmed by plasmid restriction analysis and by sequencing. Constructs for plant functional assays were transferred to *Agrobacterium tumefaciens* electrocompetent strain GV3101 containing pSoup plasmid.

4.2 Strains and growth conditions

Escherichia coli DH5α was used for gene cloning, and *Agrobacterium tumefaciens* strain GV3101 with pSoup was used for plant agroinfiltration and transformation experiments, as described in (Sarrion-Perdigones et al., 2011a).

4.3 Plant transient transformation

Agroinfiltration was performed as previously described in (Orzaez et al., 2006). Briefly, overnight grown bacterial cultures (5 mL) were sedimented by centrifugation (15 min, 3000× *g*), resuspended in agroinfiltration buffer (AB) (10 mM MES pH 5.6, 10 mM MgCl2, 200 µM acetosyringone) and incubated for 2 h at room temperature (RT) on a horizontal rolling mixer. Bacterial cultures were diluted in AB to an optical density of 0.2 at 600 nm. Co-infiltrations of each construct with both pGreen_P19 (bushy stunt virus-TBSV-P19, suppressor of silencing) (Sarrion-Perdigones et al., 2011a) and pGreen_Luciferase (*Firefly Luciferase* Genetic reporter under the control of a Nopaline synthase promoter and terminator) were performed by mixing equal volumes of the corresponding bacterial suspensions. Inoculations were carried out by syringe-agroinfiltration in leaves of 4–5 weeks old *Nicotiana benthamiana* plants (growing conditions: 24 °C day/20 °C night in a 16 h light/8 h dark cycle). Samples were collected 5 days post-infiltration and examined for transgene expression.

4.4 Plant material and sample preparation

For sample preparation, *Nicotiana benthamiana* leaves were ground with a mortar and a pestle to a fine powder under liquid nitrogen, and the total soluble protein (TSP) was extracted with 1:3 (*w*:*v*) phosphate buffer saline (PBS) pH = 7.4 complemented with 0.5 mM PMSF protease inhibitor. After mixing, the suspension was centrifuged twice at 2 °C at 16,000× *g*, and the supernatant, referred to as clarified crude extract, was immediately used for analysis. Samples were equalized with the luciferase reporter system as an internal standard. Luciferase activity was determined with the Dual-Luciferase Reporter Assay System (Promega), following the manufacturer's procedures, and luminescence was measured with a Glomax 96 microplate luminometer (Promega).

4.5 VP8* Rotavirus surface protein production

Recombinant VP8* was obtained as described in (Juarez et al., 2012). Briefly, *Escherichia coli* M11 were transformed with Plasmid pQEVP8*, kindly provided by Monedero from Instituto de Agroquímica y

Tecnología de Alimentos (IATA, Valencia, Spain). Purification was performed using HisTrap HP affinity columns (GE Healthcare, Buckinghamshire, UK), following the manufacturer's procedures.

4.6 ELISAs for the quantification and detection of VP8* binding activity of IgA and sIgA

Plates (Corning, New York, NY, USA) were coated overnight at 4 °C in coating buffer (50 mM carbonate buffer pH 9.8) either with anti-IgA capture antibody 1:500 (Sigma-Aldrich, St-Louis, MO, USA) for IgA/sIgA quantification or with 10 µg/mL of recombinant VP8* for the detection of VP8* binding activity. Plates were then washed four times in PBS and blocked with a 2% (*w*/*v*) solution of ECL AdvanceTM 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 h at room temperature. After incubation, plates were washed four times in PBS, and the antibodies for detection were added in PBS-T-2% blocking buffer (GE Healthcare). Four different antibodies were used for detection in VP8*-ELISAs: anti-HC (HRP-conjugated) 1:5000 (Sigma Aldrich, St. Louis, MO, USA), anti-LCλ (non-conjugated) 1:5000 (Sigma Aldrich), anti-LCκ (non-conjugated) 1:5000 (Pierce Thermo Scientific) and anti-SC (non-conjugated) 1:500 (Gentaur, Kampenhout, Belgium). Anti-rabbit-HRP (GE Healthcare) secondary antibody (1:5000) was used after the non-conjugated detecting antibodies. For quantification, anti-HC (HRP-conjugated) 1:5000 (Sigma Aldrich) and anti-SC (non-conjugated) 1:500 (Gentaur, Kampenhout, Belgium) were used. Anti-rabbit-HRP (GE Healthcare) secondary antibody 1:5000 was also used for detection after the anti-SC detecting antibody. After four PBS washes, the substrate (*O*-phenylenediamine from Sigma Aldrich) was added, and the reactions were stopped with 3 M HCl. Absorbance was determined at 492 nm. The mean and SD of three samples of each combination were calculated for every VP8*-ELISA. A standard curve of HC content in a commercial IgA obtained from human colostrums (Sigma-Aldrich) was used to calculate the HC content in IgA/sIgA samples. The same standard was used to estimate the sIgA content in sIgA-containing samples using anti-SC as the secondary antibody. To facilitate comparisons, all antibody concentrations were provided as equivalents of HC. Three replicates were analyzed per each experimental point, and the mean ± SD was calculated.

4.7 SDS-PAGE and Western blot analysis

Proteins were separated by SDS/PAGE in 10% denaturing gels (Invitrogen, Carlsbad, NM, USA). Gel staining was carried out with coomassie following standard procedures. For Western blot analysis, blots were incubated with 1:20,000 anti-HC (Sigma Aldrich), 1:10,000 anti-LC (Sigma Aldrich) or anti-SC 1:5,000 (Gentaur), followed by 1:20,000 µg/mL HRP-conjugated anti-rabbit IgG secondary antibody (GE Healthcare) for the detection of the LC and SC. Blots were developed with an ECL Plus Western Blotting Detection System (GE Healthcare).

4.8 SSL7 affinity purification

The SSL7 protein specifically binds the hydrophobic interface between the Cα2 and Cα3 domains of the Fc portion of the HC. Protein extracts, prepared as explained previously, were further clarified using a 0.22 µm Stericup (Millipore, Billerica, MA, USA) on ice. Purification steps were performed as previously described by (Juarez et al., 2012).

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7. Supplementary material

Suplementary Table 1: Oligo List

BASIC PART	PRIMER NAME	PRIMER SEQUENCE 5' – 3'
35s	A10JUL09	CCCGTCTCACCACACTAGAGCCAAGCTGATCTC
	A10MAY111	CCCGTCTCACATCGATATCACTAGTGCGGCCGC
SP	A10SEP05	CCCGTCTCAGATGGGCACTTCCTCTGTTTT
	A10JUL10	CCCGTCTCAGGCAAGGAGGGACGGGAGAAGGAG
VH	A10JUL16	CCCGTCTCATGCCCAGGTGCAGCTGTTGCAGT
	A10JUL17	CCCGTCTCAATGCGACGGTGACCAGGCTACCTT
VL	A10JUL11	CCGTCTCATGCCTCTTCTGAGCTGACTCAGGA
	A10JUL12	CCGTCTCAACCTAGGACGGTCAGCTTGGTCC
CLλ	A10JUL13	CCCGTCTCAAGGTGGTCAACCAAAGGCCGCCCCCTCTGTCACTCT
	A10JUL03	GGCGGGAGTGGAGACAACCAAACCC
	A10JUL04	GGGTTTGGTTGTCTCCACTCCCGCC
	A10JUL14	CCCGTCTCACTCATGAACATTCTGTAGGGGCCA
	A10JUL14KDEL	CCCGTCTCACTCAAAGTTCATCTTTTGAACATTCTGTAGGGGCCA
CH1	A10JUL18	CCCGTCTCAGCATCCCCGACCAGCCCCAAGGTC
	A10JUL19	CCCGTCTCACTCAGTAGCAGGTGCCGTCCACCT
	A10JUL20	CCCGTCTCACTCAGAGTTCGTCCTTTGAACATTCTGTAGGGGCCA
CH2	A10JUL18	CCCGTCTCAGCATCCCCGACCAGCCCCAAGGTC
	A10AGO01	ACCATGGGGAAACCTTCACC
	A10AGO02	TGCAGGTGAAGGTTTCCCCATGGTTCCATGG
	A10JUL19	CCCGTCTCACTCAGTAGCAGGTGCCGTCCACCT
	A10JUL20	CCCGTCTCACTCAGAGTTCGTCCTTTGAACATTCTGTAGGGGCCA
SC	A10SEP07	GGGGTCTCATCATCGTCTCATGCCAAGAGTCCCATATTTGGTCC
	A10SEP08	GCATATTTGCTGGAAACGTAGC
	A10SEP09	TCCTCGGAGGGCTACGTTTC
	A10SEP11	GGGGTCTCACTCACCTGGGATCCTGAATGGCTTTGT
	A10SEP10	GGGGTCTCACTCAGAGTTCGTCCTTCCTGGGATCCTGAATGGCTT
Tnos	A10ABR03	CCCGTCTCATGAGGGAATGGATCTTCGATCCCGATCGT
	A10JUL15	CCCGTCTCAGACGGCGAGTCGGTCCCATTATTGA

General Discussion

Today, infectious diseases are the world's biggest killers of children and young adults. They account for more than 13 million deaths a year - one in two deaths in developing countries (WHO, 1999). Among the six most important infectious diseases (Diarrhoea, HIV/AIDS, Malaria, Measles, Pneumonia and Tuberculosis), diarrhoeas caused by enteric pathogens kill approximately 2.5 million infants worldwide and more than 450.000 of these deaths are caused by Rotavirus itself (Parashar et al., 2003).

Passive immunization strategies have a great potential for prevention and treatment of infectious diseases. The current high cost of recombinant antibodies coupled with the high antibody quantities required, limits the wide spectrum use of passive immunization. Plants are emerging platforms for inexpensive recombinant antibody production and, in particular, expression in edible fruits is a promising strategy to achieve high antibody quantities for oral administration. Despite this, at the time this work was started there were no experimental indications on the feasibility of this strategy. The experimental work conducted here has served as an initial proof of concept, showing that anti-rotavirus neutralizing immunoglobulin A antibodies can be stably produced in the fruit at moderate levels without apparently inducing deleterious changes in its edible composition. Moreover, a deeper understanding of the requirements for sIgA production and assembly in plants was obtained with the combinatorial analysis of different sIgA isoforms. From the standpoint of the experimental data produced here, new technical and ethical considerations arise that will be discussed next and that will need to be addressed in the future in order to bring forward a fruit-based OPI.

1. IgA expression levels

The first part of this work shows that considerably high yields of recombinant IgA against the model rotavirus strain SA11 are produced in tomato fruits. The homozygous T3 elite IgA producing-tomato line accumulated on average around 40 µg of IgA per gram of tomato fresh weight, which corresponds to roughly 0,7 mg/g DW. However it is difficult to conclude whether the current levels would be sufficient for ensuring a protective effect in the target mucosa as there is no commercialised pharmaceutical based on monoclonal antibodies for OPI. According to a recent work developed by Virdi et al. (2013), to protect one piglet of 1 month of age and ≈8 Kg of weight it is necessary to supply 20 mg of an antibody cocktail per day. The extrapolation of these quantities to 2 years old children (12 Kg in weight) yields an estimate daily dose of 43.0 g of tomato powder, approximately equivalent to 3-4 fresh tomatoes per day. Although these quantities can vary considerably considering the neutralizing activity of each antibody, it is evident that a more concentrated product will undoubtedly facilitate the success of the fruit-OPI strategy.

An obvious direction for improvement is the increase in the *in planta* expression levels. Our elite line produces IgA approximately at a 4% TSP rate. This was a remarkable improvement from the 1-2% TSP levels found in T1, particularly considering the relatively small population employed in our minibreeding strategy,

and indicates that breeding can substantially bring up antibody yields in stable transformants. By comparing with other systems (Hadlington et al., 2003), it is reasonable to believe that by generating large number of initial transformants and through an intense breeding strategy it would be possible to reach up to 8-10% TSP as reported e.g. for sIgA in tobacco. In this regard, it has been also suggested that sIgA stability can contribute to boost *in planta* accumulation (Hadlington et al., 2003). As a rule of thumb, an 8-10% TSP would be equivalent to a 20 mg dose per flesh tomato (200 g each), which could be close to the daily dose required for protection.

Additional strategies could be considered in order to boost IgA expression in fruits above the 10% threshold. Higher expression levels have been reported using viral vectors (Werner et al., 2011) or viral-derived transcriptional enhancers (Sainsbury and Lomonossoff, 2008; Sainsbury et al., 2009) in leaves. An interesting possibility is to trigger the expression upon ripening of a viral vector integrated in the plant genome, taking advantage of this natural gene expression switch. Although the technology investment is considerable, it is technically feasible to adapt these technologies to the fruit. Alternatively, to reach higher antibody concentration it is possible to use partially purified formulations which do not disturb the GRAS status of the final composition. This could be accomplished by industrial processes for protein concentration as e.g. partial precipitation (Giese et al., 2013; Li et al., 2013; Oelmeier et al., 2013), cross flow (micro) filtration (Baruah et al., 2005; Lee et al., 1992), or membrane chromatography (Zhou and Tressel, 2006), yielding IgA-enriched formulations.

Alternative edible plant organs should also considered as production platforms. Among them, seeds seem most promising as they are natural protein producers, permit long-term storage, ease of handling and transporting without cold chain maintenance. Among the different seed options, rice seeds are the most popular choice (Kurokawa et al., 2013; Nojima et al., 2011; Steere et al., 2012; Suzuki et al., 2011; Tokuhara et al., 2010; Yuki et al., 2009). Recent works show that with both in *Arabidopsis* and rice seeds high antibody levels can be produced, which can reach 40 fold the production levels achieved after drying tomato fruits. Virdi et al. (2013) and Tokuhara et al. (2013) show levels up to 30 mg/g of *Arabidopsis* seeds and 5,6 mg/g rice seeds respectively. The disadvantage of producing antibodies for OPI in seeds is that generally seeds cannot be consumed uncooked. With llama-heavy chain antibodies (VHH) as an exception, antibodies are degraded and/or deactivated if subjected to high cooking temperatures which makes seed-based pharmaceuticals not that appropriate for oral therapies.

A current trend in antibody therapy consists on the use of antibody cocktails made of a small number of monoclonal antibodies as a way to increase the efficiency of the treatment (Bakker et al., 2008; Both et al., 2013; Koefoed et al., 2011; Logtenberg, 2007; Robak et al., 2012). Synergistic neutralizing effects have been reported when two or more antibodies are combined against infectious agents. Moreover, the use of antibody cocktails could contribute to reduce the risk of inducing resistance. In the case of fruit-based

OPI, the preparation of antibody cocktails will require the establishment of separate production lines, one per each antibody, and the subsequent mixed formulation of the cocktail. The co-expression of several recombinant antibodies in the same transgenic plant is an alternative strategy; however it is likely that chain shuffling among the different idiotypes would limit the viability of this approach.

2. Formulation and stability in the mucosa

Two tomato fruit-based antibody formulations of facile preparation were shown to neutralize rotavirus infections *in vitro* as efficiently as purified IgA. These preparations consist of tomato juice (clarified crude extract) and tomato powder (freeze-dried ground tomato). Dried formulations are most convenient not only for long-term storage and transportation but also for antibody concentration. However, to achieve a successful protection against infectious diseases by means of OPI, a critical limitation is the inactivation or degradation of antibodies by physiological temperature, low pH pepsin-rich environment of the stomach and by digestive enzymes in the intestine (Harmsen et al., 2005; Hussack et al., 2011; Lee et al., 2012). It can be argued that OPI imitates native maternal passive protection and therefore recombinant sIgA is best suited to endure the aggressive digestive environment. However, maternal OPI is most effective in newborns whose digestive system is not fully developed. It is therefore questionably that unprotected antibodies could provide an effective OPI in a mature digestive tract. The plant matrix has been seen to offer protection against degradation (Virdi et al., 2013), and this could play in favour of a tomato-based formulation. However, to study the stability of antibodies during digestion, the tomato-derived formulations should be extensively assayed in conditions which mimic the environment of the digestive tract, therefore subjecting them to physiological temperature, agitation and simulated gastric and intestinal fluids (Hussack et al., 2011; Lee et al., 2012; Liu et al., 2011).

Several engineering, selection-based approaches and formulations could be made to improve thermal and pH stability and protease resistance. Some of them could be taken into account in further attempts of producing recombinant antibodies, for instance, engineered disulfide bonds can increase the thermal stability of recombinant antibody fragments (Gong et al., 2009; Hagihara et al., 2007; Hussack et al., 2011; Saerens et al., 2008; Young et al., 1995). Moreover, selection of antibodies from libraries in the presence of proteases, denaturants, extreme pH and elevated temperatures can lead to the isolation of more resistant clones with favourable characteristics (Jermutus et al., 2001). Alternatively, downstream processing can also be used to improve stability of antibodies. Several formulations have shown to protect different recombinant proteins from degradation when incubated under digestion conditions. Microencapsulation of avian Immunoglobulin Y (IgY) with a pH-sensitive methacrylic acid copolymer and also with chitosan-alginate microcapsules were found to protect IgY from gastric inactivation *in vitro* (Kovacs-Nolan and Mine, 2005; Li et al., 2007). An advantage of using tomato fruit as a production platform is that

downstream processing can also benefit from the "state of the art" tomato processing technology. For instance, spry drying (a method used in food industry for production of tomato powder) which has been historically a non-popular process for handling delicate biotechnological products, is gaining acceptance incessantly (Bowen et al., 2013; Maa et al., 1998). Moreover, companies like Upperton in UK have succeeded in spray drying more than 30 biological formulations including peptides and monoclonal antibodies without significant loss of activity (Upperton, 2013).

3. Safety considerations: Identity preservation and transgenic unintended effects.

The IgA-producing tomato lines were transgenically labelled with anthocyanins in order to make them easily distinguishable from wild type lines. Ros1/Del purple tomatoes bred up to F3 showed comparable IgA levels and neutralizing activity than equivalent red tomatoes, indicating that the antocyanin background compromise neither the expression nor the functionality of the IgA. Labelling edible OPI biofactories with distinctive phenotypic features could contribute to ease the opposition against the use of edible crops for the production of therapeutic proteins, an attitude that arose after the Prodigene episode in 2002 (Prodigene, 2002). Engineering of the phenylpropanoid biosynthesis pathway could provide a range of possible color labels not only in tomato but also in other edible and not edible crops for which labelling could be also an advantage as e.g. open field GMOs. Also in tomato, alternative labels could be obtained by manipulating phenylpropanoid but also carotenoid biosynthesis, (e.g. yielding yellow or white tomatoes by blocking phytoene desaturase), or by combining both anthocyanin and carotenoid manipulation, as demonstrated using Virus Induced Gene Silencing in the fruit (Orzaez et al., 2009).

A potential problem of the transgenic labelling strategy through metabolic engineering is that the GRAS status of the resulted product is not guaranteed. Approval of anthocyanin-enriched tomatoes, as other biofortification strategies in plants (Beyer et al., 2002; Ortiz and Swennen, 2013) is an ongoing effort and yet a partially unexplored area in terms of regulatory issues. The possibility of using metabolic engineering as a labelling strategy in edible fruits will very much depend on the regulatory conditions imposed to related biofortification attempts. Alternatively, non-transgenic, minority GRAS varieties and cultivars showing distinctive features (e.g, white, yellow, pink, green fruits) (Paran and van der Knaap, 2007), are also available that could be considered as recipients for transgenic labelling.

The uncertainty about the GRAS status of purple tomatoes led us to evaluate the presence of unintended effects associated with IgA production using only red tomatoes varieties. Edible plant organs are considered as such because at the mature stage they contain essential nutrients but lack toxicants, allergens or anti-nutrients in their composition that could result harmful for the majority of the population. This does

not exclude, in the case of allergens, that a small portion of the population could be harmed, even severely, by the presence of a particular compound. The objective of this work relies on the assumption that recombinant production of IgA in edible tomatoes does not alter substantially fruit composition and consequently its edibility. This hypothesis had never been corroborated experimentally, and the availability of IgA tomatoes represented a unique opportunity to confirm or reject the assumption. The proteomic and metabolomic profiles performed on IgA and non-IgA tomatoes failed to identify substantial differences associated with IgA production beyond the natural variation between tomato lines and cultivars. All the significant differences found in the proteome corresponded to antibody chains or antibody fragments. In the metabolomic analysis, although a model could be created that separates IgA lines from their wild type relatives, these differences resulted from a combination of multiple variables and could not be associated with the presence of abnormal levels of any particular secondary metabolites in the IgA fruits. Taking together, these data support the initial hypothesis and provides experimental support to the assumption that edible formulations derived from IgA tomatoes are not less safe for consumption than equivalent formulations derived from wild type tomatoes.

4. Production of sIgA in fruits.

The feasibility of producing functional IgA in the fruit was addressed in Chapter 1. However, as already described in the introduction, secretory IgA (sIgA) is more convenient for OPI. For that reason, sIgA expression was set as the following goal (Chapter 3). The selection of the most appropriate isotype was addressed by performing a combinatorial analysis where sixteen different sIgA combinations were constructed and tested. With the use of multigene engineering technologies as the Goldenbraid assembly system (Sarrion-Perdigones et al., 2011; Sarrion-Perdigones et al., 2013), this task was relatively effortless. The best performing sIgA complex in transient expression analysis comprised α1 Heavy Chain (HC) and λ Light Chain (LC) and was retained in the ER by means of a KDEL peptide attached to the C-terminal end of the SC. The superior stability of constructs carrying α1 HC were somehow unexpected as this has an elongated hinge region, which is susceptible to proteolytic degradation. However, it seems that a long hinge region does not suppose a serious drawback to the antibody.

The hinge region of the α1 HC is O-glycosylated in animal cells with mucine-like sugars, a decoration that cannot be achieved natively in plant cells. Instead, arabinose residues are described to attach to the hinge region of α1 HC when produced in corn (Karnoup et al., 2005). Mucine-like sugars are thought to protect the hinge region against proteolitic degradation (Yoo and Morrison, 2005); moreover, mucophilic properties conferred by mucin may facilitate the removal of attached antigens via the mucus. However, whether plant-specific glycosylation patterns confer comparable properties to the hinge region is still

unknown (Twyman et al., 2003). On the other hand, SC is a heavily N-glycosylated protein, this probably conferring additional resistance to the complex. The humanization of the N- and O-glycosylation patterns of the sIgA should be considered in the future as it might improve biological activity and efficacy. In fact, collaboration has already been established with Assoc. Prof. Richard Strasser in BOKU, Vienna, to address these issues.

As mentioned in Chapter 3, two thirds of the measurable IgA activity do not form secretory complexes. Additionally, relatively large amounts of free SC have also been detected. These facts suggest that the expression of the JC could be a limiting factor in sIgA complex formation, since SC only binds to dimeric IgA complexes containing a J chain. A similar phenomena was observed by Virdi et al. (2013) when co-transformed *Arabidopsis* with a T-DNA bearing VHH-IgA coding sequences and a second T-DNAs bearing the coding sequences of the JC and SC, resulting in all three possible assembled formats: mIgA, dIgA and sIgA. On the whole, a higher proportion of secretory complex would be desirable, provided that sIgA is considered to be the most stable form in the mucosa. A possible approach to achieve this would be to modulate the expression ratios of all the elements comprising the complex (HC, LC, JC and SC) via careful selection of the activity of the different promoters involved. Stable transformation of tomato and/or an alternative edible platform would be the next step to carry out once the expression of sIgA is improved.

5. Final remarks

Despite all the mentioned advantages that plants can offer as antibody expression systems, very few antibodies have been subjected to clinical trials (Obembe et al., 2011; Yusibov et al., 2011). The regulatory issues associated with genetically modified (GM) plants and the already established mammalian systems for production of antibodies are most likely obstructing the natural development of this technology (Paul and Ma, 2011; Virdi and Depicker, 2013). The first issue can be partially solved by growing plants in contained glass facilities instead of on large open fields. To obtain the required permission for crop-growing in greenhouse facilities is easy and not as expensive as for in field production (legislation 2009/41/EC – contained use). Moreover, expression levels are currently sufficiently high to generate large antibody quantities from reasonably small plant biomass (Paul and Ma, 2011; Whaley et al., 2011; Xu et al., 2012). The second issue is a question of placing each technology in the most appropriate niche. Most probably, plant expression systems are not going to replace mammalian antibody expression as this is well established with antibodies which have already gone through the cGMP production and drug regulation. Production of "biosimilars" in plant production platforms is probably not worthwhile. However, there are certain advantages that plant platforms provide that could be the clue for production of "biobetters" (therapeutics with enhanced features and/or efficacy). Among others, plants are human-pathogen free, are quick in

antibody production with transient expression systems, glycosylation patterns can be currently engineered *à la carte* and last, the possibility of producing complex sIgA and IgM antibodies which can be exploited for oral therapies. Thus, plant antibody expression technology has its own niche, which might be far more suitable than conventional platforms for the production of certain antibodies (Virdi and Depicker, 2013).

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Conclusions

1. Neutralizing human immunoglobulin A (IgA) antibodies against rotavirus can be produced using transgenic tomato fruits as biofactories, reaching levels up to 0,7 mg of IgA per gram of dry weight. Minimally processed tomato-derived products suitable for oral intake showed strong anti-rotavirus binding activity and inhibited virus infection *in vitro*.
2. It is possible to label IgA-producing tomato fruits with a distinctive purple colour by engineering the anthocyanin biosynthesis pathway in the fruit. Minimally-processed products derived from IgA purple tomatoes overexpressing Rosea1 and Delila transcription factors showed anti-rotavirus activities similar to their red counterparts.
3. No substantial differences beyond natural variation associated with IgA production were found in IgA-tomatoes when their proteomic and metabolomic profiles were analyzed. This supports the assumption that formulations derived from IgA-tomatoes maintain the edible status.
4. A fully human secretory IgA (sIgA) can be assembled in plants by co-expressing all four required genes in the same cell. Moreover, it is feasible to optimize sIgA production in plants by means of a combinatorial analysis assisted by GoldenBraid technology.