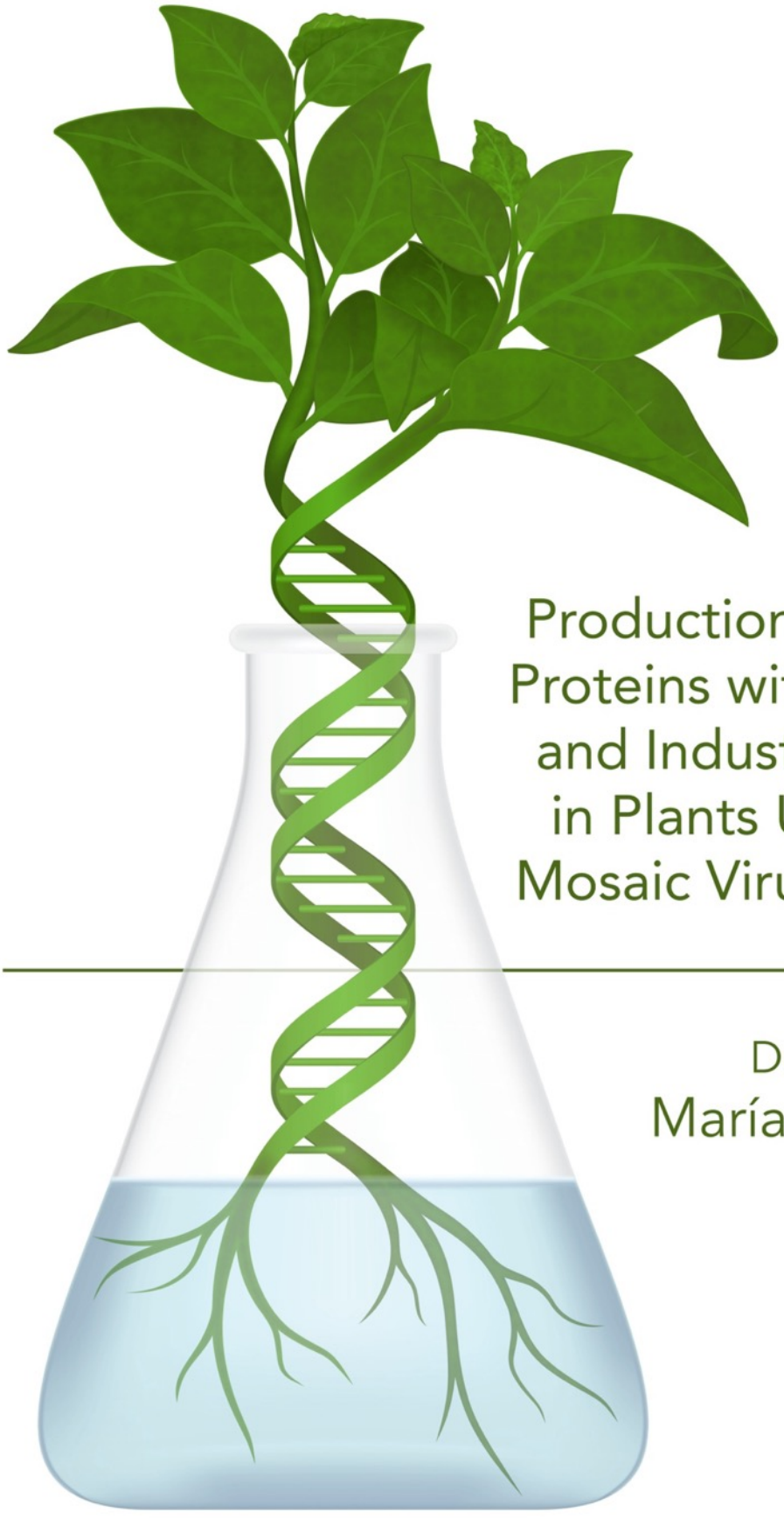

Production of Recombinant Proteins with Pharmaceutical and Industrial Applications in Plants Using a Tobacco Mosaic Virus-Derived Vector

Doctoral Thesis

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Valencia, July 2023

UNIVERSITAT POLITÈCNICA DE VALÈNCIA

PhD in Biotechnology

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CERTIFICA:

Que Dña. María Nicolau Sanus, Graduada en Biotecnología por la Universidad Católica de Valencia San Vicente Mártir, ha realizado bajo su dirección el trabajo con título "Production of Recombinant Proteins with Pharmaceutical and Industrial Applications in Plants Using a Tobacco Mosaic Virus-Derived Vector" que presenta para optar al grado de Doctora en Biotecnología por la Universitat Politècnica de València.

Y para que así conste a los efectos oportunos, firma el presente certificado en Valencia a 27 de Julio de 2023.

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Dr. José Antonio Daròs Arnau

A la meua estrella
Als meus pares

Summary

Plants are emerging as an attractive alternative to conventional heterologous production systems, including bacteria, mammalian cell cultures, yeast, or fungi, for the synthesis of high-value products, such as secondary metabolites and proteins. The demand for these products is often limited by production capacity and associated costs. However, using plants as production platforms offers advantages in terms of affordability, sustainability, scalability, and the absence of human and livestock pathogens, making them an increasingly appealing choice. Despite limitations in cargo capacity, transient gene expression using viral vectors provides an efficient and reproducible method for producing recombinant proteins in plants, unlike stable transformation, which is more labor-intensive and time-consuming. Vectors derived from tobacco mosaic virus (TMV), particularly those in which the viral coat protein (CP) gene is mostly replaced with the gene of interest, are classic in plant biotechnology and frequently use for large-scale production of recombinant proteins. In this work, we first aimed to optimize a TMV vector to improve production. Translational fusion of the amino-terminal end of TMV CP to the recombinant protein of interest led to increased accumulation of the green fluorescent protein, interferon alfa-2a and a nanobody against the Spike protein of SARS-CoV-2 in *Nicotiana benthamiana* leaves. Insertion of a specific cleavage site, based on the nuclear inclusion *a* proteases (NIaPro) form two potyviruses, along expression of the cognate proteases led to, in addition to production of the mature recombinant protein, a further increase in the accumulation of the aforementioned recombinant proteins. Second, we aimed to set up strategies to produce recombinant proteins of industrial and pharmaceutical interest in N. benthamiana using a TMV vector. We successfully produced large amounts of a thermophilic xylanase, active under extreme temperature and alkaline pH conditions, in *N.*

benthamiana using a TMV vector. The enzyme that accumulated rapidly in plant tissues was targeted the apoplast, which enormously facilitated purification, and avoided any adverse effect on plant growth. This plant-made enzyme was shown to be useful for the production of probiotic xylooligosaccharides. We also produced large amounts of an engineered glucose oxidase (GOX) in *N. benthamiana* leaves using a TMV vector. The plant-made GOX that was also easily purified from apoplastic fluids exhibited potent antimicrobial properties against *Staphylococcus aureus* and *Escherichia coli*.

Resumen

Las plantas están emergiendo como una alternativa atractiva a los sistemas convencionales de producción heteróloga, como bacterias, cultivos celulares de mamíferos, levaduras u hongos, para la síntesis de productos de alto valor, como metabolitos secundarios y proteínas. La demanda de estos productos a menudo se ve limitada por la capacidad de producción y los costos asociados. Sin embargo, utilizar las plantas como plataformas de producción ofrece ventajas en términos de accesibilidad económica, sostenibilidad, escalabilidad y la ausencia de patógenos humanos y de animales, lo que las convierte en una opción cada vez más atractiva. A pesar de las limitaciones en la capacidad de carga, la expresión génica transitoria utilizando vectores virales proporciona un método eficiente y reproducible para la producción de proteínas recombinantes en plantas, a diferencia de la transformación estable, que requiere más mano de obra y tiempo. Los vectores derivados del virus del mosaico del tabaco (TMV), particularmente aquellos en los que el gen de la proteína de la cubierta viral (CP) se reemplaza principalmente por el gen de interés, son clásicos en la biotecnología vegetal y se utilizan con frecuencia para la producción a gran escala de proteínas recombinantes. En este trabajo, nuestro primer objetivo fue optimizar un vector de TMV para mejorar la producción. La fusión traduccional del extremo amino-terminal de la CP del TMV con la proteína recombinante de interés mostró un aumento en la acumulación de la proteína verde fluorescente, interferón alfa-2a y un nanobody contra la proteína Spike del SARS-CoV-2 en hojas de *Nicotiana benthamiana*. La inserción de un sitio de clivaje específico, basado en las proteasas de inclusión nuclear (NIaPro) de dos potyvirus, junto con la expresión de las proteasas correspondientes, además de la producción de la proteína recombinante madura, aumentó aún más la acumulación de las proteínas recombinantes mencionadas anteriormente. En segundo lugar, nuestro objetivo fue establecer estrategias para producir proteínas

recombinantes de interés industrial y farmacéutico en *N. benthamiana* utilizando un vector de TMV. Logramos producir grandes cantidades de una xilanasa termofílica, activa en condiciones extremas de temperatura y pH alcalino, en *N. benthamiana* utilizando un vector de TMV. La enzima que se acumuló rápidamente en los tejidos de la planta se dirigió al apoplasto, lo que facilitó enormemente la purificación y evitó cualquier efecto adverso en el crecimiento de la planta. Se demostró que esta enzima producida en planta es útil para la producción de xilooligosacáridos probióticos. También produjimos grandes cantidades de una glucosa oxidasa (GOX) modificada en hojas de *N. benthamiana* utilizando un vector de TMV. La GOX producida en planta, que también se purificó fácilmente a partir de fluidos apoplásticos, exhibió potentes propiedades antimicrobianas contra *Staphylococcus aureus* y *Escherichia coli*.

Resum

Les plantes están emergint com una alternativa atractiva als sistemes convencionals de producció heteròloga, com ara bacteries, cultius cel·lulars de mamífers, llevats o fongs, per a la síntesi de productes d'alt valor, com son metabòlits secundaris i proteïnes d'interés industrial i farmacéutic. La demanda d'aquests productes sovint es veu limitada per la capacitat de producció i els costos associats. No obstant això, utilitzar les plantes com a plataformes de producció ofereix avantatges en termes d'accessibilitat econòmica, sostenibilitat, escalabilitat i l'absència de patògens humans i animals, la qual cosa les converteix en una opció cada vegada més atractiva. Malgrat les limitacions en la capacitat de càrrega, l'expressió gènica transitoria mitjançant vectors virals proporciona un mètode eficient i reproducible per a la producció de proteïnes recombinants en plantes, a diferència de la transformació estable, que requereix més mà d'obra i temps. Els vectors derivats del virus del mosaic del tabac (TMV), particularment aquells en els quals el gen de la proteïna de la coberta viral (CP) és principalment reemplaçat pel gen d'interès, són clàssics en la biotecnologia vegetal i s'utilitzen sovint per a la producció a gran escala de proteïnes recombinants. En aquest treball, el nostre primer objectiu va ser optimitzar un vector de TMV per a millorar la producció. La fusió traduccional de l'extrem amino-terminal de la CP del TMV amb la proteïna recombinant d'interès va mostrar un augment en l'acumulació de la proteïna verda fluorescent, interferó alfa-2a i un nanobody contra la proteïna Spike del SARS-CoV-2 en fulles de *Nicotiana benthamiana*. La inserció d'un lloc de tall específic, basat en les proteases d'inclusió nuclear (NIaPro) de dos potivirus, juntament amb l'expressió de les proteases corresponents, a més de la producció de la proteïna recombinant madura, va augmentar encara més l'acumulació de les proteïnes recombinants esmentades anteriorment. En segon lloc, el nostre objectiu va ser

establir estratègies per a produir proteïnes recombinants d'interés industrial i farmacèutic en *N. benthamiana* utilitzant un vector de TMV. Vam aconseguir produir grans quantitats d'una xilanasa termofílica, activa en condicions extremes de temperatura i pH alcalí, en N. benthamiana utilitzant un vector de TMV. El enzim que es va acumular ràpidament en els teixits de la planta es va dirigir a l'apoplast, la qual cosa va facilitar enormement la purificació i va evitar qualsevol efecte advers en el creixement de la planta. Es va demostrar que aquesta enzima produïda en planta és útil per a la producció de xilooligosacàrids probiòtics. També vam produir grans quantitats d'una glucosa oxidasa (GOX) modificada en fulles de *N. benthamiana* utilitzant un vector de TMV. La GOX produïda en planta, que també es va purificar fàcilment a partir de fluids apoplàstics, va exhibir potents propietats antimicrobianes contra *Staphylococcus aureus* i *Escherichia coli*.

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Abbreviations

Viruses

CaMV: cauliflower mosaic virus

PPV: plum pox virus

TBSV: tomato bushy stunt virus

TEV: tobacco etch virus

TMV: tobacco mosaic virus

ToMV: tomato mosaic virus

TuMV: turnip mosaic virus

TVCV: turnip vein-clearing virus

Others

+ss: positive single stranded

6K1: 6 kDa peptide 1

6K2: 6 kDa peptide 2

A. tumefaciens: *Agrobacterium tumefaciens*

BSA: bovine serum albumin

cDNA: complementary DNA

CI: inclusion protein

CP: coat protein

CV: column volume

DBTL: design, build, test and learn

DNA: deoxyribonucleic acid

DNS: dinitro salicylic acid

dpi: days post-inoculation

E. coli: *Escherichia coli*

eGFP: enhanced green fluorescent protein

GFP: green fluorescent protein

FDA: food and drug administration

GMC: glucose-methanol-choline

GMO: genetic modified organism

GOX: glucose oxidase

HCPro: helper component protease

HRP: horseradish peroxidase

ICTV: International Committee on Taxonomy of Viruses

IFN-α2a: interferon alpha 2a

IgG: immunoglobulin G

kDa: kiloDalton

LacZ: lac operon beta-galactosidase

LB: left border

LB: lysogenic broth

mAb: monoclonal antibody

MCS: multiple cloning site

MP: movement protein

N. benthamiana: Nicotiana benthamiana

NIa: nuclear inclusion a

NIaPro: nuclear inclusion a protease

NIb: nuclear inclusion b

OD: optical density

ORF: open reading frame

P1: protein 1

P3: protein 3

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PFM: plant molecular farming

PIPO: pretty interesting Potyviridae ORF

PISPO: pretty small interesting Potyviridae ORF

Pro: protease

RB: right border

RBD: RNA binding domain

RdRp: RNA-dependent RNA polymerase

RNA: ribonucleic acid

SP: signal peptide

ssRNA-: negative single stranded RNA

ssRNA+: positive single stranded RNA

T-DNA: transfer-DNA

Ti: tumor-inducing

TRBO: TMV RNA-based overexpression

UTR: untranslated región

Vir: virulence genes

VPg: viral protein genome-linked

XOS: xilooligosaccharide

General Introduction

General Introduction

The planet is facing increasingly severe consequences of climate change, including food and raw material shortages. As a result, we are becoming more aware of the urgent need to protect and preserve our environment. Biotechnology has the potential to play a crucial role in addressing these challenges and making a significant contribution towards sustainable development.

MOLECULAR FARMING

Plants are becoming an attractive alternative to traditional heterologous production systems (bacteria, mammalian cell cultures, yeast or fungus) of high-value products such as secondary metabolites and proteins, whose demand is often limited by the capacity and production costs due to being cheaper and more sustainable. The discipline that studies the production of this type of high-added value compounds in plants is known as molecular farming (Lee et al., 2023). Although it is a relatively young discipline, it is booming due to the problems derived from heterologous systems and the advantages plants can bring. Plant molecular farming initially arose from the idea that plants only need light, water and some mineral salts to grow. The scalability of plant production is more straightforward than in other systems since it depends on increasing or reducing the number of plants, and the desired protein can be obtained in a few days. Additionally, plants can correctly fold and assemble complex proteins, ensuring their biological activity and efficacy. Plants also offer the potential for product safety and regulatory advantages, as they do not carry the risk of contamination with human and livestock pathogens. Production in plants is quite simple since sterility is not required in their cultivation (Buyel, 2019; Chung et al.,

2021; Fausther-Bovendo & Kobinger, 2021; Gaobotse et al., 2022; Long et al., 2022; Mett et al., 2008; Shanmugaraj et al., 2020; Y. Wang & Demirer, 2023). Many plant species have been used as a platform to produce recombinant proteins, including tobacco, potato, tomato, alfalfa, carrot, lettuce, strawberry, lentil, wheat or rice, but without doubt, the most widely used plant for the expression of recombinant proteins is *Nicotiana benthamiana*. This plant, due to its many attributes, such as fast growth, resistance, generation of large amounts of biomass, y short growth cycle, easy reproduction, scalability, and susceptibility to a large number of viruses, makes a perfect host to use as a biofactory plant (Y. Gleba et al., 2004; H. Liu & Timko, 2022; Margolin et al., 2020; Sanfaçon, 2017; Singh et al., 2021; Tsekoa et al., 2020). It is the preferred species for the agroinfiltration technique since its large leaves are easily infiltrated, allowing large amounts of transformed tissue to be obtained. It is a very versatile platform that allows small tests and large-scale processes to quickly obtain the final product (Bally et al., 2018; Jutras et al., 2020). In addition, it should be noted that plant cells, unlike prokaryotes, have an endomembrane system and a secretory pathway that allows complex proteins to fold and assemble correctly. In plants, post-translational modifications also occur that are very similar to those that occur in mammalian cells (H. Liu & Timko, 2022; Margolin et al., 2023; Mortimer, 2019; Su et al., 2023a; Vitale & Denecke, 1999).

Plant molecular farming continuously works to improve production systems. For this, optimizations have been emerging, such as, for example, the adaptation of the codons of the protein for the species of plant to be used or the humanization of N-glycans, since glycosylation is one of the most important and crucial post-translational modifications for the viability of the recombinant protein. It is also essential to adjust the external factors such as the necessary nutritional parameters of the culture or the internal parameters, co-expression of protease inhibitors or inclusion of specific signal peptides that direct the protein to the

corresponding subcellular location. This allows the protein to be practically ready for use without performing additional complex purification steps that increase costs and production times. All these advances have permitted, not only to improve performance, but also to meet the standards of industrial manufacturing and clinical application in the case of pharmaceutical products (Fausther-Bovendo & Kobinger, 2021; Liu & Timko, 2022; Long et al., 2022; Schillberg & Finnern, 2021).

Plant molecular farming encompasses a variety of different technologies for the expression of recombinant proteins; it can be carried out by two modalities, stable transformation by transgenic plants that can be in turn of the nuclear genome or chloroplasts and transient transformation (**Fig. 1A**) (Fischer & Buyel, 2020; Lee et al., 2023; Lico et al., 2020). The difference between the two techniques is that in the stable, the exogenous gene or genes are integrated into the genome with which heritability is achieved to subsequent generations of the plant, while if the transformation is transient (**Fig. 1B**), the expression of the protein occurs for a short period of time and does not affect all the cells of the plant. Stable transformation generally has a lower transformation efficiency compared to transient transformation. It is often more challenging to achieve stable integration of DNA into the plant genome. In addition, the transient system has some additional advantages over the stable one since the accumulation of the desired product is achieved much faster, only in a few days, and it is easily scalable to reach appropriate production levels. In general, a higher yield can be obtained in production. In contrast, the stable transformation is time-consuming and resource-intensive (Lee et al., 2023; Pasin et al., 2019; Sainsbury, 2020; Sainsbury & Lomonossoff, 2014; Sirirungruang et al., 2022).

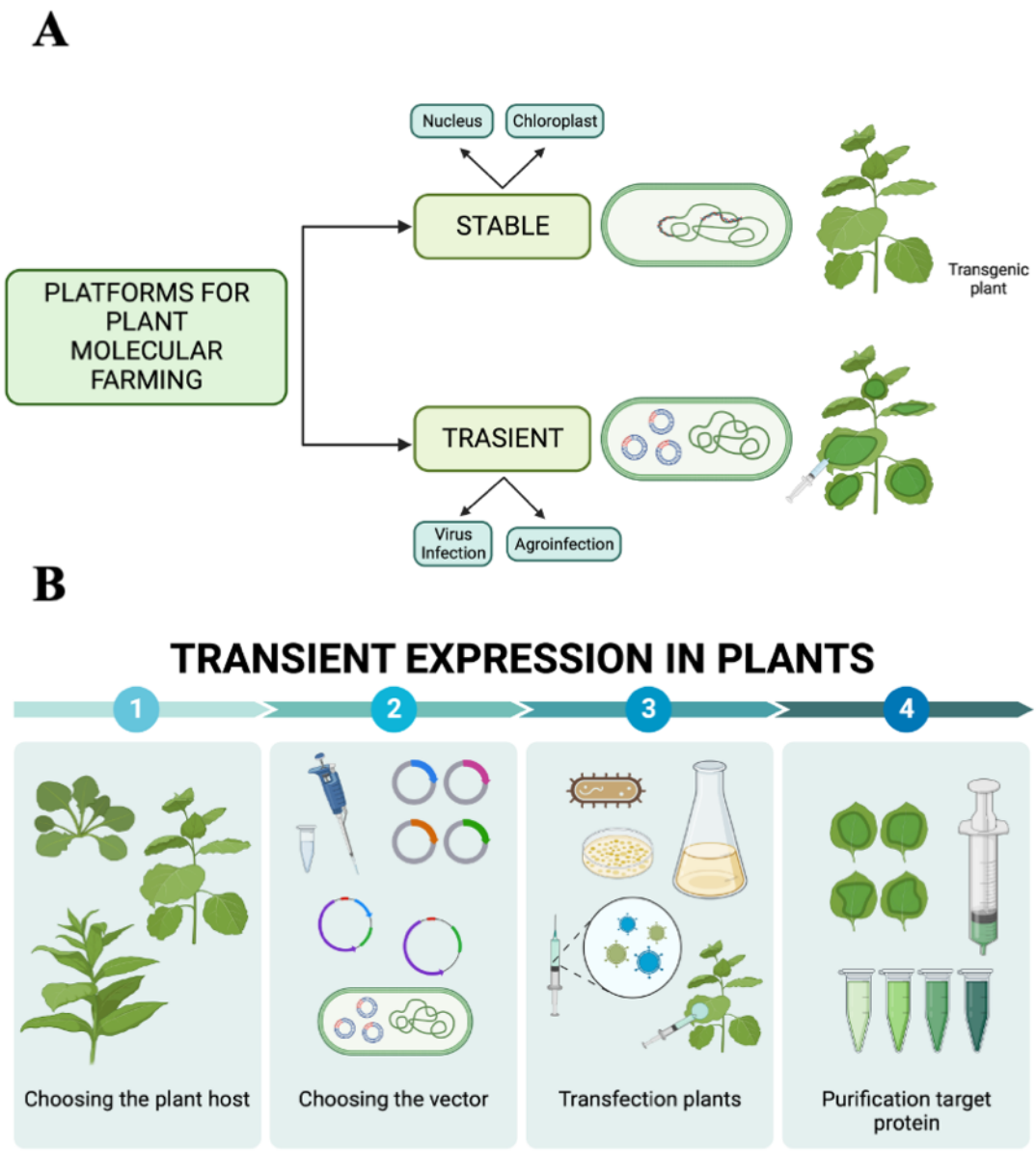

Fig. 1. (A) Transformation approaches to produce heterologous recombinant proteins in plant molecular farming. (B) Summary of the steps to perform a transient expression in plants: 1, choosing the best plant host for the purpose; 2, selecting the correct vector to express the heterologous protein and cloning; 3, transfecting the plants using *Agrobacterium tumefaciens*; 4, several days after inoculation, harvesting the leaves and purifying the target protein. Adapted from Shanmugaraj et al., 2020.

The most used technique employs *Agrobacterium tumefaciens* as a vector in the transient expression of recombinant proteins. This bacterium has the ability to introduce genes into the plant chromosomes (Chilton et al., 1977). For this, some bacterial genes producing very particular metabolites, such as opins, are replaced by genes of interest controlled by specific promoters. Wild-type *A. tumefaciens* harbors a tumor-inducing (Ti) plasmid containing transfer-DNA (T-DNA), which is a fragment of DNA that is transferred to the plant genome and region of virulence genes (*Vir*). In order to use this bacterium as a vector for gene transfer and take advantage of its properties, an adaptation was made that consisted of mutating the Ti plasmid where the genes that control the induction of the tumor are replaced by a multiple cloning site (MCS) for the insertion of heterologous sequences. This disassembled plasmid is better known as a binary vector. It contains a resistance marker and replication source for *Escherichia coli* and *A. tumefaciens* (*OriE* and *OriA*, respectively) and right and left borders (RB and LB) from the T-DNA (**Fig. 2**). *Vir* genes are expressed from a second plasmid of the binary system better known as Ti helper (Mahmood et al., 2023; Mohammadinejad et al., 2019; Molina-Hidalgo et al., 2021).

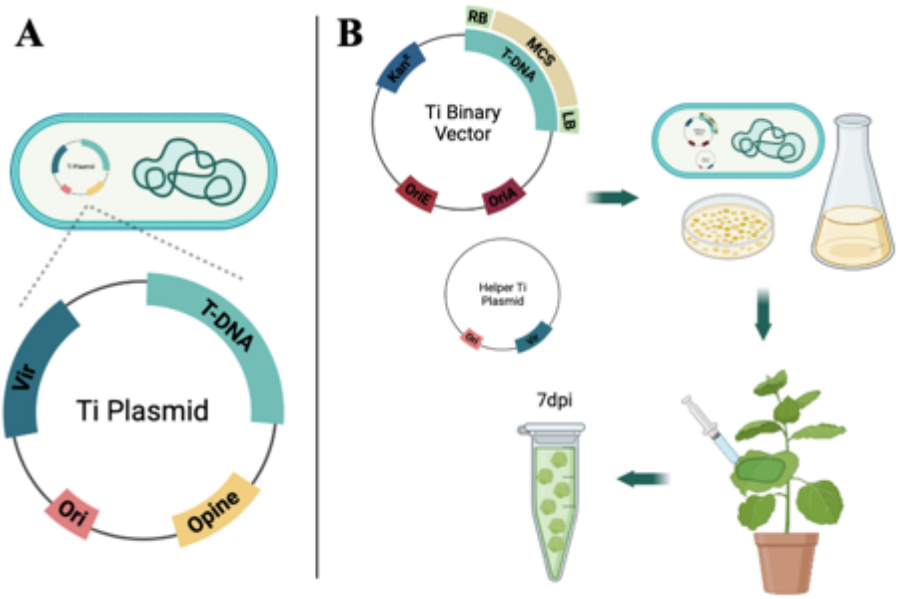

Fig. 2. Plant transformation through *A. tumefaciens*. (A) Wild-type *A. tumefaciens* harbors a tumor-inducing (Ti) plasmid that contains two elements: (1) the transfer-DNA (T-DNA), a virulent DNA molecule delivered from these bacteria into the host plant genome, and (2) the virulence (*Vir*) region. (B). Ti binary vector, an adaptation of *A. tumefaciens* as a tool for plant genetic transformation, has a multiple cloning site (MCS) for inserting the heterologous sequences. The agroinoculation occurs after transforming *A. tumefaciens* with the Ti vector that encodes the recombinant protein. Then, the culture is delivered into the intercellular space of plant leaves using a needle-less syringe or applying vacuum.

Although plant molecular farming has many advantages, the early stages of its development advanced slowly due to its lack of competitiveness for human clinical trials due to technological constraints and little knowledge. Nowadays, all the research in plant molecular farming has advanced a lot to the point that you can already find different products manufactured with this technique on the market, such as the recombinant human β-glucocerebrosidase (taliglucerase alfa) produced in carrot cells in suspension that has been the first therapeutic protein produced in plants approved by the Food and Drug Administration (FDA) as a drug to treat human diseases. Mapp Biopharmaceutical Inc. was granted fast-track designation by the FDA for their plant-made cocktails of three monoclonal antibodies (ZMapp) against Ebola virus disease (H. Liu & Timko, 2022; Tekoah et al., 2015; Zahmanova et al., 2023).

Overall, molecular farming holds great potential for the production of valuable compounds, offering a sustainable and efficient approach to meet the growing demands of the pharmaceutical and biotechnology industries. It combines the benefits of biotechnology and agriculture, paving the way for the development

of innovative and cost-effective solutions for human health and industrial applications.

PLANT SYNTHETIC BIOLOGY

Synthetic biology is an emerging field that arises from the mixture of molecular biology and engineering principles. There are different definitions for this discipline; one of the most used is the one that came from the joint opinion of three scientific committees from different areas of the European Commission. According to them, it is "the application of science, technology and engineering to facilitate and accelerate the design, manufacture and/or modification of genetic materials of living organisms" (Breitling & Takano, 2015; Shapira et al., 2017). Synthetic biology aims to design and construct new tools or parts that allow gene products to modulate biological processes at will. Just as electrical circuits are modulated, they are also intended to do so at the biological level. However, modulating biological processes is more complex because of the complexity of cellular interactions. Despite this, circuits are being created that can be predictable by implementing them in biological systems and living organisms capable, for example, of addressing food and energy challenges, as well as gene-based methods to improve human medical conditions and insect-borne diseases (RA et al., 2016; Shapira et al., 2017). Synthetic biology has significant differences from conventional genetic engineering. While genetic engineering directly manipulates gene material using molecular biology techniques, synthetic biology tries to go further. Its objective is to design or redesign biological systems and improve their qualities or add new ones making processes easier and cost-effective (X. Liu et al., 2023). Thus, more data such as transcriptomics, proteomics, metabolomics or even mathematical models are integrated to understand how an organism works and modify it according to

needs. The outstanding achievements that have been obtained with synthetic biology applied to single-cell systems have driven the creation of iterative cycles of design, construction, testing and learning. (DBTL) (Y. Wang & Demirer, 2023). (Fig. 3).

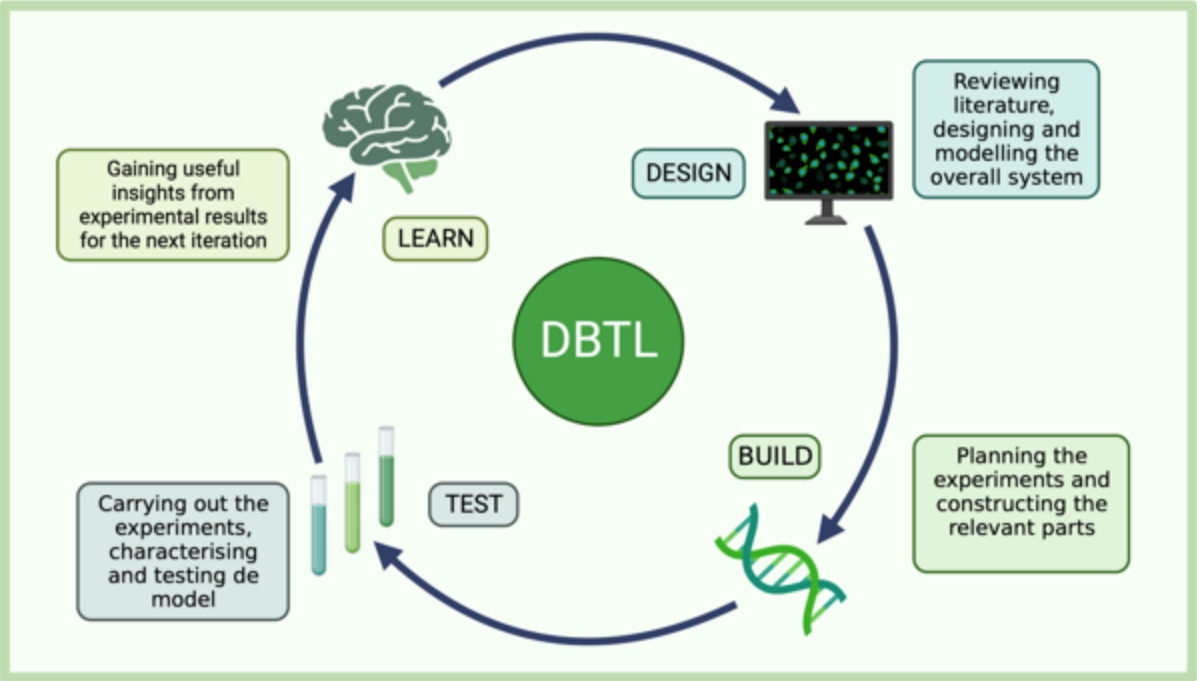

Fig. 3. Iterative design-build-test-learn (DBTL) cycle in synthetic biology. Adapted from D. Gupta et al., 2021; Mortimer, 2019.

The DBTL cycle links all the stages that are necessary to create a genetic circuit that can be efficient. At the "design" phase, the objective is first defined, and the host organism and the building blocks to be used for the system are selected based on existing prior knowledge. The next stage is the "construction" phase, where the experiments are planned, the assembly of the different components is carried out, and they are integrated into the host that has been selected, so perform the constructions and the DNA assembly. The phase that continues is "testing". Necessary tests are performed to evaluate the effect of the genetic circuit on the host and data collected. Finally, in the "learning" phase, the data obtained in the test phase are analyzed to observe what can be improved, or what improvements have been achieved with the insertion of the genetic circuit

and use for the next design (Freemont, 2019; D. Gupta et al., 2021; Mortimer, 2019; Pouvreau et al., 2018; Raman et al., 2021; Y. Wang & Demirer, 2023).

The first genetic circuits were described in 2000 (Michalodimitrakis & Isalan, 2009). In 2019, a synthetic *E. coli* was designed using the 59 reduced codons to encode the 20 essential amino acids. The first computer-based synthetic bacterial genome representing Caulobacter enthesis-2.0 was also created. However, there is still a need to develop new technologies and methods to accelerate the use of synthetic biology in fields that have yet to be reached. A significant and striking example of synthetic biology is the hamburger of the future which will be made from a consortium of bacteria and hormones, like yoghurt is produced. They are working on synthesizing molecules produced by fungi/bacteria to adjust these burgers' flavor, fragrance and nutrition (Voigt, 2020). Living cells have also been designed that could be integrated into architectural materials and would have an air pollution-cleaning function (Freemont, 2019). Another example is the coupling of living cells to design electronic devices that allow brain-computer interfaces to create robots. Soon we expect to see many advances in synthetic biology that provide solutions to current problems and are easily transferable from the laboratory to industrial, field or clinical practice levels (Freemont, 2019; Gilbert & Ellis, 2019; Singh, V., 2022; Tseng et al., 2020; Voigt, 2020).

Although synthetic biology is advancing very rapidly in microorganisms or cultured cells, there is much to explore in the field of plants because they have very different characteristics in terms of physiology, metabolism, life cycles and environmental niches. This makes it challenging to use tools already described for other microbiological organisms. Now the objective of researchers working in plants is focused on creating new tools that adapt to them since these tools will be an essential resource for food safety, obtaining recombinant proteins,

substances of interest and solutions to the exploitation of limited resources. In addition, plants are the intermediate system between microorganisms and other higher organisms, such as animals, since they have a lower ethical component and are complex multicellular eukaryotic systems (Cook et al., 2014; W. Liu & Stewart, 2015; X. Liu et al., 2023). It is expected that applying synthetic biology in plants will create an extensive toolbox that brings significant advances to the scientific community that can be used in all areas and thus achieve a more complex vision of life. There are already some relevant advances resulting from synthetic biology in plants. They have managed to improve plant biomass to make it easier to decompose by microbial conversion for the subsequent production of biofuels. Synthetic biology strategies are also being used in agriculture to reduce dependence on fertilizers. As the precision with which plants can be manipulated increases, so will the number of possible applications. Among the prospects of synthetic plant biology is to improve food crops by increasing their nutritional value and yield, reducing environmental pollution or even developing plants that can survive space travel. One of the most ambitious goals is the complete redesign of the plant genome, as was done in the Sc2.082 yeast project. There are, however, many aspects to discover and explore in the world of synthetic plant biology. Still, it offers innovative approaches for producing valuable compounds, developing biosensors, and establishing plant-based biomanufacturing platforms. As research and technology continue to advance, synthetic biology holds promise for addressing global challenges and transforming various sectors through sustainable and customizable plant-based solutions (Aldulijan et al., 2023; D. Gupta et al., 2021; Mortimer, 2019).

PLANT VIRUSES

Biotic stresses caused by different agents such as nematodes, fungi, oomycetes, bacteria, viruses or viroids challenge agricultural production worldwide. Viruses, also fungi, account among the agents that cause most losses in crops.

The beginnings of virology dates back to the late nineteenth century, specifically in 1886. Adolph Mayer, a German chemist and agronomist, published the first paper on plant viruses. This article was entitled 'Tobacco mosaic disease' and was the first study where all the disease symptoms were named and explained in detail. Mayer postulated that the disease-causing agent could be mechanically transmitted to other plants from infected plant extracts. In 1892, the Russian researcher Dimitri Ivanovski, who was investigating a disease affecting the tobacco plant, saw that the causative agent of the disease was so small that it was able to pass through the porcelain filters. Bacteria were unable to pass through them and he determined that a bacterial toxin was the cause of the tobacco mosaic disease (Gergerich & Dolja, 2006). But it was not until 1898 when the Dutch microbiologist Martinus Willem Beijerinck, using much finer filters than those used by Ivanovski, first proposed that the cause of the disease was a '*virus*', an agent that was much smaller than any known bacteria. Beijerinck was the first scientist to use the wording *'contagium vivum fluidum'*, meaning live contagious fluid, to refer to the tobacco mosaic virus. At that time, the discipline of virology was born.

There is a wide variety of plant viruses. According to the International Committee on Taxonomy of Viruses (ICTV), approximately 900 different virus species have been found, which are hosted by plants (Balke & Zeltins, 2019; Walker et al., 2021; Zahmanova et al., 2023). These are non-cellular infectious agents that have the capacity to self-assemble from their structural components. They are obligatory parasites that need to usurp the metabolism of the plant cell to

reproduce (Balke & Zeltins, 2019; Mäkinen, 2020; Sánchez Pina et al., 2021a; M. Wang et al., 2020). So, it is impossible to grow them in any culture medium. In recent studies, it has been found that not all plant viruses affect negatively, but some also have a beneficial impact on the host in the particular ecosystem in which they are found (Jones, 2021; Lefeuvre et al., 2019; Zahmanova et al., 2023). The genome of plant viruses can be RNA or DNA, with a common feature being their small size with a limited coding capacity of a set of proteins that allow them to fulfill infection. The main viral proteins are polymerases, responsible for the viral genome replication, proteins necessary for movement (movement protein, MP) from cell to cell and coat proteins (CP) that are responsible for encapsidation and protecting viral progeny (Balke & Zeltins, 2019; M. Wang et al., 2020). The imaging techniques applied to studying virus-host interactions have allowed to understand the infection cycles better. It is also known that, despite having limited genomes, they are able to quickly complete complex infective cycles by coping with the defensive mechanisms of the host plant (Sánchez Pina et al., 2021b).

TOBAMOVIRUS: PLANT VIRUS EXPRESSION VECTORS

The possibility of using viruses as vectors open a huge window to the expression of proteins of therapeutic and industrial interest. Viruses are easily manipulated and, in addition, have an easy infection process that makes them a desirable alternative to transgenic systems. Plant viral vectors have been designed from the genomes of both positive and negative sense RNA single-stranded viruses (ssRNA +, ssRNA-) (Khakhar & Voytas, 2021). The first vectors of expression from viruses were based on using a full virus containing the entire genome. The heterologous sequence of the gene of interest was inserted. These viruses behaved like wild-type since they maintained all their genetic structure for plant

infection and replication. They also had the capacity for systemic movement in the host plant, and the size of the heterologous gene negatively affected generating lower productivity when large proteins were introduced. Some advances in the development of viral vectors have consisted of maintaining only sequences necessary for their replication, although sacrificing the ability to move (Y. Gleba et al., 2004, 2007; Y. Y. Gleba et al., 2013).

Tobamovirus is a genus of viruses whose genome is single-stranded positive-sense RNA (ssRNA+) and belongs to the family *Virgaviridae*. The name *Tobamovirus* comes from the host and symptoms of the first virus discovered, tobacco mosaic virus (TMV). Many plants, such as tobacco, potato, tomato and pumpkin, are natural hosts of this virus. TMV has 2130 copies of CP and was one of the first viruses to be engineered as a vector (Y. Gleba et al., 2004; K. L. Hefferon, 2012; Zahmanova et al., 2023). The TMV genome was around 6400 nt, and its RNA sequence was published in 1982. It was one of the first complete viral genomes to be published. Icon Genetics, a biotechnology company based in Germany, created an expression system called magnICON, a hybrid system between TMV and turnip vein-clearing virus (TVCV) that also belongs to the genus *Tobamovirus*. This three-part system has 5' parts, which is composed of the essential elements of the two tobamoviruses and a promoter for the expression of the gene of interest (Fig. 4); a 3' part, which is where the gene of interest is inserted; and finally, the part in which recombinase is expressed (Marillonnet et al., 2004; Venkataraman & Hefferon, 2021; M. Wang et al., 2020). The expression vector of this system lacks CP, so it cannot move systemically through the plant. *A. tumefaciens* is responsible for carrying the viral vector to the nucleus of plant cells. Once the plant is agroinoculated with magnICON vectors, each the 5' and 3' parts of the vector are fused by recombination that forms the DNA replicon and transcription begins. With this system, authors also developed a some massive agroinfiltration techniques. Using this technique of

mass inoculation, yield of the production of a complete immunoglobulin G (IgG) was raised to 4.8 g/kg of fresh weight (GLEBA et al., 2005; Y. Gleba et al., 2004; K. Hefferon, 2017; Venkataraman & Hefferon, 2021). Different signal peptides can be fused into plant expression viral vectors, allowing recombinant proteins to target desired subcellular compartments (Gils et al., 2005). With non-competitive viral vectors of the magnICON system, two different proteins could be expressed in the same cell (Giritch et al., 2006), and the expression of complete monoclonal antibodies was also achieved (Huang et al., 2006; Santi et al., 2006; Webster et al., 2009). Another gene expression system based on TMV is TMV RNA-based overexpression (TRBO). Expression is increased several times, becoming comparable to magnICON regarding recombinant protein accumulation; this vector's design is simpler. Most of the coding region of TMV CP is replaced by a multiple cloning site in which the gene of the protein to be expressed is placed near the 3' terminal end of the TMV RNA (Lindbo, 2007a, 2007b). This vector has been able to express the antigen HBcAg from the hepatitis B virus developing oral and parenteral immunogenicity in mice (Huang et al., 2006; Rybicki, 2020). Also, a TMV vector has successfully produced a transient expression of human myoglobin in *N. benthamiana* (Carlsson et al., 2020). Different antigens and antibodies against COVID-19 have also been made in the last few years, highlighting the vaccine produced by BioProcessing with the conjugated domains of RNA binding domain (RBD) (Monroy-Borrego & Steinmetz, 2022; Royal et al., 2021). These results show the great potential that plants have for producing proteins with high added value, both industrial and pharmaceutical since it was possible to verify that they are fully active and, therefore, valid for their biological function. Another heterologous protein expression system based on TMV was designed in 2019. The novelty it introduced with respect to those already described above was the ease of cloning by Gibson assembly (Gibson et al., 2009). This system greatly facilitates

the manipulation of the viral genome (Shi et al., 2019). The system consists of two cloning vectors; a small intermediate of effortless handling for inserting the gene of interest, which contains a linker with only two restriction sites and the marker of selection alpha peptide of beta-galactosidase (LacZ). DNA digestion or polymerase chain reaction (PCR) amplification of an intermediate plasmid fragment transfers the complementary DNA (cDNA) of interest to the final vector. Depending on the intermediate plasmid used, two types of final vectors are achieved, a vector of local expression in which a large part of the CP of the TMV is replaced by the corresponding cDNA of the recombinant protein of interest, and therefore does not allow it to move systemically through the infected plant. It remains only in the agroinfiltrated halo, and a vector that does not have the CP deleted, and that can move systemically through the plant. This last vector of expression complements the lack of the CP of the TMV with a fragment of the genome of the tomato mosaic virus (ToMV) that includes the promoter, the cDNA that encodes its CP and the 3' Untranslated region (UTR), providing an alternative CP so that it is possible the encapsidation of the recombinant virus that does move through the plant (Shi et al., 2019).

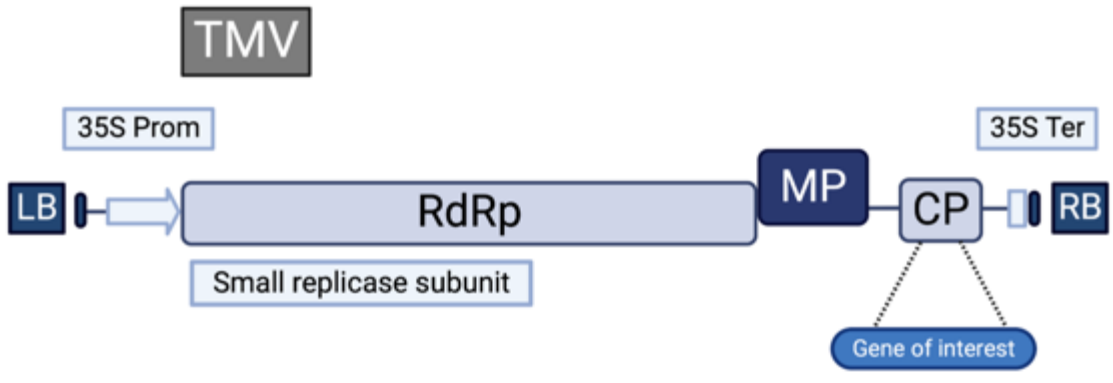

Fig. 4. Schematic representation of a TMV-derived vector to produce recombinant proteins. The constructs are driven by the cauliflower mosaic virus (CaMV) 35S promoter and terminator. LB and RB left and right borders of the *A.*

tumefaciens T-DNA; RdRp, RNA-dependent RNA polymerase; MP, movement protein; CP, coat protein.

POTYVIRUSES: A SOURCE OF SPECIFIC PROTEASES FOR BIOTECHNOLOGICAL APPLICATIONS

One of the most extensive families of plant viruses is the *Potyviridae* which contains 228 species classified into 12 genera. Potyvirids (viruses in *Potyviridae*) are positive-stranded single-stranded RNA viruses (+ss) with a flexuous morphology whose length is between 680 and 900 nm and between 11 and 20 nm wide. It is one of the families with the highest number of viruses that affect plants and entails more economic losses (Inoue-Nagata et al., 2022; Saha et al., 2022; Wylie et al., 2017; Xiao et al., 2022; Yang et al., 2021). Within this family, the most numerous genus is *Potyvirus*, comprising 183 species. Potyviruses contain a single RNA molecule of about 10 kb and are polyadenylated at its 3' end. These viruses are formed by a single open reading frame (ORF) that encodes a large polyprotein (340-370 kDa) that is finally processed into about 10 functional proteins by viral proteases (**Fig. 5**). Also, another small ORF is synthesized within the coding region of protein 3 (P3) in reading frame -1, known as N-terminal region of P3 pretty interesting Potyviridae ORF (P3N-PIPO). In some potyviruses, a second short ORF called N-terminal region of protein 1 (P1) pretty small interesting Potyviridae ORF (P1N-PISPO) occurs in the coding region of P1 (Gadhave et al., 2020; Gibbs et al., 2020; Rajamäki et al., 2014; Xiao et al., 2022; Yang et al., 2021).

The N-terminal region of the polyprotein is the least conserved and contains P1 and helper component protease (HC-Pro). P1 is a serine protease that can self-cleave from polyproteins. It is not an essential protein for viral viability. Although it is not required for viral infectivity, it does promote the amplification of the

virus. The evolutionary diversification of P1 has an essential role in adapting potyvirids to different hosts (Gibbs et al., 2020; Yang et al., 2021). HC-Pro is next in polyprotein and is a multifunctional cysteine protease involved in other potyvirus infection processes. To be functional, HC-Pro must be in dimer form in infected plants; it is present in the infection process, in the transmission by aphids and in the suppression of gene silencing considered as a mechanism of resistance to viruses. It is also involved in genome replication and long-distance movement of the virus through the plant. In addition, it is able to interact with the rest of the proteins of the polyprotein. Ultimately, it acts as a protease to self-cleave the polyprotein (Saha et al., 2022).

In the central region of the protein are protein 3 (P3), 6 kDa peptide 1 and 2 (6K1 and 6K2) and cylindrical inclusion protein (CI). P3 consists of two hydrophobic domains; one is in the N-terminal half and the other in the C-terminal part. It is a membrane protein. This protein is essential for viral replication, interacts with NIb, CI and NIa, and is a determinant of viral pathogenicity. In part, it is responsible for the symptoms derived from viral infection. As mentioned above, a different small reading frame, which appears when the ORF moves one unit (-1), is P3N-PIPO, a fusion protein with the N-terminal region of P3. PIPO stands for 'pretty interesting Potyviridae ORF' (Chai et al., 2020; Miao et al., 2022; Rajamäki et al., 2014; Revers & García, 2015). The 6K1 protein was thought to have no function on its own, but when bound to P3, it appears to be related to the presence/absence of viral symptoms. Studies using a clone of cDNA from plum pox virus (PPV) have shown that 6K1 maturation is essential for viral viability and for deleting any short pieces of 6K1 that can be lethal to the virus (Cui & Wang, 2016). It is involved in structures that are usually associated with replication vesicles. So, it is probably a membrane protein that plays a crucial role in forming such viral replication vesicles.

CI is the largest potyviral protein with four distinct domains: the N-terminal, two helicases and the C-terminal. There is a role in RNA binding, amplification and cell-to-cell movement of the viral complex. 6K2 is an integral membrane protein consisting of an N-terminal domain containing an α helix essential for vesicle formation and subsequent export to the endoplasmic reticulum, a hydrophobic transmembrane central domain and a C-terminal domain. 6K2 induces endoplasmic reticulum proliferation to form the viral replication complex. In cells infected by potyvirus complexes such as CI-6K2 and 6K2-VPg-Pro have been seen, which indicates that 6K2 probably has other functions that are not yet known. This protein could be involved in the systemic movement and possible development of symptoms of the infected plant (Urcuqui-Inchima et al., 2001; Yang et al., 2021).

Finally, the C-terminal region is the most conserved of the polyprotein of the potyviruses; NIa proteins (VPg and NIaPro), NIb and CP are found in it. NIa is the nuclear inclusion protein. It has two domains, the N-terminal, the viral protein genome-linked (VPg), and the C-terminal, the protease (NIaPro). VPg is present at the beginning of viral RNA and has essential functions in viral genome replication. It can act as a primer, it is a potent regulator of gene expression, and it can inhibit host translation to promote the translation and accumulation of viral RNA. VPg has a very flexible structure that allows it to perform protein-protein interactions. NIaPro is a cysteine protease; the most important of the potyviruses. It is responsible for the proteolytic processing of seven cleavage sites in the central and C-terminal regions of the viral polyprotein. The NIaPro processing site is strictly specific, so it is a handy biotechnological tool for removing tags or peptide signals. NIaPro interacts with other proteins that help regulate its activity and increase its functional diversity (Xiao et al., 2022; Yang et al., 2021). In addition, it acts as a suppressor of the host's antiviral defenses since it interferes with the DNA methylation pathway. Potyviral NIb is a viral RNA-

dependent RNA polymerase (RdRp). It is indispensable for viral genome replication and helps to form inclusion bodies in the nucleus of infected plant cells. NIb interacts with the viral proteins NIa-VPg, NIaPro, CP, and several proteins from the infected host. The last protein of the polyprotein of potyviruses is CP which contains 3 domains. The variable domains are the N- and C-terminal exposed on the surface of the viral particle; the N-terminal region contains most of the specific epitopes of the virus and is more conserved than those found in the central region. The CP is structurally very conserved. It is involved in various functions, including transmission by aphids, systemic movement and cell-to-cell movement, the encapsidation of viral RNA to form the virion and finally, the regulation of viral RNA amplification. Although the CP is indispensable for viral intercellular transport, it is not essential for viral genome replication. It has been seen that CP is also involved in seed transmission, host adaptation, and activation of plant defense mechanisms (Gadhave et al., 2020; Gibbs et al., 2020; Martínez-Turiño & García, 2020; Yang et al., 2021).

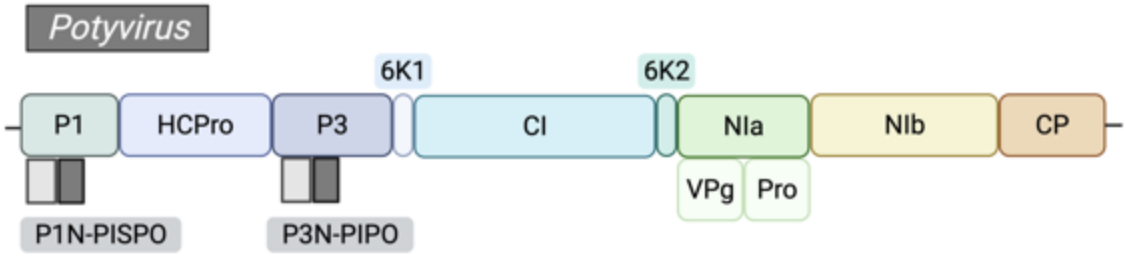

Fig. 5. Genome organization of potyviruses. The long ORF is represented with a box for each final product. Additional ORFs corresponding to out-of-frame P1N-PISPO and P3N-PIPO are also indicated.

With the potyvirus strategy of a single major ORF encoding a polyprotein cleaved by viral proteases to obtain functional proteins, many approaches have been developed to manipulate a wide variety of plant defense and development processes. With all the new technologies that have emerged in recent years

around potyviruses, it has been possible to generate excellent knowledge about them and biotechnological tools derived from their proteins and proteases.

XYLANASES

Xylan is a complex polysaccharide, a major hemicellulose component, present in plant cell walls. It consists of a backbone of β-1,4-linked xylose units with various side chains attached. It can be further decorated with multiple side groups such as arabinose, glucuronic acid, and acetyl groups. Xylan provides structural support to plants and plays a significant role in cell wall overall composition and architecture (Curry et al., 2023; G. K. Gupta et al., 2022; Walia et al., 2017). Xylan has gained considerable interest in various industries due to its potential as a renewable resource to produce value-added products. Xylanase enzymes can hydrolyze it into smaller sugar molecules, such as 2 to 10 units of xylooligosaccharides (XOS) or individual xylose units. Most xylanases exhibit their optimal pH in the range of 4.0 to 9.0, indicating that they work better under slightly acidic to slightly alkaline conditions. In terms of temperature, the optimal range for xylanase activity is typically between 30°C and 60°C (Alokika & Singh, 2019; Thakur et al., 2022; Verma, 2021).

Xylanases have gained significant attention due to their potential applications in various industries, including food, pulp and paper, biofuel production, and animal feed (Alokika & Singh, 2019; Walia et al., 2017). These enzymes are produced in large amounts by a wide range of heterologous systems like microorganisms, including bacteria, yeast or fungi, due to their inexpensive culture conditions and the generation of high yields of the desired enzyme. However, these heterologous systems sometimes have some shortcomings like high variability in the production, no suitability for producing secreted eukaryotic enzymes and scaling-up challenges for factors like oxygen transfer, nutrient

availability. Fermentation conditions may need to be carefully optimized to maintain high productivity (Karbalaei et al., 2020; Sainz-Polo et al., 2013; Y. Wang & Demirer, 2023). Xylanases from extremophilic bacteria can exhibit favorable properties, such as stability at extreme temperatures and pH levels, which make them attractive for industrial applications. The production of xylanase in plants holds great potential for various applications mentioned above. It can be used in the food industry to improve the digestibility of plant-based materials, enhance the nutritional value of animal feed, and produce prebiotic xylooligosaccharides that promote gut health. Additionally, xylanase can be employed in the pulp and paper industry to facilitate the delignification process during paper production (Alokika & Singh, 2019; Curry et al., 2023; Šuchová et al., 2022; Walia et al., 2017).

GLUCOSE OXIDASE

Glucose oxidase (GOX, β-D-glucose: oxygen 1-oxidoreductase, EC 1.1.3.4) is an enzyme that catalyzes the oxidation of β-D-glucose to gluconic acid, producing hydrogen peroxide. The enzyme functions by binding to glucose and using molecular oxygen as a co-substrate. It converts glucose into gluconic acid, releasing hydrogen peroxide as a byproduct. The hydrogen peroxide produced can be further used in various enzymatic reactions or serve as a signal in diagnostic tests. GOX exhibits optimal activity at pH 3.5-6.5 and a 30-60 °C temperature range. It shows high specificity towards β-D-glucose compared to other sugars. The enzyme kinetic properties and stability have been extensively studied (Bankar et al., 2009; Bauer et al., 2022; Khatami et al., 2022; F. Wang et al., 2022).

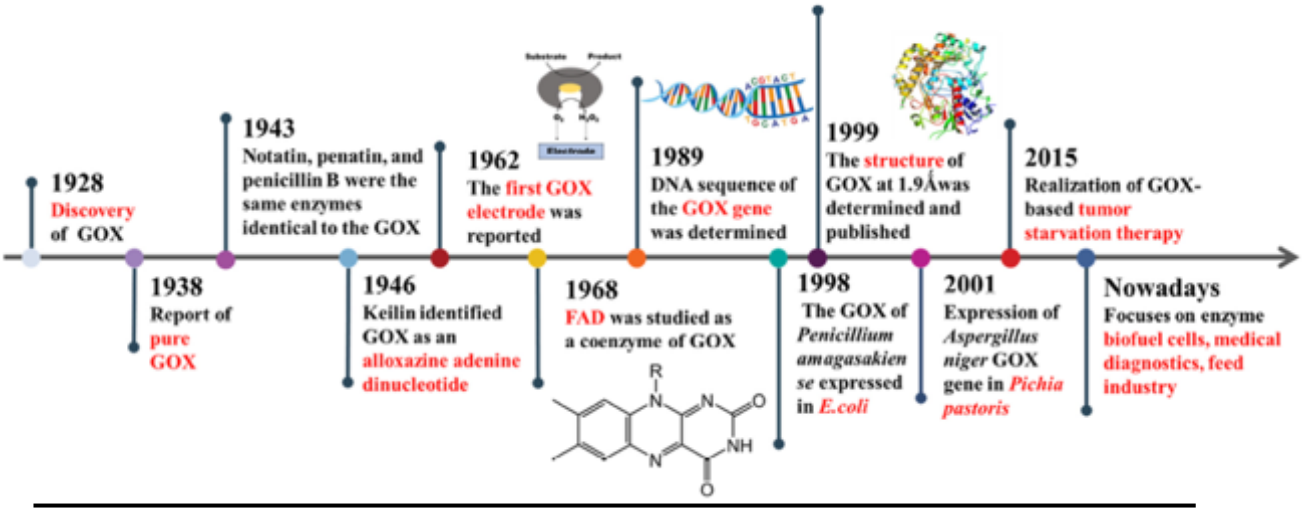

Fig. 6. The development process of glucose oxidase taken from F. Wang et al., 2022

GOX stands out as an exceptional enzyme from an industrial perspective, thanks to its numerous and significant applications. GOX has found applications in various industries, including chemical synthesis, pharmaceutical drug synthesis, food and beverage processing, clinical diagnostics, and bioprocessing. Additionally, GOX exhibits antimicrobial effects against pathogens. Its unique properties, high stability, and specificity make it an attractive candidate for various biotechnological applications. For example, in the food industry, GOX is used to improve the shelf life of certain products by converting glucose into gluconic acid, which acts as a natural preservative (Bankar et al., 2009; Bauer et al., 2022; Khatami et al., 2022). It is also employed in the production of lactose-free dairy products, which help to convert lactose into glucose and galactose (Czyzewska & Trusek, 2022). In clinical diagnostics, GOX is utilized in glucose biosensors for the measurement of blood glucose levels, commonly used by individuals with diabetes to monitor their blood sugar (Bauer et al., 2022).

The production of GOX involves fermentation and recombinant methods, primarily using microorganisms such as *Penicillium* and *Aspergillus*. Specifically, the industrial production of GOX involves the use of genetically engineered *Aspergillus niger* strains to enhance productivity (Lambré et al., 2023; F. Wang et al., 2022). Although different yeast species and *E. coli* have been tested as production hosts, they have shown limited success thus far (Bauer et al., 2022; F. Wang et al., 2022). However, the current yield is insufficient for large-scale industrial production (Marín-Navarro et al., 2015; Ostafe et al., 2014). The utilization of alternative hosts would offer enhanced versatility in GOX production. In this regard, *N. benthamiana* can be a great alternative to increase the production yield of GOX.

Objectives

Objectives

In the context of taking advantage of the potential of plants as a biofactory to produce recombinant proteins of industrial and pharmaceutical interest, this doctoral thesis was focused on harnessing the biological properties of plant viruses for that purpose. Their small genomes, easy manipulation, and great capacity to invade the host plant can contribute to the production of recombinant proteins and metabolites of interest. At the same time, the functional richness of some viral proteins may contribute to the development of complex genetic circuits in synthetic biology. To address this general objective, the following specific objectives were defined:

1. To analyze whether a TMV vector in which the gene of interest replaces most of the viral CP gene can be improved by translational fusion of TMV CP termini.

2. To develop an *in vivo* system based on potyviral proteases to remove tags in recombinant products expressed from a TMV vector.

3. To produce the extremophilic xylanase Xyn11 in *N. benthamiana* using a vector derived from TMV with an export signal peptide to the apoplast, which will facilitate protein purification.

4. To set up a large-scale production system of GOX in *N. benthamiana* using the TMV vector and to analyze the antimicrobial properties of the plant-produced enzyme.

CHAPTER I

An augmented tobacco mosaic virus vector to improve production of recombinant proteins in plants

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Author contribution:

Both authors conceived the project and designed the experiments. MNS performed the experiments. Both authors analyzed the data. JAD wrote the manuscript with input from MNS.

Abstract

Background: Plant bioengineering usually relies on stable genetic transformation, which is a labor-intensive and time-consuming process of frequent unpredictable results due to random integration of transgenes in the plant genome. In addition, many plant species are recalcitrant to transformation. Although with cargo limitations, viral vectors are an easy, efficient and reproducible alternative to transiently express genes of interest in plants. A vector derived from tobacco mosaic virus (TMV), in which the viral gene encoding the coat protein (CP) is mostly replaced with the gene of interest, is frequently adopted in projects aiming to production of recombinant proteins in plants due to unprecedented large accumulation of the product of interest. In this work, we asked if accumulation of recombinant proteins expressed from a TMV vector could still be improved by translationally fusing the amino and carboxyl termini of the viral CP to the protein of interest.

Results: Several recombinant proteins, such as the green fluorescent protein, interferon alfa-2a or a nanobody specific to SARS-CoV-2 Spike protein incremented accumulation in *Nicotiana benthamiana* biofactory plants when expressed from a TMV vector with amino-terminal extensions corresponding to the TMV CP 15 amino-terminal end. Insertion of a potyvirus nuclear inclusion *a* protease (NIaPro) cleavage site between the protein of interest and the amino-terminal extension, and co-expression of a NIaPro led to further increment accumulation of these recombinant proteins.

Conclusion: Production of recombinant proteins in plants using a TMV vector is improved by fusing the amino-terminal end of TMV CP to the protein of interest and using a potyvirus protease to *in vivo* release the mature protein.

Keywords: viral vector, recombinant protein, biofactory plant, tobacco mosaic virus, interferon alfa-2a, Nanobody 72

Introduction

Plants may be excellent biofactories to produce recombinant proteins, metabolites or nanomaterials for pharmaceutical and industrial applications (Chung *et al.*, 2020; Molina-Hidalgo *et al.*, 2020; Schillberg and Finnern, 2021; Shanmugaraj *et al.*, 2020). Plants essentially require sunlight and water to grow; they are free of human and livestock pathogens, and production can be easily and economically scaled up when required. Biofactory plants are typically engineered by stable transformation to generate lines that express the genes of interest. However, stable genetic transformation in plants is a time-consuming and labor-intensive process of relatively unpredictable results due to random insertion of transgenes and intricate regulation of gene expression. In addition, not all plant species can be genetically transformed in a stable manner. Transient expression using virus-derived vectors is an alternative to stable genetic transformation to improve reproducibility and yield in plant bioengineering. Genome amplification of the viral vector and cellular movement, along with the co-expression of viral RNA silencing suppressors, usually contribute to improved accumulation of products of interest in biofactory plants (Wang *et al.*, 2020).

Tobacco mosaic virus (TMV), a plus-strand RNA virus from genus *Tobamovirus* (family *Virgaviridae*) can be easily transformed into an exceptional vector to produce recombinant proteins in plants, due to the relatively small genome, rapid replication and spread through the host, stability of the viral particles, easy mechanical transmission and, above all, the extraordinary transcription activity from the viral coat protein (CP) promoter and the efficient translation of the

resulting subgenomic mRNA (Dawson, 2014; Lindbo, 2007; Lomonossoff and Wege, 2018). In many TMV vector assemblies, the sequence corresponding to the open reading frame (ORF) of the protein of interest replaces most, although not all, the ORF of the viral CP (Lindbo, 2007; Marillonnet *et al.*, 2004; Shi *et al.*, 2019). As a consequence of this replacement, the resulting TMV recombinant clones retain the RNA-to-RNA genome amplification and the virus cell-to-cell movement abilities, but loose the long-distance movement capacity. TMV CP ORF cannot be completely replaced with the gene of interest because the 5' and 3' terminal sequences are required for subgenomic RNA transcription and genomic RNA replication, respectively (Grdzelishvili *et al.*, 2000; Shivprasad *et al.*, 1999). Mutation of the original CP start codon and insertion of a stop codon after the ORF corresponding to the gene-of-interest avoid the fusion of the amino and carboxy-terminal fragments of the viral CP to the recombinant protein of interest.

Genomes of all living beings are the result of a complex evolutionary process that frequently lead, among others, to overlapping and context-dependent performance of biological components (Porcar and Peretó, 2016). This is particularly true in the highly constrained genomes of plant viruses. In this work, we asked whether the translational fusion of the amino and carboxy-terminal remains of the TMV CP to the recombinant protein of interest in a TMV vector may improve accumulation in plant tissues. Expressing an enhanced version of the jellyfish green fluorescent protein (GFP), we observed that fusion of the TMV CP amino terminal residues significantly increases accumulation of the recombinant product. Then, we aimed to stablish a system in which inoculation of the TMV vector is accompanied with expression of a highly-specific protease, a potyvirus protease (Cesaratto *et al.*, 2016), to remove *in vivo* the CP-derived terminal extension from the recombinant protein. Interestingly, our results showed a further incremented accumulation of the recombinant product by co-

expression and cleavage in the cases of two different potyviral NIaPro, those from tobacco etch virus (TEV) and turnip mosaic virus (TuMV). We exemplify the use of this augmented TMV vector producing human interferon alfa 2a (IFN-α2a) and SARS-CoV-2 Spike-specific Nanobody 72 in *Nicotiana benthamiana* biofactory plants.

Results

Fusion of the amino and carboxyl termini of CP improves production of a recombinant protein in plants using a TMV vector

To analyze whether the fusion of the amino- and carboxy-terminal ends of TMV CP has an effect on the accumulation of a recombinant protein that is expressed in plants using a TMV vector in which the ORF of the protein of interest replaces most of viral CP, we built four constructs to express GFP alone or GFP fused to CP amino terminus (NCP-GFP), carboxyl terminus (GFP-CCP) or both termini (NCP-GFP-CCP). The resulting recombinant virus clones (TMVΔCP-GFP, TMVΔCP-NCP-GFP, TMVΔCP-GFP-CCP and TMVΔCP-NCP-GFP-CCP; **Fig. 1A** and **Fig. S1**) were expressed in *N. benthamiana* leaves using *Agrobacterium tumefaciens*. Infiltrated tissues were harvested 7 days post-inoculation (dpi). GFP was purified and quantified by fluorescence analysis. Preparations from tissues infiltrated with the vectors that produced NCP-GFP and NCP-GFP-CCP displayed a significant green fluorescence increase of 1.7 and 1.9 fold, respectively, with respect to those infiltrated with the vector that produced GFP with no extension (**Fig. 1B**). No significant difference was observed in preparations from tissues infiltrated with the vector that produced GFP-CCP (**Fig. 1B**). This result suggested that, in this TMV vector, the translational fusion of the

amino terminus of viral CP to the recombinant protein of interest, such as GFP, improves accumulation.

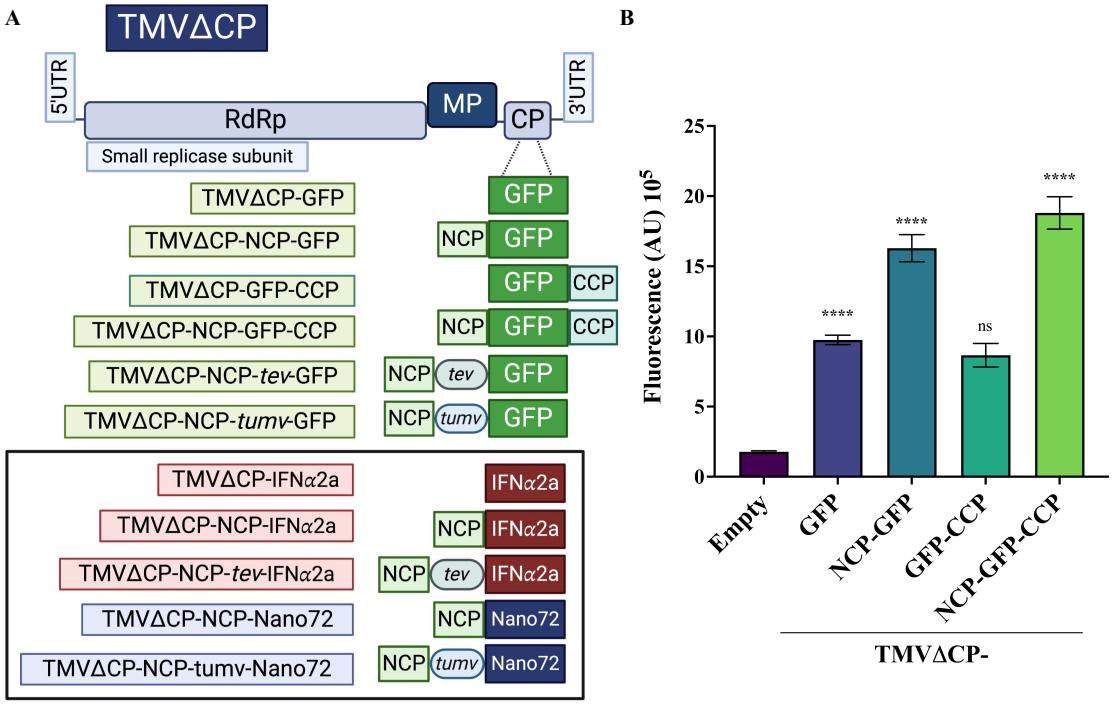

Fig. 1. Production of GFP in biofactory plants using different versions of a TMV vector in which amino- and carboxyl termini of virus CP are translationally fused to GFP. (A) Schematic representation of viral vectors. Boxes represent virus 5' and 3' untranslated regions (5' and 3'UTR), the RNA-dependent RNA polymerase (RdRp), the movement protein (MP), and the coat protein (CP). Amino and carboxy-terminal CP moieties (NCP and CCP, respectively) are also represented by boxes. The cDNAs corresponding to recombinant proteins GFP, IFNα2a and Nano72 are represented by green, red and blue boxes, respectively. TEV and TuMV NIaPro cleave sites are represented by green and blue elapses, respectively. Viral vector elements are not represented at scale. (B). Accumulation of different version of recombinant GFP with or without amino and carboxyl TMV CP extensions in *N. benthamiana* plants at 7 dpi. The column graph represents average green fluorescent in three independent plants. Error bars represent standard deviation ($p<0.0001$).

NIaPro-mediated post-translational cleavage of the TMV CP amino terminal extension further increases accumulation of GFP

For many biotechnological applications, extension of the recombinant proteins of interest with a TMV CP amino-terminal moiety should not raise any concern. However, in other applications, such as those in the pharmaceutical sector, these extra sequences may entail regulatory complications. To remove undesired extra sequences from the protein of interest, we explored the co-expression of a potyviral NIaPro, such as that from TEV, a highly active and specific protease that recognizes a motif of seven amino acids cleaving between positions -6 and +1 (Carrington *et al.*, 1993). We built a new construct in which the NIaPro cleavage site –we chose the seven amino acids (ENLYFQ/S) that mediate nuclear inclusion *b* (NIb) and CP processing in TMV– was inserted between the amino-terminal TMV CP fragment and GFP. This new fusion protein was termed NCP-*tev*-GFP to indicate that the TEV NIaPro cleavage site is inserted between the NCP extension and GFP. Using *A. tumefaciens*, this new viral clone (TMVΔCP-NCP-*tev*-GFP; Fig.1A and Fig. S1) was expressed in leaves of *N. benthamiana* along with a construct to produce TEV NIaPro (Cordero *et al.*, 2018). A series of controls, including the TMVΔCP-NCP-GFP clone (no cleavage site) and an empty vector, were used in the experiment. Quantification of GFP fluorescence at 7 dpi in preparations from infiltrated tissues showed a further 2.3-fold increase in those in which NCP-*tev*-GFP and NIaPro were co-expressed in comparison to those in which no protease was expressed or the recombinant protein did not contain the TEV cleavage site (NCP-GFP) (Fig. 2A). Pictures of *N. benthamiana* leaves at 7 dpi in which NCP-GFP or NCP-*tev*-GFP and TEV NIaPro were expressed clearly revealed the increased green fluoresce when TEV NIaPro was co-expressed (Fig. 2B). This result opened the possibility that, in addition to tag cleavage, TEV NIaPro co-expression may have an unexpected positive effect on accumulation of the recombinant protein of interest.

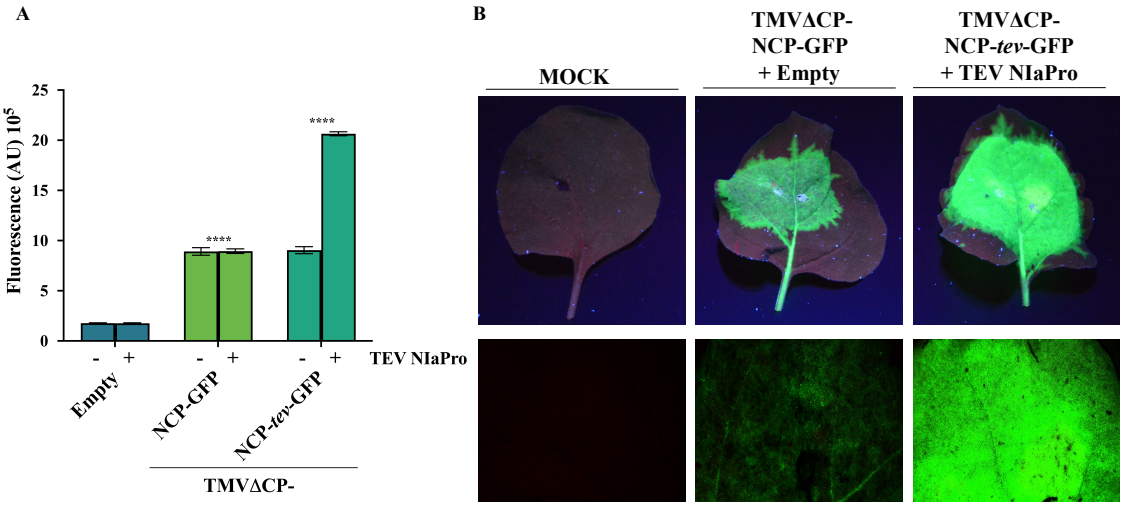

Fig. 2. Production of GFP in biofactory plants using a TMV vector in which the amino terminus of virus CP is translationally fused and cleavage of the extension by TEV NIaPro. (A). Accumulation of two versions of a recombinant GFP without (NCP-GFP) or with TEV NIaPro (NCP-*tev*-GFP) cleavage site in the absence or presence of TEV NIaPro, as indicated. The column graph represents average green fluorescent in three independent *N. benthamiana* plants at 7 dpi. Error bars represent standard deviation ($p<0.0001$). (B) Representative leaves from *N. benthamiana* plants mock-inoculated or inoculated with recombinant virus TMVΔCP-NCP-GFP or TMVΔCP-NCP-*tev*-GFP, as indicated. TEV NIaPro was transiently expressed or not in the plant tissues using *A. tumefaciens* as indicated. Leaves were photographed under UV light using a standard camera or a fluorescence stereomicroscope (upper and lower raw, respectively).

To confirm this observation, we repeated the experiment using an alternative potyvirus protease, specifically that from TuMV, a different virus species belonging to same genus. Both GFP quantification (Fig. 3A), visual inspection of infiltrated leaves (Fig. 3B), and Western blot analysis (Fig. 3C) confirmed the positive effect of potyvirus protease co-expression on TMV-based GFP accumulation.

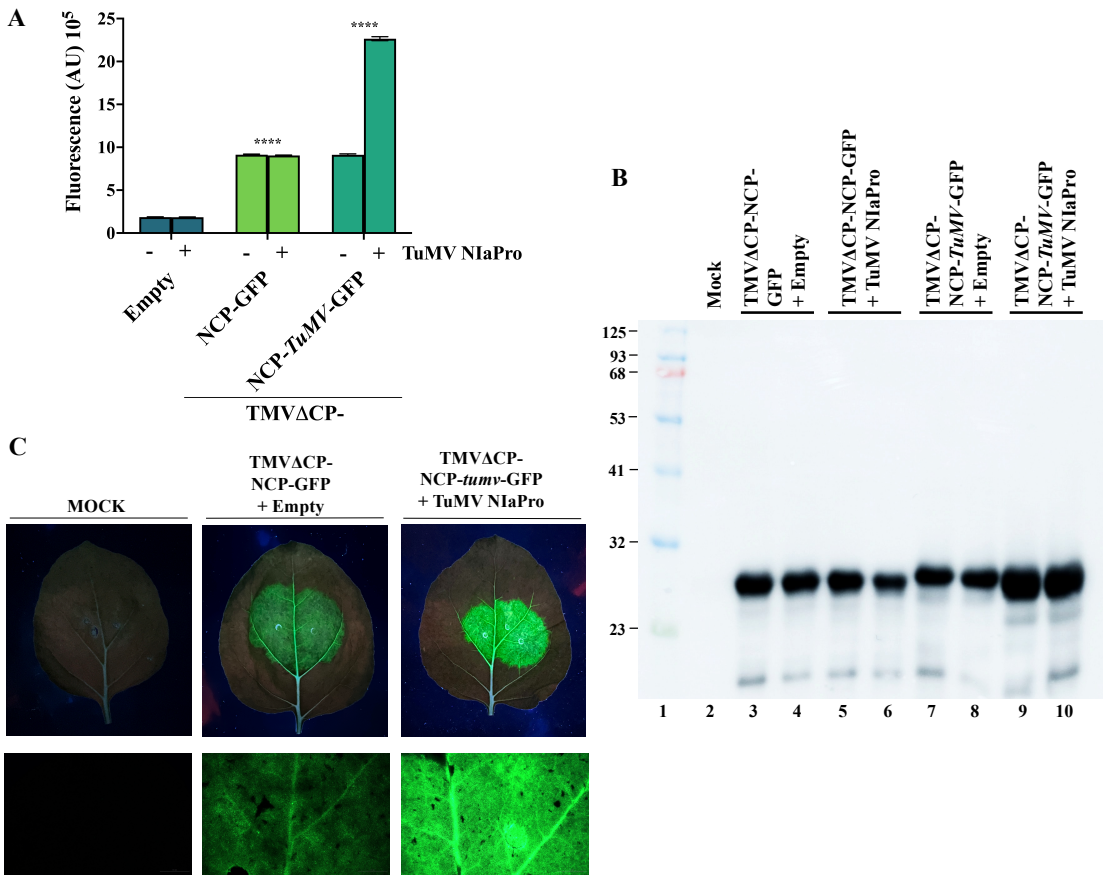

Fig. 3. Production of GFP in biofactory plants using a TMV vector in which the amino terminus of virus CP is translationally fused and cleavage of the extension by TuMV NIaPro. (A). Accumulation of two versions of a recombinant GFP without (NCP-GFP) or with TuMV NIaPro (NCP-*tumv*-GFP) cleavage site in the absence or presence of TuMV NIaPro, as indicated. The column graph represents average green fluorescent in three independent *N. benthamiana* plants at 7 dpi. Error bars represent standard deviation ($p<0.0001$). (B) Western-blot analysis of the different versions of recombinant GFP that accumulate in *N. benthamiana* tissues in the absence or the presence of NIaPro TuMV, as indicated. Lane 1, protein standards with sizes in kDa on the left; lane 2, mock-inoculated plant; lanes 3 to 6 and 7 to 10, tissues infected with recombinant viruses TMVΔCP-NCP-GFP and TMVΔCP-NCP-*tumv*-GFP, respectively. Lanes 3, 4, 7, 8 and 5, 6, 9, 10, tissues in which TuMV NIaPro was not or was co-expressed,

respectively (C) Representative leaves from *N. benthamiana* plants mock-inoculated or inoculated with recombinant virus TMVΔCP-NCP-GFP or TMVΔCP-NCP-*tumv*-GFP, as indicated. TuMV NIaPro was transiently expressed or not in the plant tissues using *A. tumefaciens* as indicated. Leaves were photographed under UV light using a standard camera or a fluorescence stereomicroscope (upper and lower raw, respectively).

Production of IFNα2a and SARS-CoV-2 Nanobody 72 in plants using an augmented TMV vector

Next, we asked whether the improvements in the TMV vector that were found using the marker protein GFP are general and can be applied to other recombinant proteins of interest. To test this notion, we chose human IFNα2a, a glycoprotein with antitumor, antiviral, and immunomodulatory activity, widely used in the treatment of viral infections, hematological disorders and solid tumors (Lewczuk *et al.*, 2019), and Nanobody 72 against the Spike protein of SARS-CoV-2 (Wrapp *et al.*, 2020). We built two new TMV clones to produce IFNα2a with and without NCP extension (TMVΔCP-IFNα2a and TMVΔCP-NCP-*tev*-IFNα2a; Fig. 1A and Fig. S1). Both recombinant IFNα2a versions contained a carboxy-terminal FLAG tag to facilitate detection by Western blot analysis using a specific monoclonal antibody conjugated to horseradish peroxidase (HRP). Proteins were extracted from three plants co-infiltrated with *A. tumefaciens* to express the TMV clones and TEV NIaPro or the corresponding empty vector. Western blot analysis confirmed efficient cleavage of the NCP tag when NIaPro is co-expressed (Fig. 4A). Quantitative analysis of the signals indicated an increased accumulation of IFNα2a when the TMV NCP is translationally fused (2.6 fold), and particularly when the TEV NIaPro cleavage site is inserted and NIaPro is co-expressed (6.9 fold) (Fig. 4B). Band inspection

in this Western blot analysis showed that mature IFNα2a was partially produced from NCP-IFNα2a in plant tissues in the absence of TEV NIaPro and its cleavage site (**Fig. 4A**, lanes 5 to 7), and that TEV NIaPro did not quantitatively released all the mature IFNα2a from NCP-*tev*-IFNα2a. Finally, we also confirmed the positive effect of TuMV NIaPro co-expression in Nanobody 72 production using the TMV vector (**Fig. 5** and **Fig. S1**). In contrast to what we observed with NCP-*tev*-IFNα2a, in this case, TuMV NIaPro quantitatively released the mature Nanobody 72 from the precursor NCP-*tumv*-Nano72 (**Fig. 5**, lanes 9 and 10).

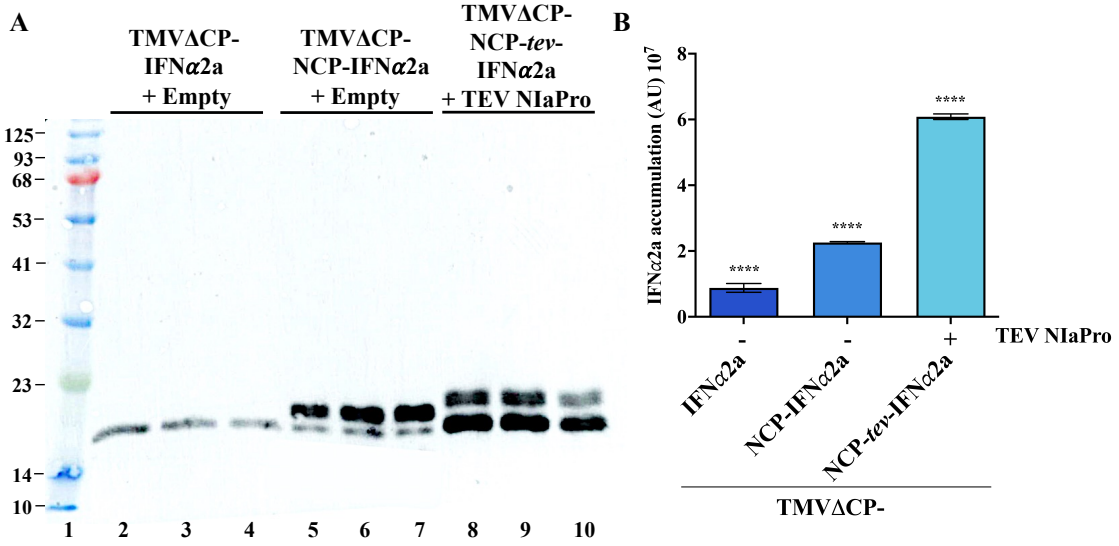

Fig. 4. Production of recombinant interferon alfa-2a (IFNα2a) in *N. benthamiana* plants using a TMV vector with TMV CP amino-terminal extension and TEV NIaPro cleavage of the tag. (**A**) Western-blot analysis of the different versions of recombinant IFNα2a that accumulate in *N. benthamiana* tissues in the absence or the presence of NIaPro TuMV, as indicated. Lane 1, protein standards with sizes in kDa on the left; lanes 2 to 3, tissues infected with recombinant virus TMVΔCP-IFNα2a with no TEV NIaPro co-expressed; lanes 5 to 7, tissues infected with recombinant virus TMVΔCP-NCP-IFNα2a with no TEV NIaPro co-expressed; lanes 8 to 10, tissues infected with recombinant virus TMVΔCP-NCP-*tev*-IFNα2a with TEV NIaPro co-expressed. Each group of three lanes containing the same

type of tissue correspond to three independent plants. (B) Accumulation of two version of a recombinant IFNα2a without (NCP-IFNα2a) or with TEV NIaPro (NCP-*tev*-IFNα2a) cleavage site in the absence or presence of TEV NIaPro, as indicated. The column graph represents average green luminescent signal in the Western-blot analyses (A) in three independent *N. benthamiana* plants at 7 dpi. Error bars represent standard deviation ($p<0.0001$).

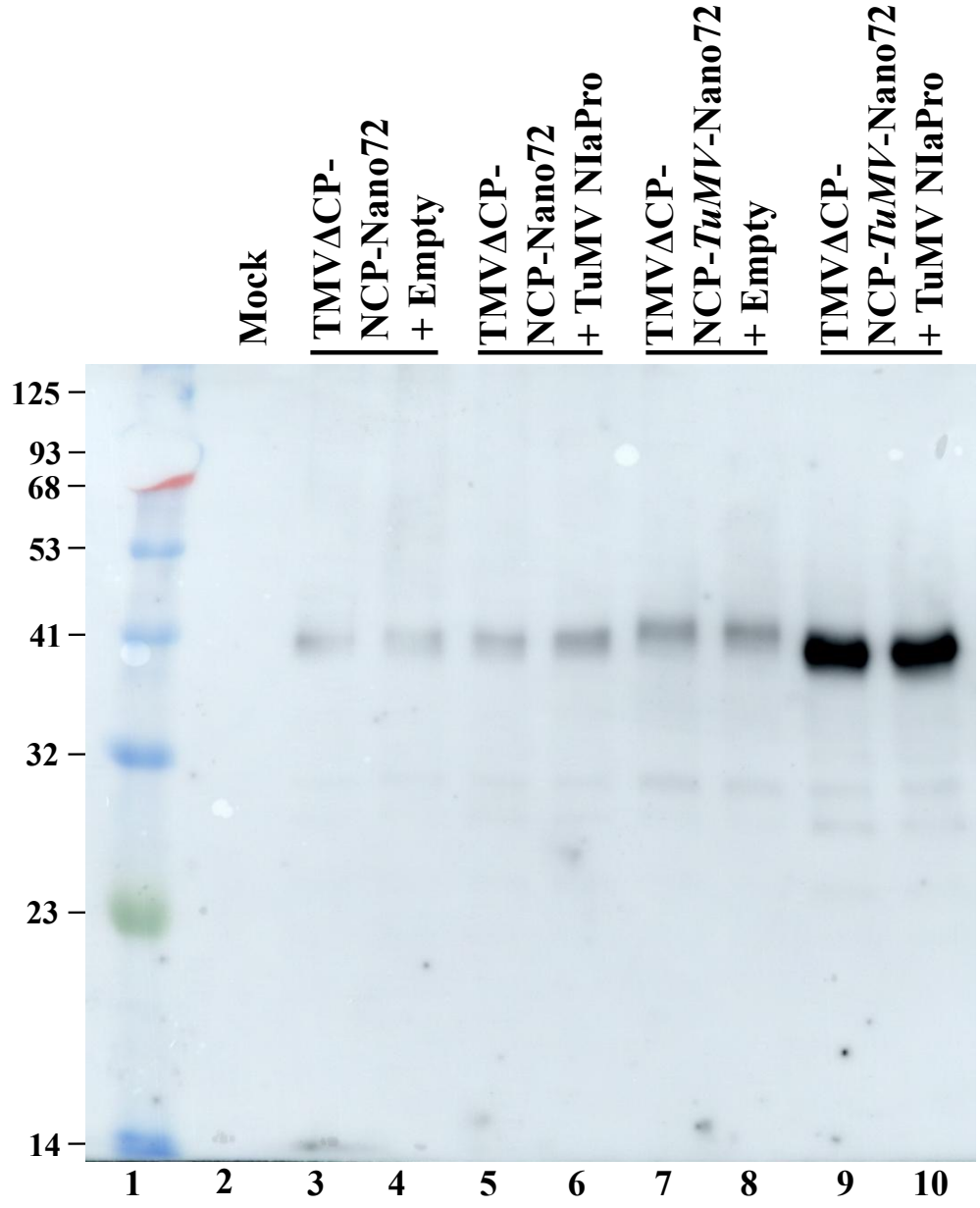

Fig 5. Production of recombinant Nano72 in *N. benthamiana* plants using a TMV vector with TMV CP amino-terminal extension and TuMV NIaPro cleavage of the tag. (A) Western-blot analysis of the different versions of recombinant Nano72 that accumulate in *N. benthamiana* tissues in the absence or the presence of TEV NIaPro, as indicated. Lane 1, protein standards with sizes in kDa on the left; lane 2, tissues mock-inoculated; lanes 3 to 6 and 7 to 10, tissues infected with recombinant virus TMVΔCP-NCP-Nano72 and TMVΔCP-NCP-*tumv*-Nano72, respectively; lanes 3, 4, 7, 8 and 4, 5, 9, 10, tissues in which TuMV NIaPro was not or was, respectively, co-expressed using *A. tumefaciens*. Each pair of lanes containing the same type of tissue correspond to two independent plants.

Discussion

TMV vectors are highly appreciated in plant biotechnology due to their capacity to produce large amounts of recombinant proteins in a very short period of time (Dawson, 2014). This capacity results from the extraordinary transcriptional activity from the viral CP promoter and the translation efficiency of the corresponding subgenomic mRNA. In this research, we interrogate whether the production capacity of a TMV vector in which the gene of interest replaces most of viral CP ORF could still be improved. To answer this question, we investigated the effect of translationally fusing the amino- and carboxy-terminal ends of TMV CP to the recombinant protein. Results indicated that, while the carboxyl terminus had no significant effect, fusion of the amino terminus substantially improved accumulation of two different recombinant proteins, GFP and IFNα2a (Figs. 1 to 4). This improvement may result from an increased translation efficiency of the recombinant mRNA. Another factor that may impact on higher product accumulation could be a higher transcription rate which thanks to the presence in the 5' region of RNA mediates its higher accumulation by RNA

polymerase. Selection of initiation site is a crucial step in eukaryotic translation and sequences around the start AUG codon –the so-called Kozak consensus– are known to strongly influence translation efficiency (Kozak, 1986; Hernández *et al.*, 2019). By fusing the amino-terminal end of CP to the recombinant protein, we regenerated the original sequence of the viral subgenomic mRNA around the AUG start codon. This may explain the improved translation efficiency. Alternatively, the higher accumulation may also result from stabilization of the chimeric recombinant protein, as the amino-terminal residues or their modification are known to regulate protein degradation (Bachmair *et al.*, 1986; Dissmeyer *et al.*, 2018). Both mechanisms are not necessarily exclusive and could contribute to the observed improved accumulation of both recombinant proteins. However, our observation that the NIaPro cleavage products of the recombinant proteins, in which the NCP tag is removed, further accumulate in infiltrated tissues (**Figs. 3** to **5**) favors the translation efficiency alternative.

The positive effect of the TMV NCP moiety on recombinant protein accumulation opens the possibility to tag genes of interest with this sequence to improve accumulation. Although we have only observed this positive effect using a TMV vector in which the gene of interest replaces most of CP ORF, we wonder if this effect is conserved in different configurations of the same vector or using different viral vector or expression platforms. In any case, this positive effect on recombinant protein accumulation was not surprising, since viral genomes are the result of a complex evolutionary process that includes an intricate host-pathogen arms race, and they are not a combination of components with discrete biological roles that can be simply exchanged in synthetic biology approaches (Porcar and Peretó, 2016). In this sense, by restoring the 5' end of the viral subgenomic mRNA, a positive effect on protein accumulation was reasonably expected.

Next, we designed a system to automatically remove most of these extra TMV CP residues from the recombinant protein of interest in the same plant tissues where it is produced. This system consisted of co-expressing a highly specific potyvirus NIaPro –we assayed both TEV and TuMV– along with the TMV vector. To induce processing of the NCP moiety, we inserted the specific seven-amino-acid cleavage sites of the corresponding protease between the amino-terminal tag and the recombinant protein. While we confirmed the release of the mature recombinant proteins in plant tissues (**Figs. 3** to **4**), we made a really unexpected observation. Our experimental results, with three independent recombinant proteins, GFP, IFNα2a and Nanobody72, indicated that insertion of the cleavage site and co-expression of the cognate NIaPro have a remarkable positive effect on accumulation of the recombinant protein. The sole co-expression of NIaPro without a cleavage site between the NCP moiety and the protein of interest did not affect accumulation, indicating that our observation does not result from an indirect effect of NIaPro on the host cell, but a process in which the NIaPro cleavage site is recognized and cleaved in the recombinant protein by the protease.

We are not aware that this has ever been described, and we have no much clue about the origin of this improved accumulation. However, we observed that NCP-*tev*-IFNα2a cleaves to some extent to the mature IFNα2a form even in the absence of NIaPro (**Fig. 4**, lanes 5 to 7). This suggests that an endogenous plant protease may still recognize the NIaPro cleavage site and partially cleave the chimeric recombinant protein. This raises the possibility that being recruited into a host cell proteolytic processing maturation process may result in an improved accumulation of the chimeric recombinant protein. Notably, potyviral NIaPro are trypsin-like serine proteases (Cesaratto *et al.*, 2016; Dolja *et al.*, 1993), with many cellular homologs (Di Cera, 2009; Page and Di Cera, 2008).

Conclusions

This research shows that the usually efficient production of recombinant proteins in plants using TMV-derived vectors can still be improved by different strategies. First, translational fusion of the amino-terminal fragment of the TMV CP into the recombinant protein of interest increases accumulation in plant tissues. Second, insertion of a potyvirus NIaPro cleavage site between TMV CP amino-terminal moiety and the recombinant protein further improves accumulation when the potyvirus NIaPro is co-expressed in the plant tissue, the TMV CP amino-terminal tag is substantially cleaved and, interestingly, overall accumulation of recombinant protein is multiplied.

Material and methods

Viral clones and plasmid constructs. Plasmids containing viral clones TMVΔCP-GFP, -NCP-GFP, -GFP-CCP, NCP-GFP-CCP, -NCP-*tev*-GFP, -NCP-*tumv*-GFP, -NCP-IFNα2a, -NCP-*tev*-IFNα2a, -NCP-Nano72 and -NCP-*tumv*-Nano72 (**Fig. S1**) were built by standard molecular biology techniques starting from pGTMV and pMTMVi-N (Addgene plasmids no. 118755 and 118756) (Shi *et al.*, 2019). Polymerase chain reaction (PCR) products were obtained using Phusion high-fidelity DNA polymerase (Thermo Scientific) (**Table S1**) and assembled using the NEBuilder HiFi DNA assembly master mix (New England Biolabs). Plasmids pG53CPMVZ and pGNIaPro have been previously described (Cordero *et al.*, 2018). pGNIaPro allows expression of TEV NIaPro (from nt 6256 to nt 6981 from GenBank accession number DQ986288, plus amino-terminal ATG and carboxy-terminal TAA codons) under the control of cauliflower mosaic virus (CaMV) promoter and terminator, and modified cowpea mosaic virus RNA-2 5' and 3' untranslated regions (Cordero *et al.*, 2018). pG53CPMVZ is the corresponding empty vector (Cordero *et al.*, 2018). pGNIaPro-TuMV is similar to pGNIaPro but

expresses the TuMV NIaPro from position 6479 to 7207 of NC_002509.2, plus amino and carboxy-terminal ATG and TAA, respectively.

Plant infiltration. *A. tumefaciens* GV3101:pMP90, containing the helper plasmid pCLEAN-S48 (Thole *et al.*, 2007), were transformed with the different plasmids. Overnight liquid cultures were brought to optical density (600 nm) 0.5 in 10 mM MES, 10 mM $MgCl_2$, 150 µM acetosyringone and further incubated for 3 h at 28ºC. These cultures were used to infiltrate leaves of four-to-five-week old *N. benthamiana* plants grown at 25ºC under a 12 h light/12 h dark photoperiod. Each plant was infiltrated in two separated areas of two different leaves.

GFP extraction and fluorescence analysis. All infiltrated tissues of each plant were harvested (7 dpi), weighted and frozen in liquid nitrogen. Tissues were homogenized in the presence of 10 volumes of 0.1 M sodium carbonate pH 9.6 using a TissueRuptor device (Qiagen). Extracts were clarified by centrifugation and 200 µl aliquots of the supernatants loaded in 96-well plates. Fluorescence was measured with a Perkin Elmer 2030 Multilabel Reader VICTOR X5 using, respectively, 485±14 nm and 535±25 nm excitation and emission filters. Infiltrated tissues were also photographed at 7 dpi under UV light using a standard camera (Nikon) and a stereomicroscope (MZ16 F, Leica) equipped with the GFP2 filter (Leica). Results were statistically analyzed using GraphPad Prism. One-way analysis of variance (ANOVA) was followed by TUKEY post-hoc test to compare the means.

Western blot analysis of proteins. Plant tissues were ground with a mortar and pestle in the presence of liquid nitrogen and homogenized in 3 volumes of 60 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM dithiothreitol, 10% glycerol and 0.01% bromophenol blue. Extracts were incubated for 5 min at 95ºC and clarified by centrifugation. Aliquots (20 µl) of the supernatants were separated by SDS-PAGE in 12.5% polyacrylamide gels, unless indicated in the figure legend. Proteins

were electroblotted to Hybond polyvinylidene difluoride membrane (GE Healthcare) and GFP, IFNα2a and Nanobody72 detected, respectively, using an anti-GFP conjugated to HRP (Invitrogen), anti-DYKDDDDK (FLAG) tag monoclonal antibody (FG4R) conjugated to HRP (Invitrogen) or a rabbit anti-human IgG conjugated to HRP (Sigma) at 1:10,000 dilution. HRP was detected using the SuperSignal West Pico PLUS chemiluminiscent substrate (Thermo Scientific). Luminiscence was quantified with an image analyzer (LAS-3000; Fujifilm).

Author contribution

Both authors conceived the project and designed the experiments. MNS performed the experiments. Both authors analyzed the data. JAD wrote the manuscript with input from MNS.

Funding

This work was supported by Ministerio de Ciencia e Innovación (MCIN; Spain) through Agencia Estatal de Investigación (AEI; grant PID2020-114691RB-I00 from MCIN/AEI/10.13039/501100011033). MNS was the recipient of the predoctoral contract PRE2018-084771 from MCIN.

Data availability

The data that support the findings of this study are available on request from the corresponding author.

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Supplementary Information

Fig. S1. Sequence of plasmids containing TMV recombinant clones to express eGFP, interferon alfa-2a and Nanobody 72 in *N. benthamiana*. Plasmids were built on the basis of pGTMV that contains a TMV infectious variant (GenBank accession no. MK087763.1). TMV expression vectors contain the deletion of most of CP gene.

>pGTMV (9669 bp)

GCGGCCGCGATTCCATTGCCCAGCTATCTGTCACTTTATTGTGAAGATAGTGGAAAAGGAAGGTGGCTCC
TACAAATGCCATCATTGCGATAAAGGAAAGGCCATCGTTGAAGATGCCTCTGCCGACAGTGGTCCCAAAG
ATGGACCCCCACCCACGAGGAGCATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGTGGA
TTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCAAGACCCTTCCTCT
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GTTTAACGCTCGTGACCGCAGGCCCAAGGTGAACTTTTCAAAAGTAATAAGCGAGGAGCAGACGCTTATT
GCTACCCGGGCGTATCCAGAATTCCAAATTACATTTTATAACACGCAAAATGCCGTGCATTCGCTTGCAG
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GCTATGCCGCTTTCCACTTCTCCGAGAACCTGCTTCTTGAAGATTCATGCGTCAATTTGGACGAAATCAA
CGCGTGTTTTTCGCGCGATGGAGACAAGTTGACCTTTTCTTTTGCATCAGAGAGTACTCTTAATTACTGT
CATAGTTATTCTAATATTCTTAAGTATGTGTGCAAAACTTACTTCCCGGCCTCTAATAGAGAGGTTTACA
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TGACAGCGAGGTCCGAATGGGATGTGGACAAATCTTTGTTACAATCCTTGTCCATGACGTTTTACCTGCA
TACTAAGCTTGCCGTTCTAAAGGATGACTTACTGATTAGCAAGTTTAGTCTCGGTTCGAAAACGGTGTGC
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ACAGGAAACTTATCAGAGTGGCAGGCGACGCATTAGAGATCAGGGTGCCTGATCTATATGTGACCTTCCA
CGACAGATTAGTGACTGAGTACAAGGCCTCTGTGGACATGCCTGCGCTTGACATTAGGAAGAAGATGGAA
GAAACGGAAGTGATGTACAATGCACTTTCAGAATTATCGGTGTTAAGGGAGTCTGACAAATTCGATGTTG
ATGTTTTTTCCCAGATGTGCCAATCTTTGGAAGTTGACCCAATGACGGCAGCGAAGGTTATAGTCGCGGT
CATGAGCAATGAGAGCGGTCTGACTCTCACATTTGAACGACCTACTGAGGCGAATGTTGCGCTAGCTTTA
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TGGTGACATGCGATGATTGGAGAAGAGTAGCTGTTAGCTCTGAGTCTGTTGTTTATTCCGACATGGCGAA
ACTCAGAACTCTGCGCAGACTGCTTCGAAACGGAGAACCGCATGTCAGTAGCGCAAAGGTTGTTCTTGTG
GACGGAGTTCCGGGCTGTGGAAAAACCAAAGAAATTCTTTCCAGGGTTAATTTTGATGAAGATCTAATTT
TAGTACCTGGGAAGCAAGCCGCGGAAATGATCAGAAGACGTGCGAATTCCTCAGGGATTATTGTGGCCAC
GAAGGACAACGTTAAAACCGTTGATTCTTTCATGATGAATTTTGGGAAAAGCACACGCTGTCAGTTCAAG
AGGTTATTCATTGATGAAGGGTTGATGTTGCATACTGGTTGTGTTAATTTTCTTGTGGCGATGTCATTGT
GCGAAATTGCATATGTTTACGGAGACACACAGCAGATTCCATACATCAATAGAGTTTCAGGATTCCCGTA
CCCCGCCCATTTTGCCAAATTGGAAGTTGACGAGGTGGAGACACGCAGAACTACTCTCCGTTGTCCAGCC
GATGTCACACATTATCTGAACAGGAGATATGAGGGCTTTGTCATGAGCACTTCTTCGGTTAAAAAGTCTG

TTTCGCAGGAGATGGTCGGCGGAGCCGCCGTGATCAATCCGATCTCAAAACCCTTGCATGGCAAGATCCT
GACTTTTACCCAATCGGATAAAGAAGCTCTGCTTTCAAGAGGGTATTCAGATGTTCACACTGTGCATGAA
GTGCAAGGCGAGACATACTCTGATGTTTCACTAGTTAGGTTAACCCCTACACCGGTCTCCATCATTGCAG
GAGACAGCCCACATGTTTTGGTCGCATTGTCAAGGCACACCTGTTCGCTCAAGTACTACACTGTTGTTAT
GGATCCTTTAGTTAGTATCATTAGAGATCTAGAGAAACTTAGCTCGTACTTGTTAGATATGTATAAGGTC
GATGCAGGAACACAATAGCAATTACAGATTGACTCGGTGTTCAAAGGTTCCAATCTTTTTGTTGCAGCGC
CAAAGACTGGTGATATTTCTGATATGCAGTTTTACTATGATAAGTGTCTCCCAGGCAACAGCACCATGAT
GAATAATTTTGATGCTGTTACCATGAGGTTGACTGACATTTCATTGAATGTCAAAGATTGCATATTGGAT
ATGTCTAAGTCTGTTGCTGCGCCTAAGGATCAAATCAAACCACTAATACCTATGGTACGAACGGCGGCAG
AAATGCCACGCCAGACTGGACTATTGGAAAATTTAGTGGCGATGATTAAAAGAAACTTTAACGCACCCGA
GTTGTCTGGCATCATTGATATTGAAAATACTGCATCTTTGGTTGTAGATAAGTTTTTTGATAGTTATTTG
CTTAAAGAAAAAAGAAAACCAAATAAAAATGTTTCTTTGTTCAGTAGAGAGTCTCTCAATAGATGGTTAG
AAAAGCAGGAACAGGTAACAATAGGCCAGCTCGCAGATTTTGATTTTGTGGATTTGCCAGCAGTTGATCA
GTACAGACACATGATTAAAGCACAACCCAAACAAAAGTTGGACACTTCAATCCAAACGGAGTACCCGGCT
TTGCAGACGATTGTGTACCATTCAAAAAAGATCAATGCAATATTCGGCCCGTTGTTTAGTGAGCTTACTA
GGCAATTACTGGACAGTGTTGATTCGAGCAGATTTTTGTTTTTCACAAGAAAGACACCAGCGCAGATTGA
GGATTTCTTCGGAGATCTCGACAGTCATGTGCCGATGGATGTCTTGGAGCTGGATATATCAAAATACGAC
AAATCTCAGAATGAATTCCACTGTGCAGTAGAATACGAGATCTGGCGAAGATTGGGTTTTGAAGACTTCT
TGGGAGAAGTTTGGAAACAAGGGCATAGAAAGACCACCCTCAAGGATTATACCGCAGGTATAAAAACTTG
CATCTGGTATCAAAGAAAGAGCGGGGACGTCACGACGTTCATTGGAAACACTGTGATCATTGCTGCATGT
TTGGCCTCGATGCTTCCGATGGAGAAAATAATCAAAGGAGCCTTTTGCGGTGACGATAGTCTGCTGTACT
TTCCAAAGGGTTGTGAGTTTCCGGATGTGCAACACTCCGCAAATCTTATGTGGAATTTTGAAGCAAAACT
GTTTAAAAAACAGTATGGATACTTTTGCGGAAGATATGTAATACATCACGACAGAGGATGCATTGTGTAT
TACGATCCCCTAAAGTTGATCTCGAAACTTGGTGCTAAACACATCAAGGATTGGGAACACTTGGAGGAGT
TCAGAAGGTCTCTTTGTGATGTTGCTGTTTCGTTGAACAATTGTGCGTATTACACACAGTTGGACGACGC
TGTATGGGAGGTTCATAAGACCGCCCCTCCAGGTTCGTTTGTTTATAAAAGTCTGGTGAAGTATTTGTCT
GATAAAGTTCTTTTTAGAAGTTTGTTTATAGATGGCTCTAGTTGTTAAAGGAAAAGTGAATATCAATGAG
TTTATCGACCTGTCAAAAATGGAGAAGATCTTACCGTCGATGTTTACCCCTGTAAAGAGTGTTATGTGTT
CCAAAGTTGATAAAATAATGGTTCATGAGAATGAGTCATTGTCAGAGGTGAACCTTCTTAAAGGAGTTAA
GCTTATTGATAGTGGATACGTCTGTTTAGCCGGTTTGGTCGTCACGGGCGAGTGGAACTTGCCTGACAAT
TGCAGAGGAGGTGTGAGCGTGTGTCTGGTGGACAAAAGGATGGAAAGAGCCGACGAGGCCACTCTCGGAT
CTTACTACACAGCAGCTGCAAAGAAAAGATTTCAGTTCAAGGTCGTTCCCAATTATGCTATAACCACCCA
GGACGCGATGAAAAACGTCTGGCAAGTTTTAGTTAATATTAGAAATGTGAAGATGTCAGCGGGTTTCTGT
CCGCTTTCTCTGGAGTTTGTGTCGGTGTGTATTGTTTATAGAAATAATATAAAATTAGGTTTGAGAGAGA
AGATTACAAACGTGAGAGACGGAGGGCCCATGGAACTTACAGAAGAAGTCGTTGATGAGTTCATGGAAGA
TGTCCCTATGTCGATCAGGCTTGCAAAGTTTCGATCTCGAACCGGAAAAAAGAGTGATGTCCGCAAAGGG
AAAAATAGTAGTAGTGATCGGTCAGTGCCGAACAAGAACTATAGAAATGTTAAGGATTTTGGAGGAATGA
GTTTTAAAAAGAATAATTTAATCGATGATGATTCGGAGGCTACTGTCGCCGAATCGGATTCGTTTTAAAT
ATGTCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCTTGTCATCAGCGTGGGCCGACCCAATAGAGT
TAATTAATTTATGTACTAATGCCTTAGGAAATCAGTTTCAAACACAACAAGCTCGAACTGTCGTTCAAAG
ACAATTCAGTGAGGTGTGGAAACCTTCACCACAAGTAACTGTTAGGTTCCCTGACAGTGACTTTAAGGTG
TACAGGTACAATGCGGTATTAGACCCGCTAGTCACAGCACTGTTAGGTGCATTCGACACTAGAAATAGAA
TAATAGAAGTTGAAAATCAGGCGAACCCCACGACTGCCGAGACGTTAGATGCTACTCGTAGAGTAGACGA
CGCAACGGTGGCCATAAGGAGCGCGATAAATAATTTAATAGTAGAATTGATCAGAGGAACCGGATCTTAT
AATCGGAGCTCTTTCGAGAGCTCTTCTGGTTTGGTTTGGACCTCTGGTCCTGCAACTTGAGGTAGTCAAG
ATGCATAATAAATAACGGATTGTGTCCGTAATCACACGTGGTGCGTACGATAACGCATAGTGTTTTTCCC
TCCACTTAAATCGAAGGGTTGTGTCTTGGATCGCGCGGGTCAAATGTATATGGTTCATATACATCCGCAG
GCACGTAATAAAGCGAGGGGTTCGAATCCCCCCGTTACCCCCGGTAGGGGCCCAGGGTCGGCATGGCATC
TCCACCTCCTCGCGGTCCGACCTGGGCTACTTCGGTAGGCTAAGGGAGAAGCGCTGAAATCACCAGTCTC
TCTCTACAAATCTATCTCTCTCTATTTTCTCCATAAATAATGTGTGAGTAGTTTCCCGATAAGGGAAATT
AGGGTTCTTATAGGGTTTCGCTCATGTGTTGAGCATATAAGAAACCCTTAGTATGTATTTGTATTTGTAA
AATACTTCTATCAATAAAATTTCTAATTCCTAAAACCAAAATCCAGGGGCCCTCGACGTTCCTTGACAGG
ATATATTGGCGGGTAAACTAAGTCGCTGTATGTGTTTGTTTGAGATCCTCTAGGGCATGCAAGCTGATCT
GGATCTCATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTC
CATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAG
GACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCT
TACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTAT
CTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCT
GCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGC
CACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAAC
TACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAGAAGAG

TTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGAT
TACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAAC
GAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATT
AAAAATGAAGTTTTAAATCAATCTAAAGTATATATGTGTAACATTGGTCTAGTGA***TTAGAAAAACTCATC***
GAGCATCAAATGAAACTGCAATTTATTCATATCAGGATTATCAATACCATATTTTTGAAAAAGCCGTTTC
TGTAATGAAGGAGAAAACTCACCGAGGCAGTTCCATAGGATGGCAAGATCCTGGTATCGGTCTGCGATTC
CGACTCGTCCAACATCAATACAACCTATTAATTTCCCCTCGTCAAAAATAAGGTTATCAAGTGAGAAATC
ACCATGAGTGACGACTGAATCCGGTGAGAATGGCAAAAGTTTATGCATTTCTTTCCAGACTTGTTCAACA
GGCCAGCCATTACGCTCGTCATCAAAATCACTCGCATCAACCAAACCGTTATTCATTCGTGATTGCGCCT
GAGCAAGACGAAATACGCGATCGCTGTTAAAAGGACAATTACAAACAGGAATCGAATGCAACCGGCGCAG
GAACACTGCCAGCGCATCAACAATATTTTCACCTGAATCAGGATATTCTTCTAATACCTGGAATGCTGTT
TTCCCTGGGATCGCAGTGGTGAGTAACCATGCATCATCAGGAGTACGGATAAAATGCTTGATGGTCGGAA
GAGGCATAAATTCCGTCAGCCAGTTTAGTCTGACCATCTCATCTGTAACAACATTGGCAACGCTACCTTT
GCCATGTTTCAGAAACAACTCTGGCGCATCGGGCTTCCCATACAATCGGTAGATTGTCGCACCTGATTGC
CCGACATTATCGCGAGCCCATTTATACCCATATAAATCAGCATCCATGTTGGAATTTAATCGCGGCCTTG
AGCAAGACGTTTCCCGTTGAATATGGCTCATAACACCCCTTGTATTACTGTTTATGTAAGCAGACAGTTT
TATTGTTCATGATGATATATTTTTATCTTGTGCAATGTAACATCAGAGATTTTGAGACACAACGTGGCTT
TGTTGAATAAATCGAACTTTTGCTGAGTTGAAGGATCAGATCACGCATCTTCCCGACAACGCAGACCGTT
CCGTGGCAAAGCAAAAGTTCAAAATCACCAACTGGTCCACCTACAACAAAGCTCTCATCAACCGTGGCTC
CCTCACTTTCTGGCTGGATGATGGGGCGATTCAGGCGATCCCCATCCAACAGCCCGCCGTCGAGCGGGCT
TTTTTATCCCCGGAAGCCTGTGGATAGAGGGTAGTTATCCACGTGAAACCGCTAATGCCCCGCAAAGCCT
TGATTCACGGGGCTTTCCGGCCCGCTCCAAAAACTATCCACGTGAAATCGCTAATCAGGGTACGTGAAAT
CGCTAATCGGAGTACGTGAAATCGCTAATAAGGTCACGTGAAATCGCTAATCAAAAAGGCACGTGAGAAC
GCTAATAGCCCTTTCAGATCAACAGCTTGCAAACACCCCTCGCTCCGGCAAGTAGTTACAGCAAGTAGTA
TGTTCAATTAGCTTTTCAATTATGAATATATATATCAATTATTGGTCGCCCTTGGCTTGTGGACAATGCG
CTACGCGCACCGGCTCCGCCCGTGGACAACCGCAAGCGGTTGCCCACCGTCGAGCGCCTTTGCCCACAAC
CCGGCGGCCGGCCGCAACAGATCGTTTTATAAATTTTTTTTTTTGAAAAAGAAAAAGCCCGAAAGGCGGC
AACCTCTCGGGCTTCTGGATTTCCGATCCCCGGAATTAGATCCGTTTAAACTACGTAAGATCGATCT**TGG**
CAGGATATATTGTGGTGTAAACGTTCCTGCGGCGGTCGAGATGGATCT**TGGCAGGATATATTGTGGTGTA**
AACGTTCCT

TMV-MK087763 is in bold. Cauliflower mosaic virus (CaMV) 35S promoter is in red with the transcription +1 nucleotide on yellow background. Hepatitis delta virus (HDV)-derived ribozyme is in red. CaMV 35S terminator is in fuchsia with the processing and polyadenylation site underlined. *E. coli* pUC replication origin is in blue on gray background. *Kanamycin* selection marker is in blue on dark gray background (complementary strand). *A. tumefaciens* pSa replication origin is in blue on gray background. *A. tumefaciens* T-DNA RB with overdrive (underlined) is in blue on yellow background and double LB is in blue on red background.

>pGMTVΔCP-GFP, insert between positions 6020 and 6486 of pGTMV
AGATCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCTTGTCAATGGTGAGCAAGGGCGAGGAGCTGT
TCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGG
CGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCC
GTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACA
TGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAA
GGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAG
CTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCC
ACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACAT
CGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTG
CTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACA
TGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTGA

TMV CP initiation ATG to AGA (mutations in red). Nucleotide sequence of eGFP in green.

>pGTMVΔCP-NCP-GFP, insert between positions 6020 and 6486 of pGTMV
ATGTCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCTTGTCAATGGTGAGCAAGGGCGAGGAGCTGT
TCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGG
CGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCC
GTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACA
TGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAA
GGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAG
CTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCC
ACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACAT
CGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTG
CTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACA
TGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTGA

Nucleotide sequence of eGFP in green.

>pGTMVΔCP-GFP-CCP, insert between positions 6020 and 6486 of pGTMV
AGATCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCTTGTCAATGGTGAGCAAGGGCGAGGAGCTGT
TCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGG
CGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCC
GTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACA
TGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAA
GGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAG
CTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCC
ACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACAT
CGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTG
CTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACA
TGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAG

TMV CP initiation ATG to AGA (mutations in red). Nucleotide sequence of eGFP in green.

>pGTMVΔCP-NCP-GFP-CCP, insert between positions 6020 and 6486 of pGTMV
ATGTCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCTTGTCAATGGTGAGCAAGGGCGAGGAGCTGT
TCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGG
CGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCC
GTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACA
TGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAA
GGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAG
CTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCC
ACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACAT
CGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTG
CTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACA
TGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAG

Nucleotide sequence of eGFP in green.

>pGTMVΔCP-NCP-*tev*-GFP, insert between positions 6020 and 6486 of pGTMV
ATGTCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCTTGTCA*GAGAATCTTTATTTTCAGAGT*ATGG
TGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGG
CCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATC
TGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCT
TCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCA
GGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGAC
ACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGC
TGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAA
CTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCC
ATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACC

CCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGA
CGAGCTGTACAAGTGA

TEV NIaPro cleavage site in gray italics. Nucleotide sequence of eGFP in green.

>pGTMVΔCP-NCP-*tumv*-GFP, insert between positions 6020 and 6486 of pGTMV
ATGTCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCTTGTCA*GCTTGTGTTTATCACCAGGCA*ATGG
TGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGG
CCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATC
TGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCT
TCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCA
GGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGAC
ACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGC
TGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAA
CTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCC
ATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACC
CCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGA
CGAGCTGTACAAGTGA

TuMV NIaPro cleavage site in purple italics. Nucleotide sequence of eGFP in green.

>pGTMVΔCP-IFNα2a, insert between positions 6020 and 6486 of pGTMV
AGATCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCTTGTCAATGTGTGATCTGCCTCAAACCCACA
GCCTGGGTAGCAGGAGGACCTTGATGCTCCTGGCACAGATGAGGAGAATCTCTCTTTTCTCCTGCTTGAA
GGACAGACATGACTTTGGATTTCCCCAGGAGGAGTTTGGCAACCAGTTCCAAAAGGCTGAAACCATCCCT
GTCCTCCATGAGATGATCCAGCAGATCTTCAATCTCTTCAGCACAAAGGACTCATCTGCTGCTTGGGATG
AGACCCTCCTAGACAAATTCTACACTGAACTCTACCAGCAGCTGAATGACCTGGAAGCCTGTGTGATACA
GGGGGTGGGGGTGACAGAGACTCCCCTGATGAAGGAGGACTCCATTCTGGCTGTGAGGAAATACTTCCAA
AGAATCACTCTCTATCTGAAAGAGAAGAAATACAGCCCTTGTGCCTGGGAGGTTGTCAGAGCAGAAATCA
TGAGATCTTTTTCTTTGTCAACAAACTTGCAAGAAAGTTTAAGAAGTAAGGAATGATCAGGCGGAGGTGG
GTCAGACTACAAGGACGACGATGACAAATGA

TMV CP initiation ATG to AGA (mutations in red). Nucleotide sequence of interferon alfa-2a on blue background followed by a linker (gray) and FLAG tag (bold).

>pGTMVΔCP-NCP-IFNα2a, insert between positions 6020 and 6486 of pGTMV
ATGTCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCTTGTCAATGTGTGATCTGCCTCAAACCCACA
GCCTGGGTAGCAGGAGGACCTTGATGCTCCTGGCACAGATGAGGAGAATCTCTCTTTTCTCCTGCTTGAA
GGACAGACATGACTTTGGATTTCCCCAGGAGGAGTTTGGCAACCAGTTCCAAAAGGCTGAAACCATCCCT
GTCCTCCATGAGATGATCCAGCAGATCTTCAATCTCTTCAGCACAAAGGACTCATCTGCTGCTTGGGATG
AGACCCTCCTAGACAAATTCTACACTGAACTCTACCAGCAGCTGAATGACCTGGAAGCCTGTGTGATACA
GGGGGTGGGGGTGACAGAGACTCCCCTGATGAAGGAGGACTCCATTCTGGCTGTGAGGAAATACTTCCAA
AGAATCACTCTCTATCTGAAAGAGAAGAAATACAGCCCTTGTGCCTGGGAGGTTGTCAGAGCAGAAATCA
TGAGATCTTTTTCTTTGTCAACAAACTTGCAAGAAAGTTTAAGAAGTAAGGAATGATCAGGCGGAGGTGG
GTCAGACTACAAGGACGACGATGACAAATGA

Nucleotide sequence of interferon alfa-2a on blue background followed by a linker (gray) and FLAG tag (bold).

>pGTMVΔCP-NCP-*tev*-IFNα2a, insert between positions 6020 and 6486 of pGTMV
ATGTCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCTTGTCA*GAGAATCTTTATTTTCAGAGT*ATGT
GTGATCTGCCTCAAACCCACAGCCTGGGTAGCAGGAGGACCTTGATGCTCCTGGCACAGATGAGGAGAAT
CTCTCTTTTCTCCTGCTTGAAGGACAGACATGACTTTGGATTTCCCCAGGAGGAGTTTGGCAACCAGTTC

CAAAAGGCTGAAACCATCCCTGTCCTCCATGAGATGATCCAGCAGATCTTCAATCTCTTCAGCACAAAGG
ACTCATCTGCTGCTTGGGATGAGACCCTCCTAGACAAATTCTACACTGAACTCTACCAGCAGCTGAATGA
CCTGGAAGCCTGTGTGATACAGGGGGTGGGGGTGACAGAGACTCCCCTGATGAAGGAGGACTCCATTCTG
GCTGTGAGGAAATACTTCCAAAGAATCACTCTCTATCTGAAAGAGAAGAAATACAGCCCTTGTGCCTGGG
AGGTTGTCAGAGCAGAAATCATGAGATCTTTTTCTTTGTCAACAAACTTGCAAGAAAGTTTAAGAAGTAA
GGAATGA*TCAGGCGGAGGTGGGTCA***GACTACAAGGACGACGATGACAAATGA**

TEV NIaPro cleavage site in gray italics. Nucleotide sequence of interferon alfa-2a on blue background followed by a linker (gray) and **FLAG** tag (bold).

>pGTMVΔCP-NCP-Nano72, insert between positions 6020 and 6486 of pGTMV
ATGTCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCTTGTCAATGCAGGTGCAGCTCCAAGAAAGCG
GAGGGGGTCTGGTGCAAGCTGGGGGTTCTTTGAGATTATCCTGTGCAGCTAGCGGACGTACATTTAGCGA
ATATGCAATGGGTTGGTTCCGTCAGGCCCCAGGAAAGGAGCGAGAGTTCGTTGCTACTATTAGTTGGTCC
GGTGGCTCTACATACTACACCGACTCTGTAAAAGGACGTTTTACTATTTCAAGGGATAATGCTAAGAACA
CAGTGTATTTACAAATGAATAGCCTCAAACCGGACGATACAGCAGTATACTATTGCGCTGCTGCTGGACT
TGGCACTGTAGTATCCGAATGGGATTACGACTACGACTACTGGGGTCAGGGGACTCAGGTCACGGTGTCC
TCCGCAGGGGAGCCCAAATCTTGTGACAAAACTCACACATGCCCACCGTGCCCAGCACCTGAACTCCTGG
GGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCATGATCTCCCGGACCCCTGAGGT
CACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTG
GAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCC
TCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAAGGTGTCAAACAAAGCCCTCCC
AGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCC
CCATCCCGGGAGGAGATGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTACCCCAGCG
ACATTGCGGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAAGACCACACCTCCCATGCTGGA
CTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTC
TTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGG
GTAAATGA

Nucleotide sequence of Nanobody 72 on dark orange.

>pGTMVΔCP-NCP-*tumv*-Nano72, insert between positions 6020 and 6486 of pGTMV
ATGTCTTACAGTATCACTACTCCATCTCAGTTCGTGTTCTTGTCA*GCTTGTGTTTATCACCAGGCA*ATGC
AGGTGCAGCTCCAAGAAAGCGGAGGGGGTCTGGTGCAAGCTGGGGGTTCTTTGAGATTATCCTGTGCAGC
TAGCGGACGTACATTTAGCGAATATGCAATGGGTTGGTTCCGTCAGGCCCCAGGAAAGGAGCGAGAGTTC
GTTGCTACTATTAGTTGGTCCGGTGGCTCTACATACTACACCGACTCTGTAAAAGGACGTTTTACTATTT
CAAGGGATAATGCTAAGAACACAGTGTATTTACAAATGAATAGCCTCAAACCGGACGATACAGCAGTATA
CTATTGCGCTGCTGCTGGACTTGGCACTGTAGTATCCGAATGGGATTACGACTACGACTACTGGGGTCAG
GGGACTCAGGTCACGGTGTCCTCCGCAGGGGAGCCCAAATCTTGTGACAAAACTCACACATGCCCACCGT
GCCCAGCACCTGAACTCCTGGGGGGACCGTCAGTCTTCCTCTTCCCCCCAAAACCCAAGGACACCCTCAT
GATCTCCCGGACCCCTGAGGTCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCTGAGGTCAAGTTC
AACTGGTACGTGGACGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCA
CGTACCGTGTGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACAAGTGCAA
GGTGTCAAACAAAGCCCTCCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCCGAGAA
CCACAGGTGTACACCCTGCCCCCATCCCGGGAGGAGATGACCAAGAACCAGGTCAGCCTGACCTGCCTGG
TCAAAGGCTTCTACCCCAGCGACATTGCGGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAA
GACCACACCTCCCATGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGC
AGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGA
AGAGCCTCTCCCTGTCTCCGGGTAAATGA

TuMV NIaPro cleavage site in purple italics. Nucleotide sequence of Nanobody 72 on dark orange.

Table S1. PCR primers used for cloning purposes.

Primer	Orientation	Sequence (5'-3')	Purpose

D1756	Forward	agttcgtgttcttgtcaATGGTGAGCAAGGGCGAG	Amplify GFP
D1757	Reverse	5'-ctcaagttgcaggaccCTTGTACAGCTCGTCCATG	
D1758	Reverse	ctcaagttgcaggaccTCACTTGTACAGCTCGTCCATG	Amplify GFP adding stop codon TGA
D3416	Reverse	AAAGATTCTCTGACAAGAACACGAACTGAGAT	Enter the cleavage sequence of TEV NIaPro
D3417	Forward	ATTTTCAGAGTATGGTGAGCAAGGGCGAGGAGC	
D2795	Reverse	TGACAAGAACACGAACTGAG	Amplify the plasmid leaving the GFP
D2796	Forward	GGTCCTGCAACTTGAGGTAGTCAAGAT	
D3523	Forward	ctcagttcgtgttcttgtcaATGTGTGATCTGCCTCAAAC	Amplify Interferon alfa- 2a
D3524	Reverse	ctacctcaagttgcaggaccTCATTTGTCATCGTCGTCC	
D3525	Reverse	ACTCTGAAAATAAAGATTCTCTGACAAG	Amplify Interferon alfa-2a adding TEV NIaPro cleavage sequence
D4931	Reverse	AAAGATTCTCTGACAAGAACACGAACTGAGAT	Add TuMV NIaPro cleavage sequence
D4932	Forward	ATTTTCAGAGTATGGTGAGCAAGGGCGAGGAGC	
D5005	Forward	ctcagttcgtgttcttgtcaATGCAGGTGCAGCTCCAAGAAA	Amplify Nanobody72
D4092	Reverse	ctacctcaagttgcaggaccTCATTTACCCGGAGACAG	
D5007	Forward	GCTTGTGTTTATCACCAGGCAATGCAGGTGCAGCTCCAAGAAA	Amplify Nanobody72 adding TuMV NIaPro cleavage sequence
D5012	Reverse	TGCCTGGTGATAAACACAAGCTGACAAGAACACGAACTGAGAT	Amplify the plasmid leaving the TuMV NIaPro cleavage sequence

CHAPTER II

Expression of an extremophilic xylanase in *Nicotiana benthamiana* and its use for the production of prebiotic xylooligosaccharides

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Sci Rep **12**, 15743 (2022). https://doi.org/10.1038/s41598-022-19774-5

Author contribution:

J.P. and J.-A.D. conceived the study with input from all authors. D.T.-P. and M.N.-S. performed the experiments. All authors analyzed the data. J.P. and J.-A.D. wrote the manuscript with input from all authors.

Abstract

A gene construct encoding a xylanase, which is active in extreme conditions of temperature and alkaline pH (90°C, pH 10.5), has been transitorily expressed with high efficiency in *Nicotiana benthamiana* using a viral vector. The enzyme, targeted to the apoplast, accumulates in large amounts in plant tissues in as little as 7 days after inoculation, without detrimental effects on plant growth. The properties of the protein produced by the plant, in terms of resistance to temperature, pH, and enzymatic activity, are equivalent to those observed when *Escherichia coli* is used as a host. Purification of the plant-produced recombinant xylanase is facilitated by exporting the protein to the apoplastic space. The production of this xylanase by *N. benthamiana*, which avoids the hindrances derived from the use of *E. coli*, namely, intracellular production requiring subsequent purification, represents an important step for potential applications in the food industry in which more sustainable and green products are continuously demanded. As an example, the use of the enzyme producing prebiotic xylooligosdaccharides from xylan is here reported.

Introduction

Plants have emerged as a promising alternative for heterologous protein production[1]. Plant growth is fueled by sunlight, and production can be scaled up quickly and easily. As in other eukaryotic organisms, in plants, proteins are subjected to post-translational modifications and can be targeted to the export pathway or accumulated in specific subcellular localizations. Additionally, if production is properly managed, plant products are free of human pathogens, which is particularly important in the pharmaceutical and cosmetic sectors.[2,3] Plants can be stably transformed to program production of recombinant

proteins. However, this is a time-consuming process involving, in most species, labor-intensive regeneration steps based on tissue culture techniques.[4] In addition, a high production yield is not always achieved. As an alternative, transient expression strategies that use the ability of *Agrobacterium tumefaciens* to efficiently transfer a DNA fragment (T-DNA) to plant cells have been developed.[5] These strategies, which are often based on regulatory elements derived from plant viruses, are technically simpler and frequently achieve higher yields[6]. Vectors based on tobacco mosaic virus (TMV; genus *Tobamovirus*, family *Virgaviridae*) have been used to produce recombinant proteins in plants, owing to the intrinsic capacity of this virus to produce enormous amounts of the capsid protein (CP) in plant cells. This capacity is likely based on the efficient transcription of a subgenomic RNA that encodes the viral CP and its subsequent efficient translation[7,8]. An *A. tumefaciens*-delivered TMV vector, in which most of the viral CP open reading frame (ORF) is replaced by the cDNA encoding the protein of interest, was recently developed and allowed for a high-yield production of an antifungal peptide in infiltrated *Nicotiana benthamiana* tissue[9]. Interestingly, the fusion of a signal peptide from a rice protein, which targeted the recombinant protein for export, ameliorated the toxic effect of the recombinant protein in plant tissues and facilitated purification by simple extraction of apoplastic fluid[9].

Xylan, together with cellulose and lignin, is one of the major structural polymers of plants, representing around 20-40% of their biomass. Xylan is composed of a backbone of xylose units linked by β-1,4 bonds and branched with side chains of arabinofuranose and glucuronic acid. The precise composition and abundance of these side chains are highly variable, depending on the plant species from which the xylan derives. Due to its nature and abundance, xylan is a useful compound for many different industries, including those involving pulp and paper, biofuels, food and beverages, and animal feed[10–13]. Xylanases have

relevant industrial applications; for instance, biobleaching in pulp treatment in paper industry[14,15], or the hydrolysis of polysaccharides into simple sugars (2 to 6 unit xylooligosaccharides; XOS) with important functional properties, such as prebiotics or low-calorie sweeteners[16–23]. The use of xylanases in industrial processes is often carried out under extreme pH and temperature conditions that conventional enzymes cannot withstand. Therefore, enzymes active under such extreme conditions have been identified and isolated[24,25]. Extremophilic xylanases are particularly useful for XOS production as their operational conditions greatly favor substrate and product solubility and prevent contamination in a sugar-rich environment, otherwise prone to microbial contamination[26].

Enzymes tailored for specific applications are often produced in large amounts in heterologous systems. The bacterium *Escherichia coli*, the yeast *Pichia pastoris*, and the filamentous fungus *Aspergillus niger* are the most frequently used organisms in industrial enzyme production. They are particularly suited due to their inexpensive culture conditions and good productivity[27]. However, the use of these microorganisms has some shortcomings. For instance, *E. coli* is unsuitable for producing secreted eukaryotic enzymes[28]. *P. pastoris* is useful for producing a number of enzymes, but the productivity is highly variable[29]. *A. niger* and other filamentous fungi are frequently restricted to homologous products, as their more complex genetics compared to *E. coli* or *P. pastoris* hampers heterologous production. In this context, plants can be a valuable alternative for heterologous production of proteins aimed to different biotechnological applications.

Since xylanases are in-demand enzymes for several industrial applications, particularly the digestion of xylan for producing XOS, in this work we analyzed whether the production of one of these enzymes in *N. benthamiana* using a TMV-

derived vector and an export-to-apoplast strategy to facilitate purification is a good alternative to the classic *E. coli* host. We focused on the extremophilic xylanase Xyn11 from *Pseudothermotoga thermarum* DSM 5069[25], an enzyme active under high temperature (90°C) and alkaline pH (10.5), which are the most convenient conditions in an industrial setting.

Results

TMV-based production of xylanase Xyn11 in *N. benthamiana*. To evaluate the feasibility of producing recombinant xylanase Xyn11 using plants as biofactory, we first built a series of TMV-derived vectors to express this enzyme in *N. benthamiana*. The vectors were designed to express the xylanase *Xyn11* coding ORF with codons optimized for *N. benthamiana* (**Fig. S1**), under the control of TMV CP promoter. In these constructs, heterologous cDNAs replaced most of the viral CP gene (TMVΔCP), which generates viral vectors that can move from cell-to-cell, but not long distance through the inoculated plants[9]. Three different vectors were built, to produce xylanase Xyn11 alone for cytosolic accumulation (TMVΔCP-Xyn11), xylanase Xyn11 with an amino-terminal signal peptide (TMVΔCP-SP-Xyn11) to target the recombinant protein to the export pathway and accumulation in apoplastic space[9], and the former with a carboxy-terminal peptide for arabinogalactan glycosylation (TMVΔCP-SP-Xyn11-AG) (**Fig. 1; Fig. S2**). We chose the signal peptide of the *N. tabacum* (1-3)- β-endoglucanase for its reported efficient apoplast targeting in *N. benthamiana*[30]. We also observed the effect of fusing an arabinogalactan-protein module to the carboxy-terminal end, due to reported beneficial effects on protein accumulation in tobacco cells[31]. The resulting plasmids (pTMVΔCP-Xyn11, pTMVΔCP-SP-Xyn11 and pTMVΔCP-SP-Xyn11-AG) were used to electroporate *A. tumefaciens*. The cultures of transformed *A. tumefaciens* clones were used to infiltrate leaves of *N.*

benthamiana plants. The infiltrated tissues were harvested 7 days post-inoculation (dpi). Protein extracts from whole *N. benthamiana* tissues or from apoplastic liquid were analyzed by denaturing polyacrylamide gel electrophoresis (PAGE), and the gels were stained with Coomassie brilliant blue.

Fig 1. Schematic representation of TMV-derived viral vectors to express different forms of xylanase Xyn11. Boxes represent viral 5' and 3' untranslated regions (5' and 3' UTR), RNA-dependent RNA polymerase (RdRp), movement protein (MP), coat protein (CP), xylanase Xyn11 (Xyn11), (1-3)-β-endoglucanase signal peptide (SP) and arabinogalactan (AG) glycosylation module.

The results demonstrated an excellent accumulation of Xyn11 in the apoplastic fluid of the infiltrated leaves at 7 dpi. Samples from the tissues inoculated with TMVΔCP-Xyn11 showed an intracellular accumulation of a protein whose migration, slightly above the 32 kDa marker, matched that expected for the recombinant protein (**Fig. 2**, lanes 3 to 5). The corresponding band was absent in a mock-inoculated control (**Fig. 2**, lane 2). The same band, exhibiting an extraordinary intensity, was also observed in the samples corresponding to the apoplastic liquid of leaves infiltrated with TMVΔCP-SP-Xyn11 (**Fig. 2**, lanes 6 to 8, red arrow). No intense band was observed in the apoplast samples from the leaves infiltrated with TMVΔCP-SP-Xyn11-AG (**Fig. 2**, lanes 9 to 11). A band with such an intensity was also absent in the apoplastic control from a mock-

inoculated plant (**Fig. 2**, lane 12). Taken together, these results support that the expression of Xyn11 with an export signal peptide in *N. benthamiana* using the TMVΔCP-SP-Xyn11 vector may be an excellent alternative to *E. coli* for the recombinant production of this enzyme of industrial interest.

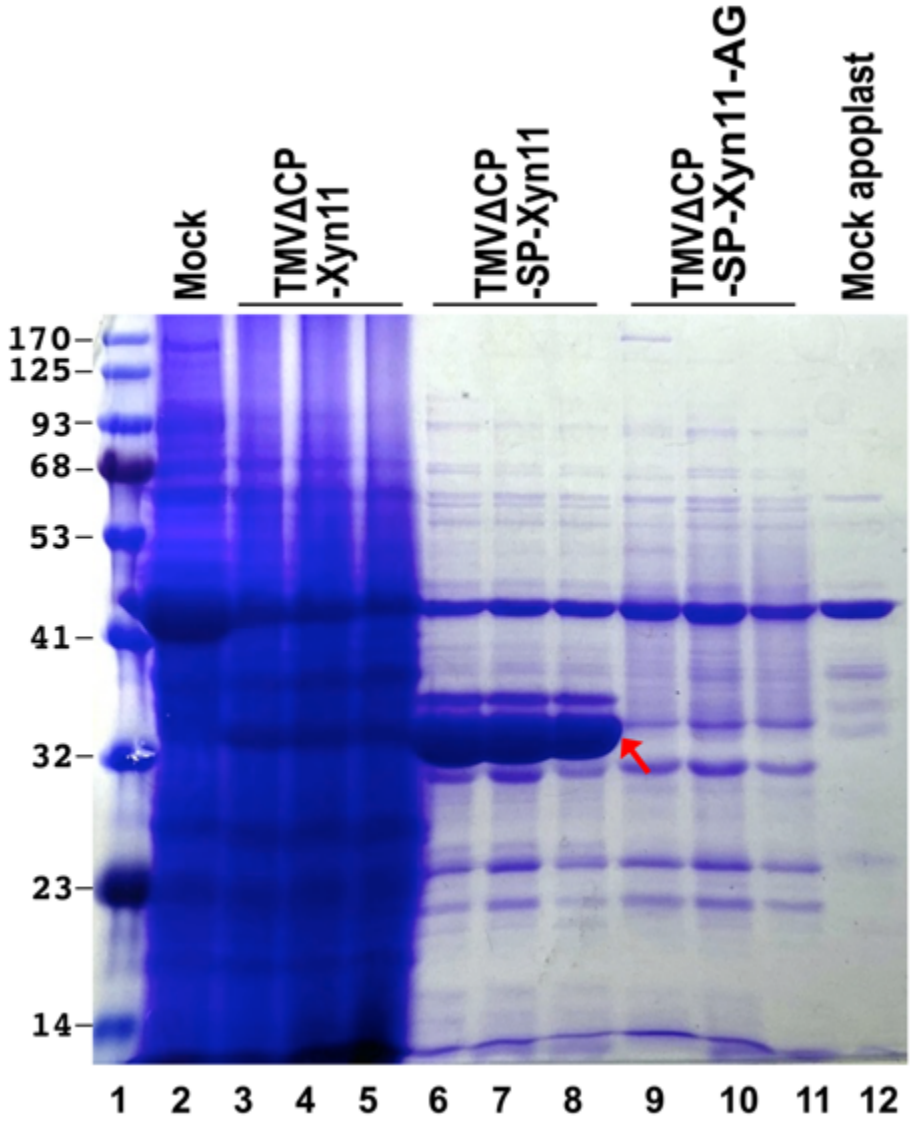

Fig 2. Electrophoretic analysis of protein extracts from *N. benthamiana* leaves infiltrated with different viral vectors. Leaves were harvested at 7 dpi and protein extracts were prepared from whole tissues or after recovery of apoplastic liquid. Proteins were separated by SDS-PAGE, and the gel was stained with Coomassie brilliant blue. Lane 1, marker proteins (BlueStar prestained protein marker, Nippon Genetics) with size in kDa on the left; lanes 2 to 5, total proteins from a

mock-inoculated plant (lane 2) or three independent plants inoculated with TMV-Xyn11 (lane 3 to 5); lanes 6 to 12, proteins from apoplastic liquid recovered from three plants inoculated with TMV-SP-Xyn11 (lanes 6 to 8), with TMV-SP-Xyn11-AG (lanes 9 to 11), or one plant mock-inoculated (lane 12). Position of recombinant xylanase Xyn11 is indicated by a red arrow.

Harvest time and the effect of an RNA silencing suppressor, namely tomato bushy stunt virus (TBSV) p19, were analyzed in the case of the TMVΔCP-SP-Xyn11 vector. Analyses confirmed 7 dpi as an optimum harvest time of plant tissues and also indicated a positive effect of p19 co-expression on Xyn11 accumulation (**Fig. S3**). Based on denaturing PAGE analysis with bovine serum albumin (BSA) standards followed by Coomassie brilliant blue staining, Xyn11 yield was estimated 0.68 ± 0.01 and 0.83 ± 0.04 µg/µl apoplastic fluid without and with p19 co-expression, respectively (**Fig. S4**). Considering 1 ml of apoplstic fluid is approximately recovered per 1 g of leaf tissue (fresh weight), these correspond to 0.68 ± 0.01 and 0.83 ± 0.04 µg Xyn11 per 1 mg *N. benthamiana* leaf tissue.

Comparative analysis of Xyn11 produced in *E. coli* and *N. benthamiana*. Xylanase Xyn11 is produced in *E. coli* with a high yield: up to 20 mg of protein per liter of culture can be recovered[25]. The thermoresistant nature of the protein makes purification easy to a high degree by subjecting the bacterial cell extract to heating for several minutes. The electrophoretic analysis of Xyn11 protein produced in *N. benthamiana* and *E. coli* (hereafter Xyn11_Nb and Xyn11_Ec, respectively; **Fig. 3**) reveals differences in the molecular mass due to differences in the genetic constructs used for expression in these two organisms. The protein produced in the bacteria contains an N-terminal extension of 20 residues, which includes a poly-His tail added to the sequence to facilitate the purification of the protein by Ni affinity chromatography, and a C-terminal extension of 9 residues

from the cloning vector[25] (**Fig. S1**). The molecular masses corresponding to the amino acid composition of Xyn11_Nb and Xyn11_Ec are 40.02 and 43.17 kDa respectively, which fits well with the migration of the proteins by SDS-PAGE (**Fig. 3**, lanes 7 and 8). This result suggests that, in *N. benthamiana*, Xyn11 is not subjected to post-translational modifications, or at least these are not significant enough to change the electrophoretic migration.

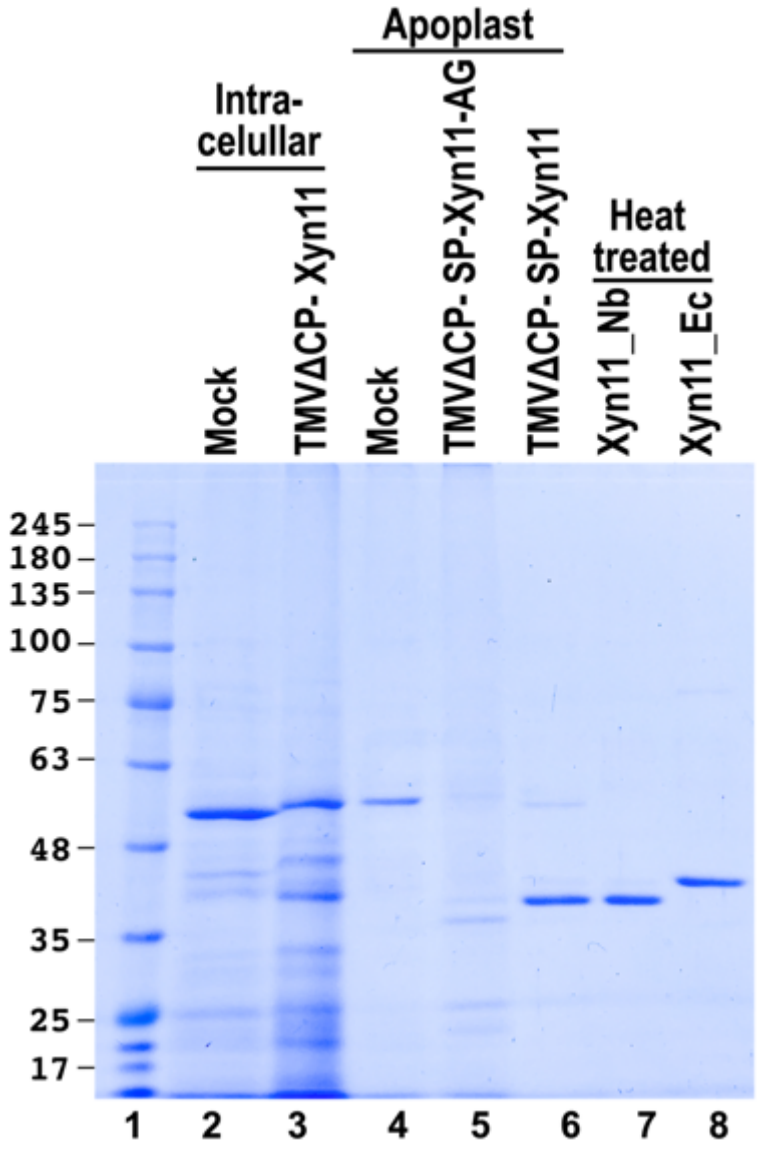

Fig. 3. Comparative analysis of recombinant xylanases produced in *N. benthamiana* and *E. coli*. Lane 1, protein markers with size in kDa on the left; lanes 2 and 3, intracellular proteins from *N. benthamiana* mock-inoculated (lane 2) and inoculated with TMVΔCP-Xyn11 (lane 3); lanes 4 to 6, apoplastic proteins

from *N. benthamiana* mock inoculated (lane 4) and inoculated with TMVΔCP-SP-Xyn11-AG (lane 5) and TMVΔCP-SP-Xyn11 (lane 6). Lanes 7 and 8, heat-treated preparations of xylanase Xyn11 purified from *N. benthamiana* apoplast (Xyn11_Nb) and *E. coli* (Xyn11_Ec).

The enzymatic (xylanolytic) activity of the proteins produced in the two hosts was compared under different conditions of pH and temperature. Assays at different pH, carried out at 90ºC (**Fig. 4A**) showed that that Xyn11_Nb and Xyn11_Ec have rather similar behavior at different pH values. Both enzymes exhibited high activity in alkaline conditions (pH 7.0 to 10.5) and lower activity in acidic conditions (pH 5.0). Remarkably, there are significant differences at pH levels of 6.0 and 10.5. At pH 6.0, Xyn11_Nb doubles the activity of Xyn11_Ec, whereas at pH 10.5, Xyn11_Ec activity is significantly higher (ca. 15%) than Xyn11_Nb. These differences may be due to the presence of supernumerary ionizable residues at the N-terminal and C-terminal regions of Xyn11_Ec, which are not present in Xyn11_Nb. These might influence the protein structure, changing the stability and activity of the enzyme. Assays at different temperatures, carried out a pH 9.0 (**Fig. 4B**) were equivalent for both enzymes, except at the highest temperature (90ºC) where Xyn11_Nb showed higher activity than Xyn11_Ec, possibly also because of the aforementioned differences related to the presence of ionizable residues at the termini of the Xyn11_Ec sequence. Further analysis was carried out to explain the pH-dependent variation of activity observed for the two enzymes (**Fig. 5**). Differences in the protein structures of Xyn11_Nb and Xyn11_Ec, and particularly differences in acidic (blue) and basic (red) ionizable residues (**Fig. 5A**), must account for significant differences in activity at some pH values. We analyzed the interdependence of the three variables, activity, pH and protein net charge (**Table S1**). Results showed that activity increases at negative values of net charge, being low at relatively high values of positive net charge

(Fig. 5B). This may explain the observed difference at pH 6.0 (Fig. 4A), likely due to the presence of the poly-His tail at the N-terminus of Xyn11_Ec.

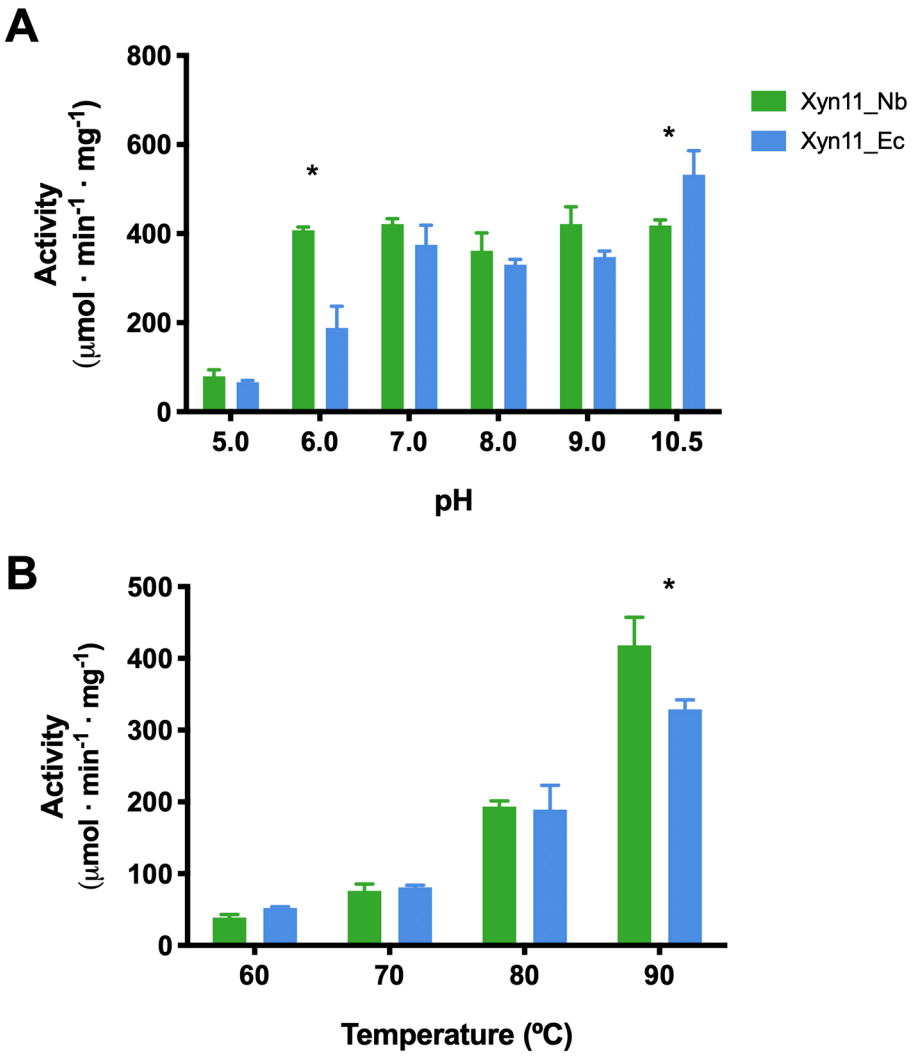

Fig. 4. Comparative xylanolytic activity of the xylanase Xyn11 produced in *N. benthamiana* (Xyn11_Nb) and *E. coli* (Xyn11_Ec). Activity was determined at different values of (A) pH or (B) temperature, as indicated. Histograms represent the average of triplicate measurements. Error bars represent standard deviation. Conditions of pH or temperature in which both recombinant enzymes exhibit a catalytic significant difference ($p<0.01$) are indicated by asterisks.

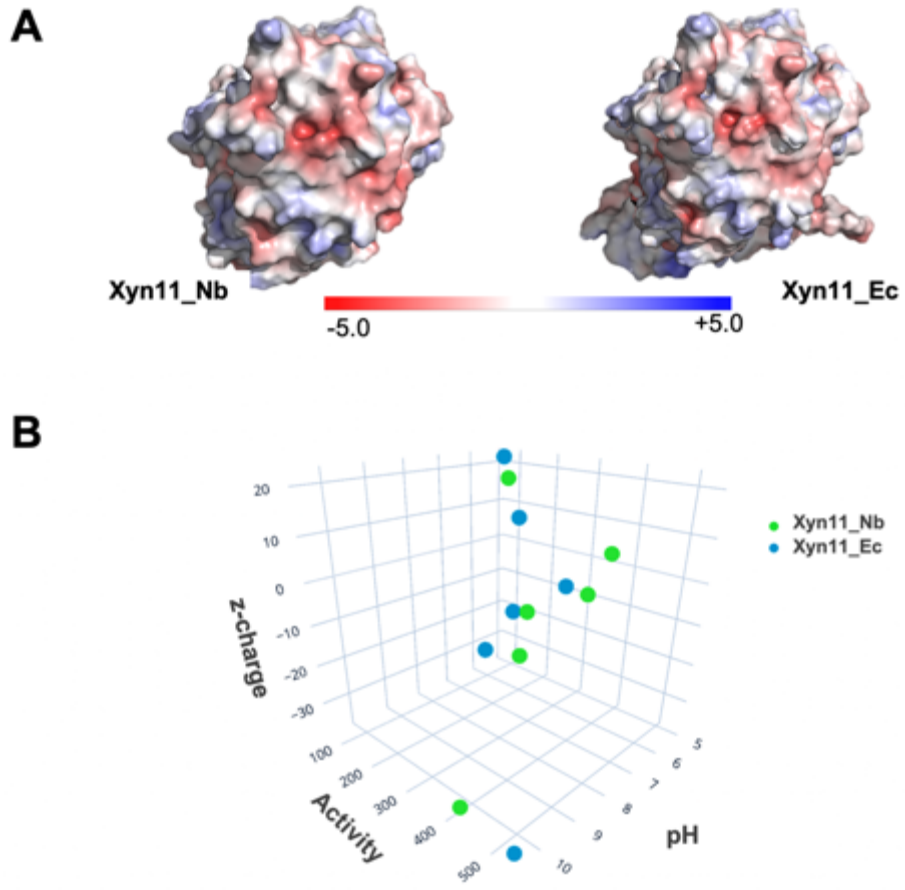

Fig. 5. Comparative structure and pH dependent activity of the xylanase Xyn11 produced in *N. benthamiana* (Xyn11_Nb) and *E. coli* (Xyn11_Ec). (**A**) Charged surface potential representation of models of the two proteins based on the crystallographic structure of Xyn11 (PDB id: 7NL2), calculated using APBS electrostatic software (https://www.poissonboltzmann.org) and rendered with Pymol 2.3.4 (https://pymol.org/). (**B**) 3D scatter plot representation of xylanase activity and protein net charge (Z) as a function of pH variation.

Overall, the two enzymes display excellent activity profiles at high temperatures and an alkaline pH. The structural stability of the enzyme is an important asset from an industrial point of view, as it implies an extended lifetime. In addition, enzyme operation under extreme conditions drastically reduces the risk of microbial contamination. The enzyme version produced in

plants shows two important additional advantages. Being secreted to the apoplast, it can be easily recovered without cell disruption, which is required to extract the enzyme produced intracellularly in *E. coli*. Furthermore, the use of a plant host is preferable to *E. coli*, particularly for food-related biotechnological applications, when considering regulatory aspects and consumer perceptions.

Use of Xyn11 for the production of XOS. Production of XOS from beechwood xylan was studied with Xyn11_Ec and Xyn11_Nb at pH 9.0 and at 90°C (reaction kinetics reported in **Fig. S5**). The chromatographic profile of the products recovered after 2 h of reaction showed equivalent result for both enzymes (**Fig. 6**). Xylose and XOS (2 to 6 units) were recovered in accordance with the endo-acting mechanism of the enzyme[25]. The chromatographic profiles of the reaction products obtained with both enzymes showed relatively higher abundances of xylobiose and xylotriose (370 mg and 150 mg XOS per gram beechwood xylan, respectively), which is important since these compounds have higher prebiotic activity whereas the presence of xylose is undesirable[32]. Xylan was largely converted into 2 to 6 unit XOS (830 mg XOS/g of xylan).

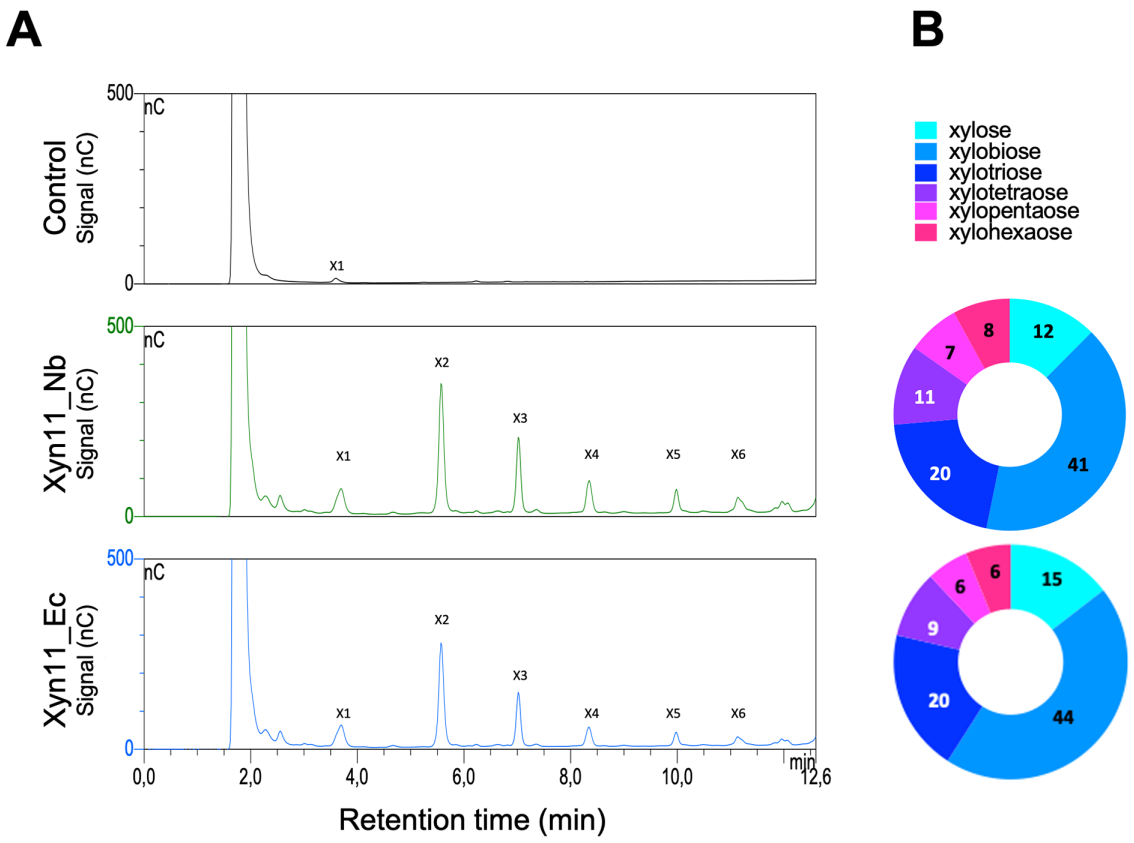

Fig. 6. Enzymatic production of XOS. (**A**) Chromatographic profile of XOS obtained by hydrolysis of xylan using Xyn11 produced in *N. benthamiana* (Xyn11_Nb) or *E. coli* (Xyn11_Ec). (**B**) Percentage of xylose and XOS obtained after enzymatic reaction with Xyn11_Nb and Xyn11_Ec.

Discussion

In this work, a combination of a TMV-derived vector[9] and *N. benthamiana* as a host plant, as well as the addition of the efficient signal peptide derived from *N. tabacum* (1-3)-β-endoglucanase[30] that targets the recombinant protein to the apoplast, results in a remarkable accumulation of the enzyme Xyn11 in the extracellular space of plant tissues (**Fig. 2**). Interestingly, this production yield, in terms of the mass of *N. benthamiana* leaves or liters of *E. coli* culture to be processed can be considered comparable (**Fig. 3**). More specifically, in our

experimental conditions for recombinant Xyn11, we estimated 20 mg per liter of *E. coli* culture and 830 mg per kg (fresh weight) of *N. benthamiana* leaves.

To date, the production of bacterial xylanases in plants for further purification and industrial use has not been largely explored. Most efforts have been directed to the expression of glycosyl hydrolases for *in situ* plant cell wall degradation and biofuel production[33–35]. Plant virus-derived vectors have been used for this purpose. Endoglucanase E1 and xylanase Xyn10A from *Acidothermus cellulolyticus* were transiently expressed in sunflower leaves using vacuum infiltration of *A. tumefaciens* cultures[36]. Three types of constructs were assayed, two of them consisting of viral vectors derived from cucumber mosaic virus (genus *Cucumomirus*, family *Bromoviridae*) and TMV. In contrast to our results, this work reported that virus-driven production of the cell wall-degrading enzymes was unsuccessful. Efficient expression was achieved using a cauliflower mosaic virus (CaMV) 35S promoter, and an improvement was accomplished by using methyl jasmonic acid in the agroinfiltration buffer and by optimizing the leaf incubation temperature[36]. A pepper mottle virus (genus *Potyvirus*) vector was also used to produce the endoglucanase D from *Clostridium cellulovorans* in *N. benthamiana*[37]. This work shows the feasibility of using plant virus vectors to systemically express bacterial enzymes to digest the cellulose of plant cells for biomass production. The production yield was much lower than reported here, which is likely related to the different capacities to produce heterologous proteins from tobamovirus and potyvirus vectors. Notably, a thermostable xylanase was efficiently produced in the chloroplasts of *N. tabacum* with no impact on photosynthetic performance of transplastomic plants[38]. Multi-organelle targeting was also used to improve xylanase accumulation in plant cells[39].

As we show in this report, the enzymatic properties of Xyn11_Nb and Xyn11_Ec are similar, particularly those related to the activity under the high temperature and pH conditions required in industrial applications (**Fig. 4**) and the specific products of reaction (**Fig. 6**). However, the production of Xyn11 in plants presents a series of advantages. First, Xyn11_Nb can be tagged as a "green" product that may be a preferred option for many consumers. Second, scaling production in plants to reach the demand peaks is easier and cheaper, compared to processes based on *E. coli* fermentation. Third, the export-to-the-apoplast strategy followed by heat treatment (**Figs. 2** and **3**) avoids cumbersome chromatographic purification of cellular extracts. This also facilitates the expression of the native amino acid sequence with no extra tags for protein purification. These tags, such as the His_6 and linkers used in Xyn11_Ec, may modify enzymatic properties and stability **(Fig. 4**). Post-purification tag removal, although possible, is undesirable due to the additional processing and production costs.

Xyn11_Nb produced 2 to 6 unit XOS, yielding up to 830 mg per gram of beechwood xylan. This yield is higher than that previously reported[19], using a crude xylanase extract from *Aureobasidium pullulans* (170.8 ± 4.0 mg XOS/g xylan). It is also higher than that reported for a thermostable xylanase from the fungus *Myceliophthora heterothallica* (234.2 mg XOS/g beechwood xylan)[40]. A yield equivalent to what we report in this communication were obtained with a commercial enzymatic cocktail[41], and with an extremophilic xylanase from *Thermotoga thermarum*[42]. In addition to their prebiotic function, XOS have other properties that make them suitable food ingredients. For example, they can be added to cheese, giving it the characteristics of a full-fat product. They also have cryoprotective properties due to their interaction with proteins that prevent denaturation during frozen storage[43]. Xyn11 high yield production in the plant

system together with the enzyme properties represent important assets for eventual uses in the food industry.

In conclusion, xylanase Xyn11 is efficiently produced in *N. benthamiana* plants using TMV as the expression vector. The purification of the recombinant protein produced in plants is highly facilitated by targeting the enzyme to the apoplast. The plant-produced Xyn11 showed excellent catalytic activity in alkaline pH and high temperature conditions, similar to the counterpart version produced in *E. coli*. However, in contrast to the bacterial version of Xyn11, the plant enzyme shows significantly higher activity at acidic pH. Remarkably, the plant enzyme exhibits higher catalytic activity than the bacterial counterpart at 90°C and pH 9.0, which are favorable reaction conditions for industrial enzymatic xylan hydrolysis. Based on the price-effective scalability of recombinant protein production in plants and the current demand of plant-derived (green) products in food industry and other sectors, Xyn11_Nb represents an attractive alternative for enzymatic xylan hydrolysis in industrial processes.

Materials and methods

Plasmid construction for xylanase expression in *N. benthamiana*. The DNA sequence encoding Xyn11 (GenBank accession no. AEH51686.1) was edited to optimize codon usage in *N. benthamiana* (**Fig. S1**) and obtained by gene synthesis (IDT). cDNAs corresponding to Xyn11 and derivatives with N-terminal signal peptide (SP-Xyn11) and a C-terminal peptide module for arabinogalactan glycosylation (SP-Xyn11-AG) (**Fig. S2**) were amplified by the polymerase chain reaction (PCR) using the Phusion high-fidelity DNA polymerase in HF buffer (Thermo Scientific) and specific primers (**Table S2**). Reaction conditions consisted of an initial denaturation at 98°C for 30 s, followed by 30 cycles of 10 s at 98°C,

30 s at 55°C and 1 min at 72°C, and a final extension at 72°C for 10 min. cDNAs were inserted into the plasmid pMTMVi-N[9], opened by digestion with *Age*I and *Xho*I (Thermo Scientific). The plasmid assembly was performed using the NEBuilder HiFi DNA assembly master mix (New England Biolabs). *Bsa*I fragments from the resulting plasmids were assembled into pGTMV (containing a TMV infectious clone, GenBank accession number MK087763.1) digested with *Nco*I and *Pfl*23II (Thermo Scientific)[9] to build pGTMVΔCP-Xyn11, pGTMVΔCP-SP-Xyn11 and pGTMVΔCP-SP-Xyn11-AG (**Fig. 1** and **Fig. S2**).

Agroinoculation of *N. benthamiana* leaves. The strain GV3101:pMP90 of *A. tumefaciens*, which harbors the helper plasmid pCLEAN-S48[44], was electroporated with the plasmids pGTMVΔCP-Xyn11, pGTMVΔCP-SP-Xyn11, and pGTMVΔCP-SP-Xyn11-AG. The transformed colonies were selected in plates with a lysogenic broth (LB) medium containing 50 µg/ml rifampicin, 50 µg/ml kanamycin, and 7.5 µg/ml tetracycline. Liquid cultures were grown for 24 h at 28°C in the same medium. Bacteria were recovered via centrifugation and brought to an optical density at 600 nm of 0.5 in agroinoculation solution (10 mM MES-NaOH, pH 5.6, 10 mM $MgCl_2$ and 150 µM acetosyringone). The cultures were incubated for 2 h at 28°C to induce the virulence genes, and used to infiltrate the leaves of 5-week old *N. benthamiana* plants. *A. tumefaciens* cultures were infiltrated at the abaxial side of the leaves using a needleless 1-mL syringe. The infiltrated plants were cultured in a growth chamber at 25°C with a 12-h day-night photoperiod. In some experiments, leaves were co-agroinfiltrated with 1:1 mixes of *A. tumefaciens* transformed with pGTMVΔCP-SP-Xyn11 and a plasmid to express TBSV p19 RNA silencing suppressor under the control of CaMV 35S promoter and terminator or the corresponding empty plasmid. Each *A. tumefaciens* culture was brought to an optical density of 1.0 and induced as stated above, and mixed 1:1 previously to infiltration. Experimental research on *N. benthamiana* complied with relevant institutional,

national, and international guidelines and legislation, and permissions were obtained.

Production and purification of Xyn11 in *E. coli*. The Xyn11 sequence was identified in a computational analysis, where xylanases active under extreme conditions of temperature and alkaline pH were searched for. A synthetic DNA fragment coding for the protein sequence, optimized for translation in *E. coli*, was cloned in an expression vector as previously described[25]. Cell extracts from the *E. coli* cultures expressing a His-tagged version of Xyn11, prepared in 20 mM phosphate buffer, pH 7.4, 10 mM imidazole, 500 mM NaCl, were heated at 85°C for 10 min, which caused the precipitation of most of the proteins, leaving the xylanase in a clarified supernatant after centrifugation. The enzyme preparation was subjected to Ni-affinity chromatography in a 1 mL HisTrap FF column (Cytiva) mounted in an AKTA-Purifier (Cytiva). Elution was carried out with a 20 mM phosphate buffer, pH 7.4, 500 mM imidazole, 500 mM NaCl. This version of the enzyme was termed Xyn11_Ec.

Purification of Xyn11 from *N. benthamiana* leaves. Infiltrated tissues from *N. benthamiana* plants were harvested at different dpi, as indicated. For total protein extraction, tissues were ground with a mortar and pestle in the presence of liquid N_2. Three volumes of the buffer TEW (60 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulphate [SDS], 100 mM dithiothreitol, 10% glycerol and 0.01% bromophenol blue) were added. The extracts were incubated for 5 min at 95 °C and clarified by centrifugation for 15 min. For apoplastic liquid recovery, the tissues were first vacuum-infiltrated with phosphate buffered saline (PBS) buffer supplemented with 0.02% (v/v) Silwet L-77, then introduced in a 50 mL syringe and squeezed. Finally, the apoplastic fractions were clarified by centrifugation for 5 min at 13,000 rpm. This version of the enzyme was termed Xyn11_Nb.

Enzyme analysis. The activity of the purified xylanases (Xyn11_Ec and Xyn11_Nb) was assayed at a range of high temperatures and alkaline pH. Enzymatic reactions at different temperatures (60, 70, 80, or 90ºC) were carried out by mixing 180 µL beechwood xylan 1% (Megazyme) with 20 µL of the enzyme in a Tris-HCl buffer (50 mM) at a pH of 9.0. The enzyme concentration was adjusted to assure a lineal response. Analyses at different pH values were carried out at 90ºC, using 50 mM buffered solutions at the following pH: 5.0 (acetate), 6.0 and 7.0 (phosphate), 8.0, 9.0 (Tris-HCl), and 10.5 (CAPS). Reactions were terminated by placing the tubes on ice. To measure sugar reduction resulting from xylan digestion, 100 µL of dinitro salicylic acid (DNS) solution was added to the reaction tubes, which were subsequently boiled for 10 min. After boiling, 900 µL of miliQ H_2O was added, and the tubes were centrifuged. A fraction of the supernatant (300 µL) from each reaction tube was transferred to a 96-well plate, and optical density at 540 nm (OD_{540}) was measured using a PowerWave HT spectrophotometer, from BioTek Instruments (Winooski, VT, USA). Statistical analysis was performed by two-way ANOVA, using a p-value <0.001.

Xylan digestion and XOS analysis. The production of XOS was carried out using as a substrate commercial beechwood xylan (Megazyme) at a 1% concentration in a 50 mM Tris-HCl buffer, pH 9.0. Xylanases, Xyn11_Ec or Xyn11_Nb, were added at 0.75 µg/mL and incubated at 90ºC for different times. The reactions were stopped by placing the tubes on ice. XOS were analyzed by exchange ion chromatography, using a DIONEX instrument equipped with a CarboPac PA100 column and a pulsed amperometric detector (Dionex, Thermo Fisher Scientific). Xylose (Sigma-Aldrich) and xylooligosaccharides, from two to six units (Megazyme), were used as the chromatographic standards.

Acknowledgements

This work was supported by grant PID2020-114691RB-I00 from the Spanish Ministerio de Ciencia e Innovación, through the Agencia Estatal de Investigación (co-financed European Regional Development Fund), and by the Bio Based Industries Joint Undertaking, under the European Union's Horizon 2020 research and innovation program (Project WOODZYMES, Grant Agreement H2020-BBI-JU-792070). M.N.-S. is the recipient of a predoctoral contract from the Spanish Ministerio de Ciencia e Innovación (PRE2018-084771).

Competing Interests

The authors declare no competing interests.

Data availability statement

Relevant DNA sequences handled in this work are deposited in GenBank under accession numbers AEH51686.1 and MK087763.1.

Additional information

Supplementary Information. Supplementary figures S1, S2 and S3, S4 and S5, and supplementary Tables S1 and S2 are included in a supplementary file.

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Supplementary Information

Fig. S1. Nucleotide and amino acid sequences of the xylanase Xyn11 versions expressed in *Escherichia coli* (Xyn11_Ec) and *Nicotiana benthamiana* (Xyn11_Nb).

>Xyn11_Ec

ATGAGAGGATCGCATCACCATCACCATCACGGATCCGCATGCGAGCTCGGGCCTCAGCCGATGTCGTCGC
AAATCCCCTCACTGAAGGATGTCTTTCTTCAAGATTTCAAAATCGGCGTGGCACTGCCGGTACGCGTCTT
CTCTAATTCCATGGATGTTGAGTTAATTACCAAACATTTCAATTCGATGACAGCGGAAAATGAGATGAAG
CCCGAATCGATTTTACGTCGTGATGCCTCGGGGAAGATTTATTATGACTTCACCGTTGCTGACCGCTACA
TCGAATTTGCACAGAAGCATGGCATGGTGGTACGCGGACACACATTAGTTTGGCACTCCCAAACACCGGA
ATGGTTTTTCAAAGATGAAAAGGGCAATTTATTGTCGCGTGAAGCAATGATTGAACGTATGCGCGAATAC
ATCCACACAGTAGTAGGCCGCTACCGTGGGAAAGTTTACGCTTGGGACGTCGTTAATGAAGCCGTGGACG
AAAACCAGCCAGACGGACTGCGTCGTTCGTTATGGTATCAGGTTATCGGCCCAGATTATATTGAGCTTGC
ATTTAAGTTTGCGCATGAGGCTGACCCAGATGCATTGTTATTTTACAACGACTATAATGAATTCTTTCCG
AAAAAACGTGACATCATCTTTAAGTTGGTTAAGGAGATGCGCGAAAAGGGCGTCCCAATTCATGGGATCG
GAATGCAACAGCATCTTACACTTGCGGACAACGTGGGGTGGATCGATATCGCAATCCAAAAATTTAAGAC
TATCAGTGGGATCCAAATCCATATTACGGAACTTGACGTATCTGTCTACAAGAGTCGTTCTCCAAGCATT
ATTTATCAGACACCTCCACTTGAAGTGCTTAAAGAACAAGCGGAATTTTATCGTAAACTTTTTGAGATTT
ATCGCAAGCACACCGACGTAATTACCAATGTTACATTTTGGGGGTTGAAGGACGACTACAGCTGGTTGCG
TTTCTTTTTTGGCCGTCGCAACGACTGGCCGCTGCTTTTTGACGAGAATTATCAACCGAAACCTGCGTTT
TGGAGTGTTATCGAATCAGTAAGCAAGGTCGACCTGCAGCCAAGCTTAATTAGCTGA

>Xyn11_Ec

MRGSHHHHHHGSACELGPQPMSSQIPSLKDVFLQDFKIGVALPVRVFSNSMDVELITKHFNSMTAENEMK
PESILRRDASGKIYYDFTVADRYIEFAQKHGMVVRGHTLVWHSQTPEWFFKDEKGNLLSREAMIERMREY
IHTVVGRYRGKVYAWDVVNEAVDENQPDGLRRSLWYQVIGPDYIELAFKFAHEADPDALLFYNDYNEFFP
KKRDIIFKLVKEMREKGVPIHGIGMQQHLTLADNVGWIDIAIQKFKTISGIQIHITELDVSVYKSRSPSI
IYQTPPLEVLKEQAEFYRKLFEIYRKHTDVITNVTFWGLKDDYSWLRFFFGRRNDWPLLFDENYQPKPAF
WSVIESVSKVDLQPSLIS*

Original **Xyn11** sequence in black, **His$_6$** tag from pQE80L (*E. coli* expression vector) in red, and **other amino acids**, besides the His$_6$ tag, from pQE80L in blue.

>Xyn11_Nb

ATGAGTTCTCAAATACCTTCTTTAAAAGATGTGTTCCTTCAGGATTTTAAGATCGGAGTCGCCCTGCCCG
TGCGAGTCTTCTCTAACTCAATGGACGTCGAGCTGATAACGAAACATTTTAACAGCATGACGGCAGAGAA
CGAGATGAAACCTGAGAGTATTCTGAGGCGAGACGCAAGTGGAAAGATATATTACGACTTTACAGTCGCT
GATAGGTATATTGAGTTTGCTCAGAAACATGGCATGGTCGTTCGAGGCCATACCCTGGTCTGGCATTCAC
AGACGCCCGAATGGTTTTTTAAAGATGAAAAGGGTAACTTGTTGTCACGAGAAGCTATGATTGAGAGGAT
GCGTGAATATATTCATACCGTTGTCGGTAGATACAGGGGTAAAGTATACGCCTGGGATGTTGTCAATGAA
GCAGTTGATGAAAATCAACCAGATGGGTTAAGAAGGTCCCTTTGGTATCAGGTAATCGGGCCCGACTACA
TTGAGTTGGCCTTTAAATTCGCTCACGAAGCTGACCCTGATGCACTGCTTTTCTATAACGACTACAATGA
ATTCTTCCCGAAAAAGAGAGATATCATATTTAAGCTTGTTAAAGAAATGAGGGAAAAGGGGGTGCCAATA
CATGGTATTGGAATGCAGCAGCACTTGACACTTGCTGATAACGTAGGTTGGATTGACATAGCCATTCAGA
AATTTAAAACGATCAGTGGCATCCAGATTCATATAACAGAACTGGATGTATCAGTCTACAAAAGCCGTTC
TCCAAGTATTATATACCAGACCCCGCCTTTAGAAGTTTTGAAAGAACAAGCCGAATTTTATCGAAAGTTA
TTCGAGATTTACAGGAAGCATACGGACGTCATCACCAATGTGACGTTCTGGGGATTGAAGGACGACTACA

GCTGGTTGAGATTCTTCTTTGGCAGAAGAAATGATTGGCCCCTGTTGTTCGACGAGAACTATCAGCCTAA
GCCGGCTTTTTGGTCCGTCATAGAGTCTGTATCAAAATGA

>Xyn11_Nb
MSSQIPSLKDVFLQDFKIGVALPVRVFSNSMDVELITKHFNSMTAENEMKPESILRRDASGKIYYDFTVA
DRYIEFAQKHGMVVRGHTLVWHSQTPEWFFKDEKGNLLSREAMIERMREYIHTVVGRYRGKVYAWDVVNE
AVDENQPDGLRRSLWYQVIGPDYIELAFKFAHEADPDALLFYNDYNEFFPKKRDIIFKLVKEMREKGVPI
HGIGMQQHLTLADNVGWIDIAIQKFKTISGIQIHITELDVSVYKSRSPSIIYQTPPLEVLKEQAEFYRKL
FEIYRKHTDVITNVTFWGLKDDYSWLRFFFGRRNDWPLLFDENYQPKPAFWSVIESVSK*

Fig. S2. Sequence of TMV recombinant clones to express different versions of xylanase Xyn11 in *N. benthamiana* (Xyn11_Nb). Vectors were built on the basis of a TMV infectious variant (GenBank accession no. MK087763.1) with deletion of most of CP gene and mutation of viral CP initiation ATG to AGA (in red). Nucleotide sequence of Xyn11_Nb is in blue, (1-3)-β-endoglucanase signal peptide (SP) in purple, and the arabinogalactan (AG) glycosylation module in gold colors.

>TMVΔCP-Xyn11
GTATTTTTACAACAATTACCAACAACAACAAACAACAAACAACATTACAATTACTATTTACAATTACAAT
GGCATACACACAGACAGCTACCACATCAGCTTTGCTGGACACTGTCCGAGGAAACAACTCCTTGGTCAAT
GATCTAGCAAAGCGTCGTCTTTACGACACAGCGGTTGAAGAGTTTAACGCTCGTGACCGCAGGCCCAAGG
TGAACTTTTCAAAAGTAATAAGCGAGGAGCAGACGCTTATTGCTACCCGGGCGTATCCAGAATTCCAAAT
TACATTTTATAACACGCAAAATGCCGTGCATTCGCTTGCAGGTGGATTGCGATCTTTAGAACTGGAATAT
CTGATGATGCAAATTCCCTACGGATCATTGACTTATGACATAGGCGGGAATTTTGCATCGCATCTGTTCA
AGGGACGAGCATATGTACACTGCTGCATGCCCAACCTGGACGTTCGAGACATCATGCGGCACGAAGGCCA
GAAAGACAGTATTGAACTATACCTTTCTAGGCTAGAGAGAGGGGGGAAAACAGTCCCCAACTTCCAAAAG
GAAGCATTTGACAGATACGCAGAAATTCCTGAAGACGCTGTCTGTCACAATACTTTCCAGACATGCGAAC
ATCAGCCGATGCAGCAATCAGGCAGAGTGTATGCCATTGCGCTACACAGCATATATGACATACCAGCCGA
TGAGTTCGGGGCGGCACTCTTGAGGAAAAATGTCCATACGTGCTATGCCGCTTTCCACTTCTCCGAGAAC
CTGCTTCTTGAAGATTCATGCGTCAATTTGGACGAAATCAACGCGTGTTTTTCGCGCGATGGAGACAAGT
TGACCTTTTCTTTTGCATCAGAGAGTACTCTTAATTACTGTCATAGTTATTCTAATATTCTTAAGTATGT
GTGCAAAACTTACTTCCCGGCCTCTAATAGAGAGGTTTACATGAAGGAGTTTTTAGTCACCAGAGTTAAT
ACCTGGTTTTGTAAGTTTTCTAGAATAGATACTTTTCTTTTGTACAAAGGTGTGGCCCATAAAAGTGTAG
ATAGTGAGCAGTTTTATACTGCAATGGAAGACGCATGGCATTACAAAAAGACTCTTGCAATGTGCAACAG
CGAGAGAATCCTCCTTGAGGATTCATCATCAGTCAATTACTGGTTTCCCAAAATGAGGGATATGGTCATC
GTACCATTATTCGACATTTCTTTGGAGACTAGTAAGAGGACGCGCAAGGAAGTCTTAGTGTCCAAGGATT
TCGTGTTTACAGTGCTTAACCACATTCGAACATACCAGGCGAAAGCTCTTACATACGCAAATGTTTTGTC
CTTCGTCGAATCGATTCGATCGAGGGTAATCATTAACGGTGTGACAGCGAGGTCCGAATGGGATGTGGAC
AAATCTTTGTTACAATCCTTGTCCATGACGTTTTACCTGCATACTAAGCTTGCCGTTCTAAAGGATGACT
TACTGATTAGCAAGTTTAGTCTCGGTTCGAAAACGGTGTGCCAGCATGTGTGGGATGAGATTTCGCTGGC
GTTTGGGAACGCATTTCCCTCCGTGAAAGAGAGGCTCTTGAACAGGAAACTTATCAGAGTGGCAGGCGAC
GCATTAGAGATCAGGGTGCCTGATCTATATGTGACCTTCCACGACAGATTAGTGACTGAGTACAAGGCCT
CTGTGGACATGCCTGCGCTTGACATTAGGAAGAAGATGGAAGAAACGGAAGTGATGTACAATGCACTTTC
AGAATTATCGGTGTTAAGGGAGTCTGACAAATTCGATGTTGATGTTTTTTCCCAGATGTGCCAATCTTTG
GAAGTTGACCCAATGACGGCAGCGAAGGTTATAGTCGCGGTCATGAGCAATGAGAGCGGTCTGACTCTCA
CATTTGAACGACCTACTGAGGCGAATGTTGCGCTAGCTTTACAGGATCAAGAGAAGGCTTCAGAAGGTGC
ATTGGTAGTTACCTCAAGAGAAGTTGAAGAACCGTCCATGAAGGGTTCGATGGCCAGAGGAGAGTTACAA
TTAGCTGGTCTTGCTGGAGATCATCCGGAGTCGTCCTATTCTAAGAACGAGGAGATAGAGTCTTTAGAGC
AGTTTCATATGGCGACGGCAGATTCGTTAATTCGTAAGCAGATGAGCTCGATTGTGTACACGGGTCCGAT
TAAAGTTCAGCAAATGAAAAACTTTATCGATAGCCTGGTAGCATCACTATCTGCTGCGGTGTCGAATCTC
GTCAAGATCCTCAAAGATACAGCTGCTATTGACCTTGAAACCCGTCAAAAGTTTGGAGTCTTGGATGTTG

CATCTAGGAAGTGGTTAATCAAACCAACGGCCAAGAGTCATGCATGGGGTGTTGTTGAAACCCACGCGAG
GAAGTATCATGTGGCGCTTTTGGAATATGATGAGCAGGGTGTGGTGACATGCGATGATTGGAGAAGAGTA
GCTGTTAGCTCTGAGTCTGTTGTTTATTCCGACATGGCGAAACTCAGAACTCTGCGCAGACTGCTTCGAA
ACGGAGAACCGCATGTCAGTAGCGCAAAGGTTGTTCTTGTGGACGGAGTTCCGGGCTGTGGAAAAACCAA
AGAAATTCTTTCCAGGGTTAATTTTGATGAAGATCTAATTTTAGTACCTGGGAAGCAAGCCGCGGAAATG
ATCAGAAGACGTGCGAATTCCTCAGGGATTATTGTGGCCACGAAGGACAACGTTAAAACCGTTGATTCTT
TCATGATGAATTTTGGGAAAAGCACACGCTGTCAGTTCAAGAGGTTATTCATTGATGAAGGGTTGATGTT
GCATACTGGTTGTGTTAATTTTCTTGTGGCGATGTCATTGTGCGAAATTGCATATGTTTACGGAGACACA
CAGCAGATTCCATACATCAATAGAGTTTCAGGATTCCCGTACCCCGCCCATTTTGCCAAATTGGAAGTTG
ACGAGGTGGAGACACGCAGAACTACTCTCCGTTGTCCAGCCGATGTCACACATTATCTGAACAGGAGATA
TGAGGGCTTTGTCATGAGCACTTCTTCGGTTAAAAAGTCTGTTTCGCAGGAGATGGTCGGCGGAGCCGCC
GTGATCAATCCGATCTCAAAACCCTTGCATGGCAAGATCCTGACTTTTACCCAATCGGATAAAGAAGCTC
TGCTTTCAAGAGGGTATTCAGATGTTCACACTGTGCATGAAGTGCAAGGCGAGACATACTCTGATGTTTC
ACTAGTTAGGTTAACCCCTACACCGGTCTCCATCATTGCAGGAGACAGCCCACATGTTTTGGTCGCATTG
TCAAGGCACACCTGTTCGCTCAAGTACTACACTGTTGTTATGGATCCTTTAGTTAGTATCATTAGAGATC
TAGAGAAACTTAGCTCGTACTTGTTAGATATGTATAAGGTCGATGCAGGAACACAATAGCAATTACAGAT
TGACTCGGTGTTCAAAGGTTCCAATCTTTTTGTTGCAGCGCCAAAGACTGGTGATATTTCTGATATGCAG
TTTTACTATGATAAGTGTCTCCCAGGCAACAGCACCATGATGAATAATTTTGATGCTGTTACCATGAGGT
TGACTGACATTTCATTGAATGTCAAAGATTGCATATTGGATATGTCTAAGTCTGTTGCTGCGCCTAAGGA
TCAAATCAAACCACTAATACCTATGGTACGAACGGCGGCAGAAATGCCACGCCAGACTGGACTATTGGAA
AATTTAGTGGCGATGATTAAAAGAAACTTTAACGCACCCGAGTTGTCTGGCATCATTGATATTGAAAATA
CTGCATCTTTGGTTGTAGATAAGTTTTTTGATAGTTATTTGCTTAAAGAAAAAAGAAAACCAAATAAAAA
TGTTTCTTTGTTCAGTAGAGAGTCTCTCAATAGATGGTTAGAAAAGCAGGAACAGGTAACAATAGGCCAG
CTCGCAGATTTTGATTTTGTGGATTTGCCAGCAGTTGATCAGTACAGACACATGATTAAAGCACAACCCA
AACAAAAGTTGGACACTTCAATCCAAACGGAGTACCCGGCTTTGCAGACGATTGTGTACCATTCAAAAAA
GATCAATGCAATATTCGGCCCGTTGTTTAGTGAGCTTACTAGGCAATTACTGGACAGTGTTGATTCGAGC
AGATTTTTGTTTTTCACAAGAAAGACACCAGCGCAGATTGAGGATTTCTTCGGAGATCTCGACAGTCATG
TGCCGATGGATGTCTTGGAGCTGGATATATCAAAATACGACAAATCTCAGAATGAATTCCACTGTGCAGT
AGAATACGAGATCTGGCGAAGATTGGGTTTTGAAGACTTCTTGGGAGAAGTTTGGAAACAAGGGCATAGA
AAGACCACCCTCAAGGATTATACCGCAGGTATAAAAACTTGCATCTGGTATCAAAGAAAGAGCGGGGACG
TCACGACGTTCATTGGAAACACTGTGATCATTGCTGCATGTTTGGCCTCGATGCTTCCGATGGAGAAAAT
AATCAAAGGAGCCTTTTGCGGTGACGATAGTCTGCTGTACTTTCCAAAGGGTTGTGAGTTTCCGGATGTG
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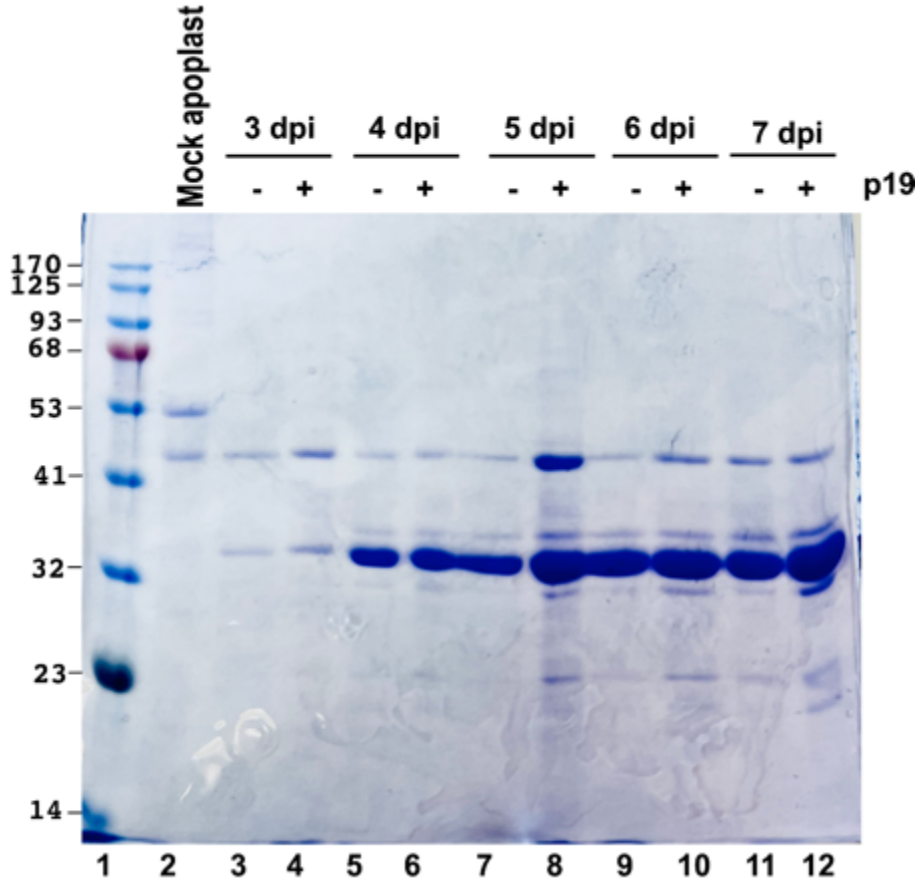

Fig. S3. Time course analysis of recombinant xylanase production in *N. benthamiana* with the viral vector TMVΔCP-SP-Xyn11 without and with co-expression of TBSV p19 RNA silencing suppressor, as indicated. Leaves were harvested from 3 to 7 dpi, as indicated, and apoplastic liquid recovered. Proteins in the apoplast were separated by SDS-PAGE, and the gel was stained with Coomassie brilliant blue. Lane 1, marker proteins with size in kDa on the left; lane 2, apoplast from a mock inoculated plant; lanes 3, 5, 7, 9 and 11, tissues harvested at 3, 4, 5, 6 and 7 dpi, respectively, from plants co-agroinoculated with TMVΔCP-SP-Xyn11 and an empty plasmid; lanes 4, 6, 8, 10 and 12, tissues harvested at 3, 4, 5, 6 and 7 dpi, respectively, from plants co-agroinoculated with TMVΔCP-SP-Xyn11 and a plasmid to express the RNA silencing suppressor p19.

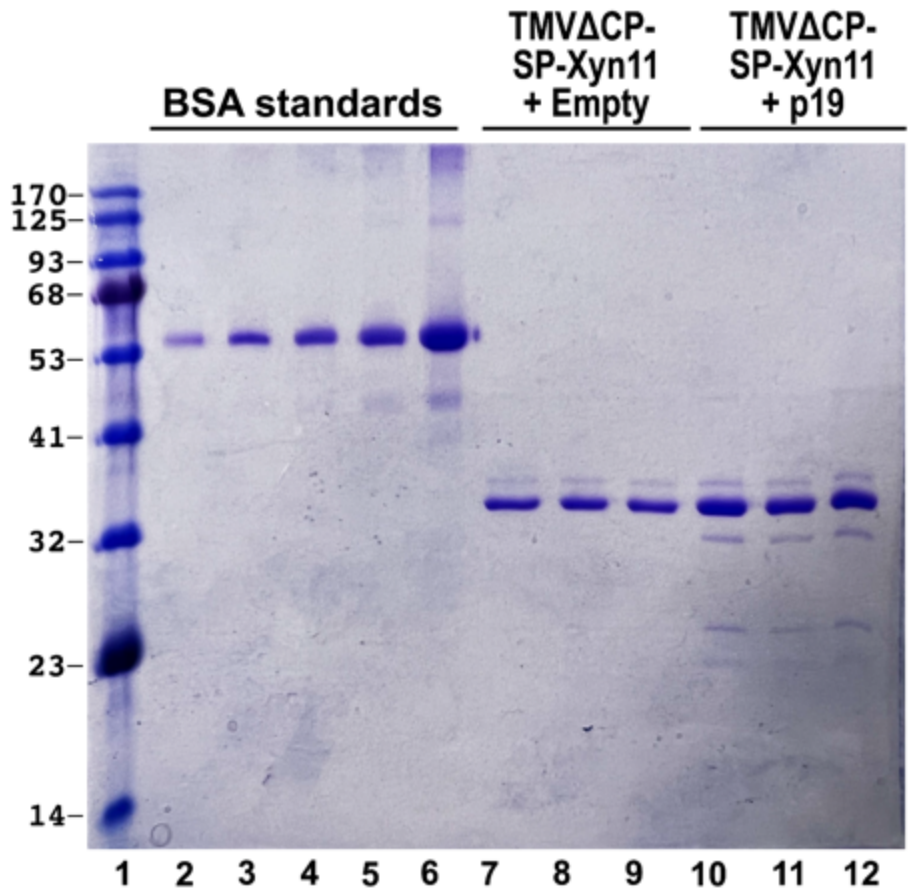

Fig. S4. Quantification of recombinant xylanase accumulating in *N. benthamiana* apoplast from plants co-agroinoculated with TEVΔCP-SP-Xyn11 and an empty plasmid or a plasmid to express TBSV p19 RNA silencing suppressor, as indicated. Leaves were harvested at 7 dpi, and protein extracts were prepared from the apoplastic fluid. Proteins were separated by SDS-PAGE, and the gel was stained with Coomassie brilliant blue. Lane 1, marker proteins with size in kDa on the left; lanes 2 to 6, BSA standards (0.24, 0.48, 0.72, 0.96 and 1.2 µg, respectively); lanes 7 to 12, biological replicates of apoplastic liquid (equivalent to 1.2 µl) harvested from plants co-agroinoculated with TMVΔCP-SP-Xyn11 and an empty plasmid (lanes 7 to 9) or TMVΔCP-SP-Xyn11 and a plasmid to express p19 (lanes 10 to 12).

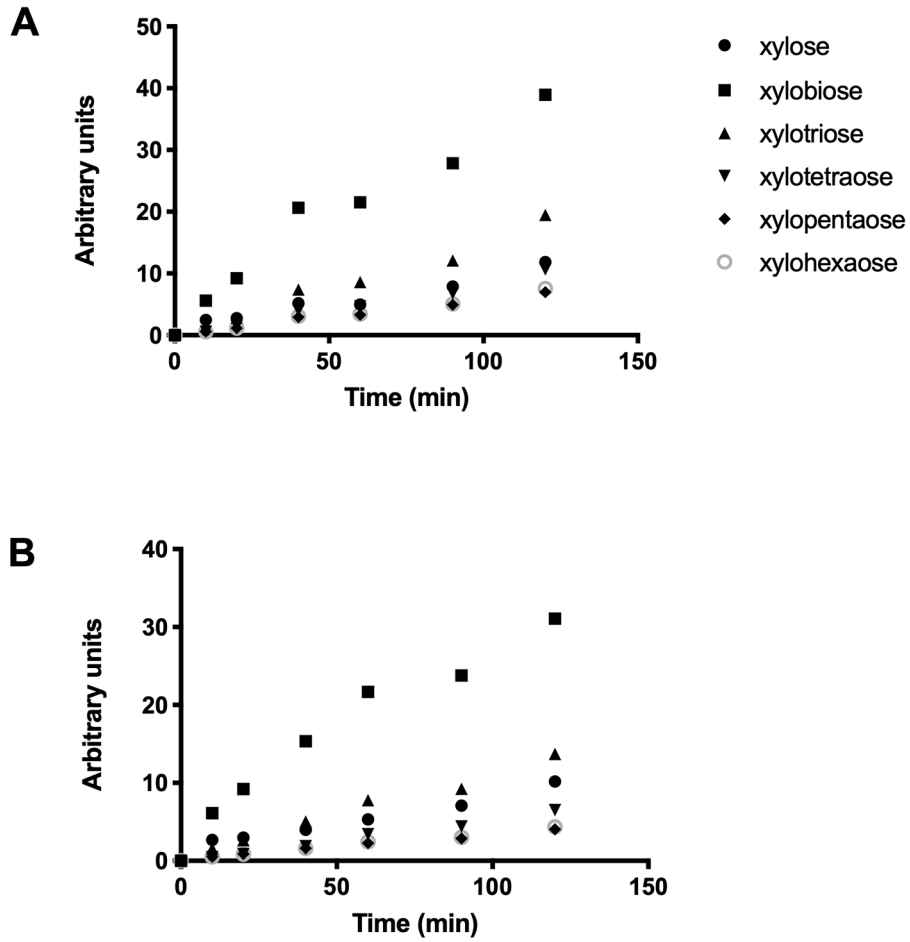

Fig. S5. Time-course production of XOS using (A) Xyn11_Nb and (B) Xyn11_Ec.

Table S1. Values of protein net charge (Z) were determined for Xyn11_Ec and Xyn11_Nb at different pH values.

pH	Xyn11_Ec	Xyn11_Nb
5.00	21.4	16.1
5.50	14.1	9.5
6.00	9.3	5.7
6.50	4.3	2.3
7.00	-0.2	-0.6
7.50	-3.0	-2.4
8.00	-4.6	-3.6
8.50	-6.3	-4.9
9.00	-9.3	-7.5
9.50	-15.5	-13.6
10.00	-26.2	-24.2
10.50	-37.0	-35.0

Table S2. PCR primers used for cloning purposes.

Primer	Orientation	Sequence	Product
D4375	Forward	5′-ctcagttcgtgttcttgtcaTGAGTTCTCAAATACCTTCTTTAAAAG-3′	Xyn11
D4376	Reverse	5′-ctacctcaagttgcaggaccTCATTTTGATACAGACTCTATG -3′	
D4377	Forward	5′-ctcagttcgtgttcttgtcaTGGCTTTGTGGTACTTGTTCAATAAAA-3′	SP-Xyn11
D4378	Reverse	5′-ctacctcaagttgcaggaccTCATTTTGATACAGACTCTATGACG-3′	
D4379	Forward	5′-ctcagttcgtgttcttgtcaTGGCTTTGTGGTACTTGTTCAATAAAAGGAG -3′	SP-Xyn11-AG
D4380	Reverse	5′-ctacctcaagttgcaggaccTCATGGGCTGGGAGAAGGGGAT-3′	

CHAPTER III

Production in *Nicotiana benthamiana* of a thermotolerant glucose oxidase that shows enzybiotic activity against *Escherichia coli* and *Staphylococcus aureus*

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Author contribution:

JP and JAD conceived the project with input from the rest of the authors. DTP, MNS and JMN performed the experiments. All authors analyzed the data. JP and JAD wrote the manuscript with input from the rest of the authors. All authors read and approved the final manuscript.

Abstract

Glucose oxidase (GOX) catalyzes the FAD-dependent oxidation of α-D-glucose to D-gluconolactone with production of hydrogen peroxide. This enzyme encounters many biotechnological applications from glucose sensors to applications in food, pharma and textile industries. For this purpose, recombinant GOX versions, usually derived from *Aspergillus niger*, are produced in fermentation systems, frequently in filamentous fungi because other production platforms such as bacteria or yeast have rendered meager results. We wondered whether *A. niger* GOX, more specifically a mutant version with superior thermotolerant properties, could be efficiently produced in *Nicotiana benthamiana* plants. To this aim, we used a tobacco mosaic virus-derived vector that is inoculated into plant tissues using *Agrobacterium tumefaciens*. Results exhibited the efficient production of the recombinant GOX in plants and the facile downstream purification when the recombinant protein is targeted to the apoplast, the space between plasma membranes and cell walls. The plant-made recombinant GOX displayed excellent catalytic properties in broad pH and temperature conditions. In addition to establishing a new strategy to produce recombinant GOX in plants as a green alternative to traditional fungal fermentation, we further investigated the potential application of this protein as an ezybiotic. Results exhibited a remarkable bacteriocide activity against *Escherichia coli* and *Staphylococcus aureus*.

Introduction

Glucose oxidase (GOX) is a FAD-dependent oxidase, typically produced by *Aspergillus niger* and other fungal species, that oxidizes α-D-glucose yielding D-gluconolactone and hydrogen peroxide. Structurally, GOX belongs to the glucose-methanol-choline (GMC) oxidase superfamily, characterized by the

presence of an N-terminal FAD-binding domain, with an α-β fold, typical of dinucleotide binding proteins, and a C-terminal substrate-binding domain consisting of a β -sheet wrapped by α-helices [1–4]. GOX is an outstanding enzyme from an industrial point of view because of its multiple and important applications, a reason why it has been dubbed the enzyme "Ferrari" [5]. Although its use in biosensors for the measurement of glucose concentration in blood [6] is undoubtedly the most popular application, GOX has many other diverse and relevant uses in medicine, and in food, pharma and textile industries [5,7].

Industrial production of GOX is carried out using *Aspergillus niger* strains genetically engineered to boost productivity [8]. Different yeast species and *Escherichia coli* have also been assayed as production hosts, although with meagre results so far [5,9]. GOX from *A. niger* is readily expressed in *Saccharomyces cerevisiae*, which makes possible to manipulate the enzyme in efforts to find engineered variants with improved properties. However, the yield is too low to allow industrial production [10,11]. The use of alternative hosts, which would provide increased versatility for traditional and new applications is therefore a relevant biotechnological goal. In this context, the use of plants as bio-factory, and specifically *Nicotiana benthamiana* [12], represents an interesting alternative.

Plants are arising as a reliable platform to produce recombinant proteins, in a technology known as molecular farming, based on price, scalability, biosecurity, and downstream processing [13]. Plants basically require sunlight and water to grow, and production can be easily scaled up; one plant can be considered a bioreactor unit that can be easily multiplied by growing more plants. No human and livestock pathogens have ever shown to infect plants, which alleviates biosecurity problems. In addition, if recombinant proteins are targeted to

particular subcellular locations, such as the apoplast, the space between the cellular membrane and cell wall, which is particularly poor in endogenous proteins, purification of the recombinant protein by mechanical means is particularly straightforward [14]

The use of enzymes as antimicrobial agents is acquiring increasing biotechnological relevance. In many instances, enzymes can represent a complement or even a convenient alternative to conventional antibiotics. Enzymes with this activity have been designed as enzybiotics, a term originally coined to describe the antibacterial activity of lytic enzymes derived from bacteriophages that disrupt the bacterial cell wall [15]. However, the concept can also be applied to other enzymes, such as GOX, whose antimicrobial activity resides in the hydrogen peroxide production that is concomitant to its catalytic action [5,16].

Improving GOX stability shall enhance its usability for different industrial applications. This is the rationale behind the isolation of mutated variants of the enzyme with increased resistance to thermal denaturation. In this work, we have used one such engineered GOX version [11] to address two important biotechnological issues. Firstly, we have implemented an efficient alternative procedure for large scale production of the enzyme using *N. benthamiana* as a host. Secondly, we show remarkable antimicrobial (enzybiotic) properties of the plant-produced engineered GOX variant.

Materials and methods

GOX expression in *N. benthamiana*

A synthetic DNA coding for an engineered version of A. niger GOX, whose codon sequence was optimized for translation in *N. benthamiana* (IDT)

(Supplementary Fig. S1), was assembled [17] (Table S1) into intermediate plasmid pMTMVi-N [18], opened by digestion with AgeI and XhoI (Thermo Scientific). The BsaI (New England Biolabs) fragment from the resulting plasmid was assembled into pGTMV [18] (containing a TMV infectious clone, GenBank accession number MK087763.1) digested with NcoI and Pfl23II (Thermo Scientific) to build pGTMVΔCP-GOX (Supplementary Fig. S2).

Agrobacterium tumefaciens (GV3101:pMP90), harboring the helper plasmid pCLEAN-S48 [19], was transformed with plasmid pGTMVΔCP-GOX. Liquid cultures were grown in lysogenic broth (LB) medium containing 50 µg/mL kanamycin for 24 h at 28°C. Cells were pelleted by centrifugation and brought to an optical density (OD) at 600 nm of 0.5 in 10 mM MES-NaOH, pH 5.6, 10 mM $MgCl_2$ and 150 µM acetosyringone. Bacterial preparations were incubated for 2 h at 28°C to induce the virulence genes, and used to infiltrate the leaves of 5-weeks old *N. benthamiana* plants. Preparations were infiltrated at the abaxial side of the leaves using a needleless 1-mL syringe. Plants were cultured in a growth chamber at 25°C with a 16/8-h day/night photoperiod.

GOX purification and enzyme analysis

Infected tissues from *N. benthamiana* plants were harvested at 7 days post-inoculation (dpi) and infiltrated with phosphate buffered saline (PBS) buffer supplemented with 0.02% (v/v) Silwet L-77. Infiltrated tissues were then introduced in a 50 mL syringe and squeezed to obtain an apoplastic liquid, which was finally clarified by centrifugation for 5 min at 13,000 rpm. GOX was further purified by ion exchange chromatography. The apoplastic fluid was dialyzed against buffer A (50 mM acetate/acetic buffer pH 5.5). The resulting sample was centrifuged at 12,000 *g* for 5 min and the supernatant was injected in a 1 mL ResourceQ column (GE-Healthcare) coupled to an ÅKTA-purifier system (GE-Healthcare). Flow-through was collected and the column was subsequently

washed with 5 column volumes (CV) of buffer A and 5 CV of 10% buffer B (50 mM acetate/acetic buffer pH 5.5, 1 M NaCl). Elution was carried out with a 5 CV linear gradient from 10% to 50% B, followed by a final wash with 5 CV of 50% B and 5 CV of 100% B. The fraction corresponding to purified GOX was quantified spectrophotometrically at 280 nm, using an extinction coefficient ($\boldsymbol{\varepsilon}^{1\%}$) of 14.8.

GOX activity was determined as previously described (Marin-Navarro et al 2015). Briefly, GOX samples were incubated at the indicated temperatures in a reaction mixture containing 85 mM glucose, 12 µg/mL horseradish peroxidase, and 0.17 mM O-dianisidine in the appropriate buffers for each pH (50 mM citrate pH 4 and 5; 50 mM phosphate pH 6 and 7; 50 mM Tris-HCl pH 8 or 50 mM borate-NaOH pH 9). Reactions were stopped by addition of 0.2 M HCl; absorbance at 400 nm was measured and the amount of oxidized glucose was determined by interpolation on a standard curve. One unit of activity was defined as the amount of enzyme oxidizing 1 µmol of glucose per minute at 37°C and pH 7.0.

Antimicrobial assay

GOX antibacterial activity was assayed using previously grown bacterial cultures, either by using a disk impregnated with the enzyme on a solid medium or by incubation with the enzyme in a liquid suspension. For the disk diffusion test, *Staphilococcus aureus* and *E. coli* were grown at 37°C to an OD of 0.6. Cultures were diluted 1/300 with buffer P (50 mM phosphate buffer pH 7) and 0.2 mL were spread on LB solid medium. Once plates were dried, 5 mm-diameter filter paper disks were placed on their surface. Four different solutions were prepared to impregnate the disks. Three of them containing 200 mM glucose in buffer P and GOX at different concentrations (0.7 to 12 U/mL). The fourth was a negative control without glucose, with GOX (2.8 U/mL). The filter paper disks were loaded with 12 µL of the different GOX treatments. For the liquid suspension test, *S. aureus* (CECT 794) and *E. coli* strain Nissle 1917 were inoculated in LB medium

and grown at 37 ^{0}C to an OD at 600 nm of 0.6 or to late stationary phase (24 h). Cells were collected by centrifugation at 12,000 *g* for 7 min, washed with 1 mL of buffer P and finally resuspended in 1.2 mL of the same buffer to an OD of 0.15. Aliquots (100 μL) were mixed with either 50 μL of GOX (5.5 U/mL) and 50 μL of 400 mM glucose in buffer P; 50 μL of GOX (5.5 U/mL) and 50 μL of buffer P; or 100 μL of buffer P, and incubated for 90 min at 37 °C. All these treatments were carried out in triplicate. Subsequently, these samples were inoculated into 300 μL of LB in a 96-multiwell plate with a final 1/50 dilution and growth was monitored by measuring OD at 600 nm every 30 min in a microplate reader (SPECTROstar Omega, BMG LABTECH). Relative number of viable cells after GOX treatment with or without glucose was estimated from the inoculum size (N_0) of the growth curves. To this end, a classic logistic model was applied according to equation [1]:

$$N = \frac{b \cdot N_0}{N_0 + [(b - N_0) \cdot e^{-rt}]} \qquad [1]$$

where N_0 and N are the cell densities at time 0 and at time t, respectively, b represents the maximum cell density in the medium, or carrying capacity, and r is the intrinsic growth rate of the culture. Parameters N_0, b and r were adjusted to fit equation [1] to experimental data through minimization of the sum of squared differences using the Solver complement within the Microsoft Excel software. Detection limit for this determination was calculated as the minimum N_0 value required to detect an OD increase of 0.1 units in 23 h.

For the disk diffusion test, *S. aureus* and *E. coli* were grown at 37^{0}C to an OD of 0.6. Cultures were diluted 1/300 with buffer P and 0.2 mL were spread on LB solid medium. After plates were dried, 5 mm-diameter filter paper disks were placed on their surface. Four different solutions were prepared to impregnate disks, all of them containing 200 mM glucose in buffer P and GOX at different

concentrations, including a control without enzyme, and 12 µL of each was spotted on the disks.

Electrophoretic and mass spectrometry analyses

Preparations of apoplastic fluid and purified protein were analyzed by SDS-PAGE and gels were stained with Coomassie brilliant blue. Protein identification was carried out through liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. Gel slices containing the band of interest were digested overnight with 200 ng of sequencing grade trypsin (Promega) at 37°C as described elsewhere [20]. The reaction was stopped with trifluoroacetic acid at a final concentration of 0.1%. An aliquot (1 µl) of the digestion mixture was diluted to 20 µL with 0.1% formic acid and loaded in an Evotip pure tip (EvoSep) according manufacturer instructions. LC–MS/MS was performed in an Evosep One system connected to a Tims TOF fleX mass spectrometer (Bruker). The sample loaded in the Evotip pure tip was eluted to an analytical column (PepSep C18 10 cm x 150 µm, 1.5 µm; Bruker) by the Evosep One system and resolved with the 100 SPD chromatographic method defined by the manufacturer.

Results

Production of glucose oxidase in *N. benthamiana*

The main goal of this work was to analyze whether plants, and particularly the outstanding biofactory plant *N. benthamiana*, was an optimal platform to produce a thermoresistant version of *A. niger* GOX, an enzyme with multiple biotechnological and industrial applications. To this end, we used a TMV-derived vector that is delivered into *N. benthamiana* cells by means of *A. tumefaciens* (agroinoculation) [14,18]. In this vector, GOX replaced most of viral coat protein (CP) coding region (**Fig. 1**), rendering a recombinant virus able to replicate and

move cell-to-cell, but unable to move systemically through the plant. Using denaturing polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie brilliant blue staining, we analyzed protein accumulation in agroinoculated tissues and compared the protein content with those of control tissues. A differential band with a position corresponding to the expected mobility of the recombinant GOX suggested efficient production of the heterologous enzyme in *N. benthamiana* at 7 dpi using the TMV vector (**Fig. 2**). Additionally, three supernumerary proteins with estimated molecular masses of 34, 25 and 22 kDa (**Fig. 2**, P2 to P4) were also detected. These proteins were also observed in tissues agroinoculated with an empty plasmid (no TMV vector) and, to a lesser extent, in the mock-inoculated plants (i.e., infiltrated with buffer with no *A. tumefaciens*), but not in the non-treated control plants. Mass spectrometry analysis of these proteins allowed their identification, showing that they correspond to peroxidase N1, acidic endochitinase Q and pathogenesis-related protein R, respectively (**Table S2**). A noticeable protein of estimated 54 kDa (P1), corresponding to Rubisco large subunit, was also present in all samples.

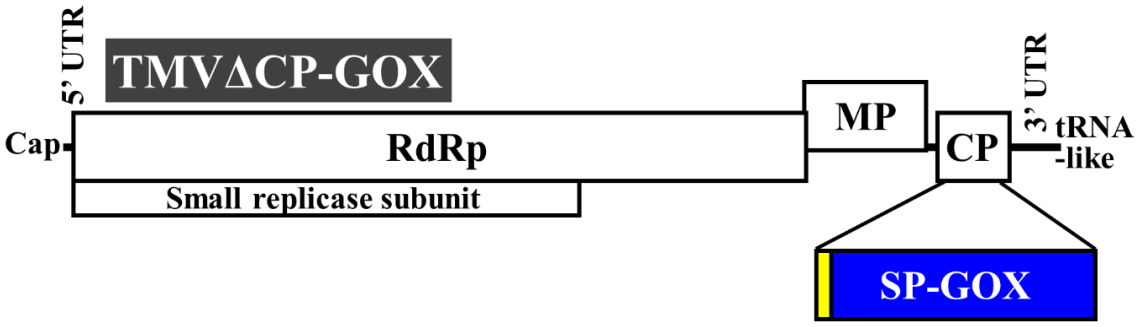

Fig. 1. Schematic representation of the TMV-derived vector to express GOX in *N. benthamiana* plants. 5' UTR and 3' UTR, 5' and 3' untranslated regions; RdRp, RNA-dependent RNA polymerase; MP, movement protein; CP, coat protein; SP, signal peptide, GOX, glucose oxidase. Other features such as a 5' cap and 3' tRNA-like structures are also indicated.

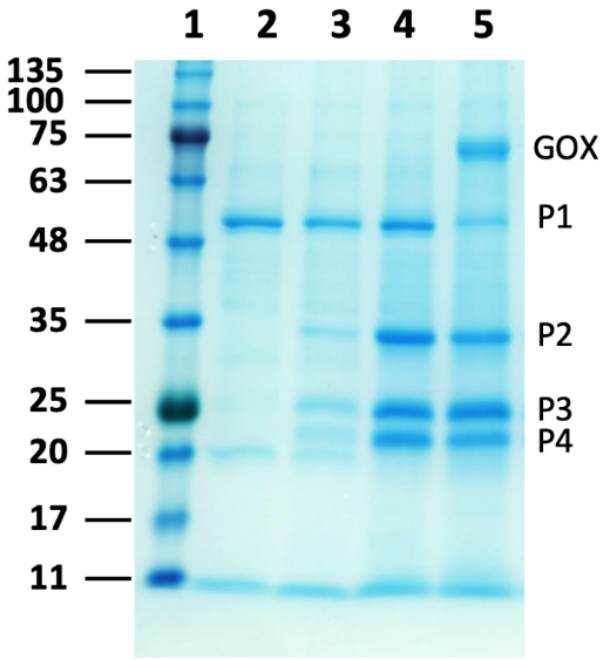

Fig. 2. Electrophoretic analysis of recombinant GOX production in *N. benthamiana* plants. Lane 1, protein standards; lane 2, non-infiltrated plant; lane 3, plant infiltrated with buffer; lane 4, plant agroinoculated with an empty plasmid; lane 5, plant agroinoculated with TMVΔCP-GOX. The positions of GOX and four supernumerary proteins (P1 to P4) are shown. Standard protein sizes in kDa are indicated in the left margin.

Purification and enzymatic analysis of GOX from *N. benthamiana*

The observed molecular weight of GOX (72 kDa) is lower than that reported for the enzyme produced by *A. niger* (86 kDa) [21], but somewhat higher than that expected for the non-glycosylated monomer (63 kDa). Therefore, some type of post-translational modification, likely glycosylation, seems to have occurred. GOX was obtained after a single step of an anion-exchange chromatography with high purity (**Fig. 3**). The specific activity of the plant-made GOX was 110.8 ± 0.5 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ of enzyme at 37°C and pH 7, which is similar to previously reported data (135 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ of enzyme at 40 °C and pH 5.5) for the wild-type version isolated from *A. niger* [22]. This indicates that the triple mutation

harbored in the GOX engineered version [11] used in this work does not impairs enzyme activity.

The activity of plant-produced GOX was evaluated at different pH values and temperatures, as shown in Figure 4. The results indicate that the recombinant GOX remains active over a broad pH range (4 to 8), with optimal activity at pH 6. Even under rather high acidic conditions (pH 4), the enzyme maintained around 50% of its maximum activity, while under basic conditions (pH 9), it retained only about 10% (**Fig. 4A**). Additionally, the enzyme exhibited maximum activity between 35°C and 50°C, with a gradual decrease in activity at lower and higher temperatures, falling to 50% and 30% of its peak activity, respectively (**Fig. 4B**). These findings suggest that plant-produced recombinant GOX is capable of functioning under optimal conditions across a wide range of pH and temperature.

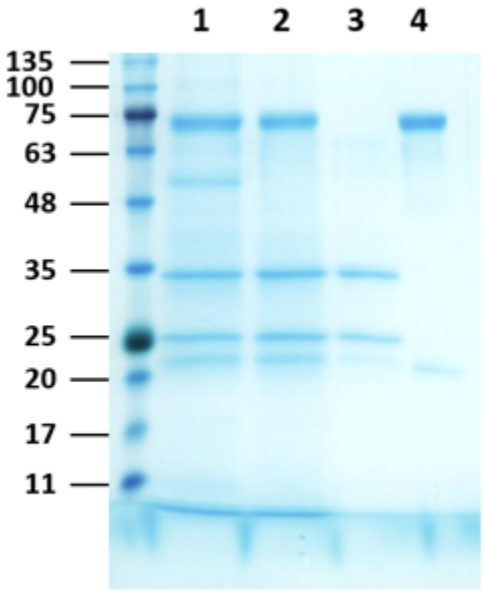

Fig. 3. Plant-made recombinant GOX purification. Aliquots of the preparations at different steps were separated by SDS-PAGE. Apoplastic fluid from *N. benthamiana* (lane 1) was dialyzed and centrifuged. The supernatant (lane 2) was

injected in an anion exchange column. The flow through (lane 3) and the elution peak (lane 4) were collected.

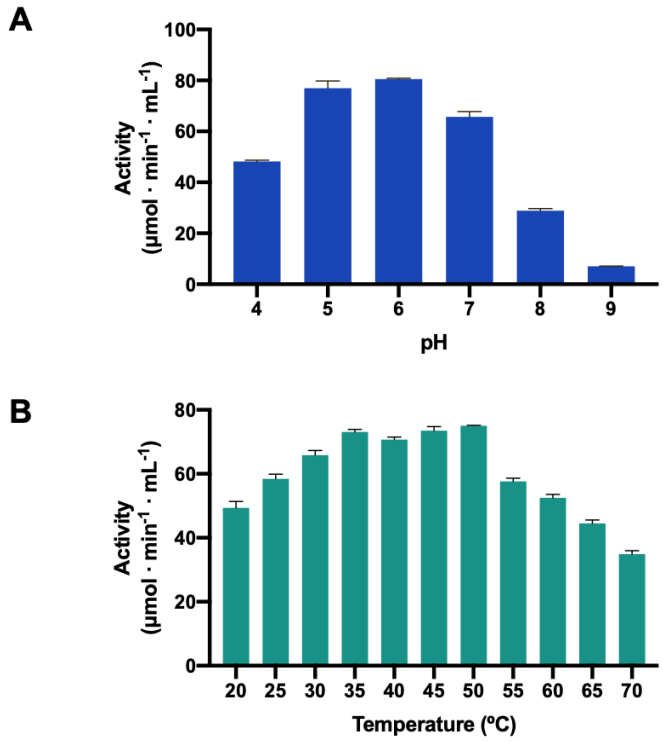

Fig. 4. Analysis of enzymatic activity of the plant-produced engineered GOX under different conditions of pH at 40ºC (A) and temperature at pH 7 (B). Histograms represent average activity and standard deviation (n = 3) at the indicated conditions.

Antimicrobial activity of GOX produced in *N. benthamiana* against gram positive and gram-negative bacteria

The antimicrobial activity of the plant-made recombinant GOX was tested against a gram positive and a gram-negative bacteria, *S. aureus* and *E. coli*, respectively. Two different assays were performed. The first consisted on a disk diffusion assay carried out on a Petri dish whereas in the second the cells were incubated with the enzyme in liquid medium. Disk diffusion tests were assayed with increasing amounts of enzyme, which correlated positively with wider halo

diameters resulting from growth inhibition (**Fig. 5**). Halo sizes were much higher for *S. aureus* than for *E. coli*, despite previous reports have described that gram-positive strains are usually less sensitive to GOX treatments. The thicker peptidoglycan of the gram-positive bacteria has been proposed to act as protective barrier to H_2O_2 and other reactive species but other enzymatic activities (catalase and superoxide dismutase) may also have this defense function [16,23]. In particular, *E. coli* produces two inducible catalases (HPI and HPII). HPI expression is induced under logarithmic growth phase by H_2O_2 whereas both enzymes are overproduced during stationary phase compared to logarithmic phase [24]. Thus, HPI induction may explain the higher apparent resistance of *E. coli* strain in this assay. Therefore, a second test was carried out where cells were previously grown to logarithmic phase (i.e., not exposed to H_2O_2) (**Fig. 6**) or up to stationary phase (**Fig. 7**) and incubated in liquid media with GOX in the presence or absence of glucose. Samples were subsequently inoculated in fresh medium and culture growth was monitored to test cell viability.

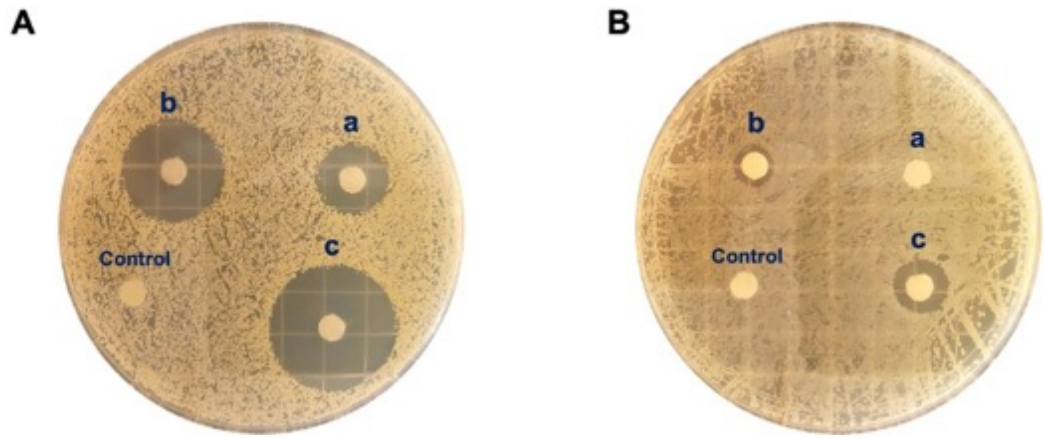

Fig. 5. Disk diffusion tests with *S. aureus* (A) and *E. coli* (B) were carried out with a reaction mixture containing 200 mM glucose and increasing amounts of GOX (a, 0.7 U/mL; b, 2.8 U/mL; and c, 12 U/mL). Control was performed with 2.8 U/mL of GOX without glucose.

When analyzing culture growth of log-phase treated cells (**Fig. 6**) a significant increase in the lag-phase was observed in the *S. aureus* cells incubated with GOX and glucose, compared to the controls, whereas no growth was detected after 24 h for *E. coli* cells subjected to the same treatment (**Fig. 6A, C**). This result is likely a consequence of the GOX-induced reduction in the number of viable cells. In both cases, cells treated with the enzyme in the absence of glucose behaved as the control, confirming that the reduction in cell viability is a specific consequence of GOX activity. An estimation of the reduction of the number of viable cells after enzymatic treatment, compared to the control without glucose, was obtained by fitting the logistic equation [1] to the experimental growth data (**Fig. 6 B, D**). According to these results, GOX treatment resulted in a 1.6 log reduction in *S. aureus* and at least a 13.6 log reduction in *E. coli*, estimation based on the detection limit ($N_0 = 1 \cdot 10^{-5}$) of the method. Compared to these results, cells grown to late stationary phase showed a higher sensitivity to GOX treatment in the case of *S. aureus* and a similar behavior in *E. coli* (**Fig. 7**).

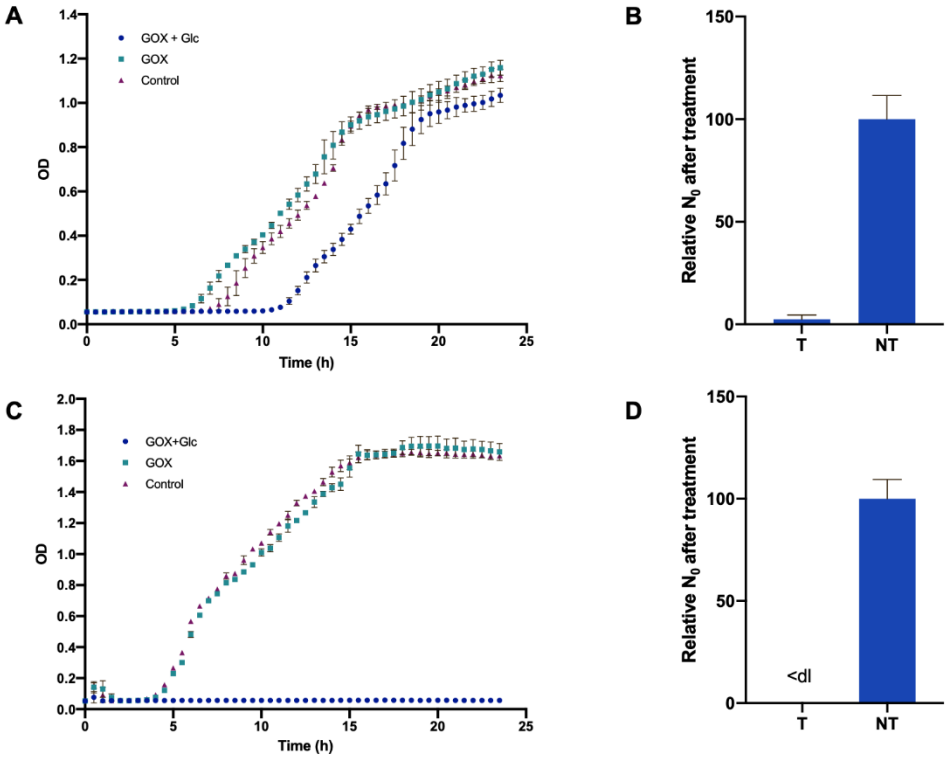

Fig. 6. Antimicrobial activity of the plant-made GOX against log-phase grown *S. aureus* (A, B) and *E. coli* (C, D). Culture growth (A, C) was monitored after incubation of liquid cell suspensions with the enzyme in the presence (GOX + Glc) or absence (GOX) of 100 mM glucose, or without any additive (Control). Relative number of viable cells (N_0) after plant-made GOX treatment with (T) or without (NT) glucose for *S. aureus* (B) and *E. coli* (C); dl = 0.03%.

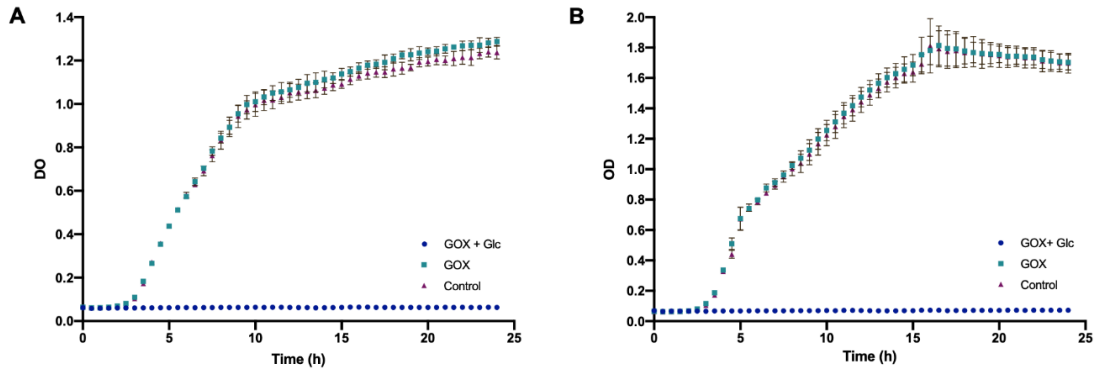

Fig. 7. Antimicrobial activity of the plant-made GOX against late stationary phase grown *S. aureus* (A) and *E. coli* (B). Culture growth was monitored after incubation of liquid cell suspensions with the enzyme in the presence (GOX + Glc) or absence (GOX) of 100 mM glucose, or without any additive (control). Plots represent the average (n = 3) optical density at 600 nm at different time points. Error bars represent standard deviation.

Discussion

Our results show that plants, and particularly N. benthamiana, are suitable hosts to produce GOX, a highly valuable industrial enzyme. A key feature of the system used here is gene expression driven by a TMV vector [18]. GOX accumulation in plant tissues is based on RNA-to-RNA replication of the viral vector and efficient

translation of the viral subgenomic mRNA in which the coding sequence of the recombinant protein is inserted. This subgenomic mRNA, which results from partial replacement of a viral coding region for that of the gene of interest, originally encodes the viral CP that reaches enormous amounts in infected tissues [25], likely as a consequence of efficient translation regulatory signals at the 5′ and 3′ termini of the mRNA, in addition to the intrinsic stability of the viral protein. In our approach, the recombinant GOX is targeted to the export pathway by fusion of an amino-terminal peptide derived from N. tabacum (1-3)-β-endoglucanase [26], which efficiently targets the recombinant protein to the apoplastic space. This exclusive plant cellular localization, between the cytoplasmic membrane and the cell wall, is particularly poor in endogenous proteins (see Fig. 3). In addition, the apoplastic content can be mechanically separated from the rest of the plant tissues by a simple buffer infiltration followed by tissue squeezing using a hand operated syringe. Under this simple process, a protein preparation enriched in the recombinant protein of interest can be easily recovered.

SDS-PAGE analysis of the apoplast fluids from N. benthamiana agroinfiltrated with exogenous DNA and, to a lesser extent in buffer-infiltrated tissues, shows the conspicuous presence of protein bands of 34, 25 and 22 kDa, corresponding to peroxidase N1, acidic endochitinase Q and pathogenesis-related protein R, respectively (Table S2). These proteins are not detected in the apoplast of non-infiltrated control plants. Additionally, a band corresponding to the large subunit of Rubisco was present in all preparations from infected, infiltrated with empty plasmid or buffer, and non-treated plants. The presence of Rubisco, an intracellular protein, in the apoplastic fluid is easily explained by the superabundance of this protein in the leaves, since even minor tissue damage can cause significant leakage to the apoplast fraction [27]. The presence of the other three proteins in the apoplast of treated leaves in GOX expressing plants,

but also in controls, can be explained by their known expression pattern. Peroxidase N1 is a secreted, defense-related protein, induced by stress conditions such as plant wounding [28]. Acidic endochitinase Q has been reported to be induced by TMV infection [29]. Pathogenesis-related protein R accumulates in the plant apoplast as a defensive mechanism against infection and is also a virus-induced protein [30]. Accumulation of these three endogenous proteins in the apoplast in our experiments seems to be a consequence of mechanical damage, rather than viral vector replication or A. tumefaciens infection, because they also accumulate in tissues infiltrated with just buffer. Interestingly, the plant response to tissue damage with expression of defense proteins does not prevent efficient GOX accumulation.

Antimicrobial activity of GOX has been well documented [5,9,16]. Our results, using an engineered GOX variant produced in N. benthamiana, confirm this property showing high bactericidal activity against E. coli and S. aureus, which was monitored in both cases by the decrease in viable cells (Fig. 6B, D). The relative higher resistance of S. aureus may be a consequence of the thicker peptidoglycan layer of the gram-positive bacteria, as previously suggested [16,23]. The apparent higher resistance of E. coli to GOX observed in the disk diffusion assay (Fig. 5) turned out not to be so, as revealed by cell viability analysis. The apparent contradiction can be due to the induction of HPI catalase, promoted by the H2O2 generated by the GOX catalyzed reaction [24]. Furthermore, scavenging effects caused by E. coli cells highly reactive to H2O2 cannot be ruled out. Interestingly, cells grown to late stationary phase show similar or even higher sensitivity to GOX treatment than those grown to log-phase, even when catalase expression may be higher.

Conclusion

In sum, these results support that *N. benthamiana* is an optimal platform for the cheap, sustainable and scalable production of an engineered GOX and that the recombinant protein can be easily purified by mechanical means from plant tissues. Notably, in addition of well-known use in diagnosis and other biotechnological applications, our results highlight the potential use of plant-made GOX as an enzybiotic.

Acknowledgements

This research was supported by Generalitat Valenciana, grant INNEST/2021/7 from Agència Valenciana de la Innovació, and from the Ministerio de Ciencia e Innovación (Spain), grant PID2020-114691RB-I00 from Agencia Estatal de Investigación. M.N.-S. is the recipient of a predoctoral contract (PRE2018-084771) from the Ministerio de Ciencia e Innovación.

Competing Interests

The authors declare there are no conflicts of interest, including financial, personal, or any other relationships with other people or organizations.

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Supplementary Information

Fig. S1. Nucleotide and amino acid sequences of the glucose oxidase (GOX) version expressed in *Nicotiana benthamiana*.

AID16306.1- Mature_GOX_T544M/Q90R/Y509E

SNGIEASLLTDPKDVSGRTVDYIIAGGGLTGLTTAARLTENPNISVLVIESGSYESDRGPIIEDLNA
YGDIFGSSVDHAYETVELATNNRTALIRSGNGLGGSTLVNGGTWTRPHKAQVDSWETVFGNEGWNWD
NVAAYSLQAERARAPNAKQIAAGHYFNASCHGTNGTVHAGPRDTGDDYSPIVKALMSAVEDRGVPTK
KDFGCGDPHGVSMFPNTLHEDQVRSDAAREWLLPNYQRPNLQVLTGQYVGKVLLSQNGTTPRAVGVE
FGTHKGNTHNVYAEHEVLLAAGSAVSPTILEYSGIGMKSILEPLGIDTVVDLPVGLNLQDQTTATVR
SRITSAGAGQGQAAWFATFNETFGDYSEKAHELLNTKLEQWAEEAVARGGFHNTTALLIQYENYRDW
IVNHNVAYSELFLDTAGVASFDVWDLLPFTRGYVHILDKDPYLHHFAYDPQYFLNELDLLGQAAATQ
LARNISNSGAMQTYFAGETIPGDNLAYDADLSAWTEYIPEHFRPNYHGVGTCSMMPKEMGGVVDNAA
RVYGVQGLRVIDGSIPPMQMSSHVMTVFYAMALKISDAILEDYASMQ

>GOX

AGTAACGGGATTGAGGCATCCCTGTTAACCGATCCGAAGGATGTCTCCGGGAGGACGGTAGACTACA
TAATTGCAGGAGGAGGACTGACAGGTCTTACTACCGCAGCAAGGCTTACAGAAAACCCAAATATATC
AGTGCTTGTAATTGAGTCTGGTAGTTACGAGTCCGACAGGGGGCCAATTATCGAGGATTTAAACGCA
TACGGGGATATTTTCGGTAGTTCAGTTGATCATGCTTATGAAACCGTTGAATTGGCCACCAACAACC
GTACCGCCTTGATTAGATCAGGAAACGGATTGGGCGGATCTACGCTTGTTAATGGTGGAACATGGAC
CAGGCCCCATAAGGCTCAGGTAGATAGTTGGGAGACTGTCTTTGGCAATGAAGGGTGGAACTGGGAC
AACGTAGCAGCTTATTCACTGCAGGCTGAAAGGGCCCGTGCCCCGAACGCTAAACAAATTGCCGCAG
GCCACTATTTCAATGCTTCCTGTCACGGCACTAACGGTACTGTACACGCAGGCCCCCGAGACACAGG
CGACGACTACTCTCCCATAGTAAAAGCACTTATGTCAGCAGTAGAAGACCGTGGGGTACCCACGAAA
AAGGACTTCGGGTGTGGCGATCCACACGGGGTTTCTATGTTCCCCAATACTCTCCACGAAGACCAAG
TTCGTTCAGATGCCGCCCGAGAATGGCTCCTGCCCAACTATCAACGACCAAATCTTCAAGTTCTTAC
TGGCCAGTACGTGGGCAAAGTTCTCCTCTCACAGAATGGTACAACACCCCGTGCAGTAGGGGTCGAA
TTTGGTACACACAAGGGAAACACGCATAATGTTTACGCCGAACATGAAGTGTTGCTGGCCGCTGGAT
CAGCCGTTTCACCGACCATCTTAGAATATTCCGGAATAGGCATGAAAAGTATACTTGAGCCGTTGGG
AATCGACACAGTGGTGGATTTACCGGTCGGGCTCAACTTACAGGATCAAACGACAGCTACGGTTCGA
TCAAGGATAACAAGTGCCGGAGCCGGCCAGGGTCAAGCAGCATGGTTTGCTACTTTCAATGAAACCT
TCGGGGACTACAGTGAAAAGGCTCACGAATTGCTCAATACGAAACTTGAGCAGTGGGCCGAAGAAGC
AGTTGCTAGGGGGGGCTTTCACAACACGACGGCCTTGCTCATCCAGTATGAAAACTATCGAGACTGG
ATAGTGAACCACAATGTGGCATACTCAGAACTGTTTTTAGACACTGCAGGCGTAGCCAGTTTCGACG
TGTGGGACTTACTTCCTTTCACGAGAGGTTATGTACATATTCTTGACAAGGACCCCTATCTCCACCA
CTTCGCTTATGATCCCCAATATTTTCTTAACGAACTGGATCTCTTAGGTCAAGCCGCTGCTACCCAA
CTCGCTCGAAATATCAGTAATAGCGGAGCTATGCAAACATATTTCGCCGGTGAGACGATTCCGGGTG
ATAATCTGGCTTACGACGCAGATTTGTCCGCCTGGACAGAGTACATTCCCGAGCACTTTAGGCCTAA
TTATCATGGAGTTGGAACGTGCTCAATGATGCCCAAGGAAATGGGAGGGGTCGTGGATAACGCTGCT
AGAGTATATGGTGTTCAAGGTCTTCGTGTAATAGATGGCTCCATCCCACCTATGCAGATGAGTAGCC
ACGTCATGACAGTTTTCTATGCCATGGCACTTAAGATCTCAGATGCTATATTGGAAGATTACGCATC
AATGCAG

Fig. S2. Sequence of TMV recombinant clones to express GOX in *N. benthamiana*. Vector was built on the basis of a TMV infectious variant (GenBank accession no. MK087763.1) with deletion of most of CP gene and mutation of

viral CP initiation ATG to **AGA** (in red). Nucleotide sequence of **GOX** is in blue, **(1-3)-β-endoglucanase signal peptide (SP)** in purple.

>TMVΔCP-SP-GOX

GTATTTTTACAACAATTACCAACAACAACAAACAACAAACAACATTACAATTACTATTTACAATTACAAT
GGCATACACACAGACAGCTACCACATCAGCTTTGCTGGACACTGTCCGAGGAAACAACTCCTTGGTCAAT
GATCTAGCAAAGCGTCGTCTTTACGACACAGCGGTTGAAGAGTTTAACGCTCGTGACCGCAGGCCCAAGG
TGAACTTTTCAAAAGTAATAAGCGAGGAGCAGACGCTTATTGCTACCCGGGCGTATCCAGAATTCCAAAT
TACATTTTATAACACGCAAAATGCCGTGCATTCGCTTGCAGGTGGATTGCGATCTTTAGAACTGGAATAT
CTGATGATGCAAATTCCCTACGGATCATTGACTTATGACATAGGCGGGAATTTTGCATCGCATCTGTTCA
AGGGACGAGCATATGTACACTGCTGCATGCCCAACCTGGACGTTCGAGACATCATGCGGCACGAAGGCCA
GAAAGACAGTATTGAACTATACCTTTCTAGGCTAGAGAGAGGGGGGAAAACAGTCCCCAACTTCCAAAAG
GAAGCATTTGACAGATACGCAGAAATTCCTGAAGACGCTGTCTGTCACAATACTTTCCAGACATGCGAAC
ATCAGCCGATGCAGCAATCAGGCAGAGTGTATGCCATTGCGCTACACAGCATATATGACATACCAGCCGA
TGAGTTCGGGGCGGCACTCTTGAGGAAAAATGTCCATACGTGCTATGCCGCTTTCCACTTCTCCGAGAAC
CTGCTTCTTGAAGATTCATGCGTCAATTTGGACGAAATCAACGCGTGTTTTTCGCGCGATGGAGACAAGT
TGACCTTTTCTTTTGCATCAGAGAGTACTCTTAATTACTGTCATAGTTATTCTAATATTCTTAAGTATGT
GTGCAAAACTTACTTCCCGGCCTCTAATAGAGAGGTTTACATGAAGGAGTTTTTAGTCACCAGAGTTAAT
ACCTGGTTTTGTAAGTTTTCTAGAATAGATACTTTTCTTTTGTACAAAGGTGTGGCCCATAAAAGTGTAG
ATAGTGAGCAGTTTTATACTGCAATGGAAGACGCATGGCATTACAAAAAGACTCTTGCAATGTGCAACAG
CGAGAGAATCCTCCTTGAGGATTCATCATCAGTCAATTACTGGTTTCCCAAAATGAGGGATATGGTCATC
GTACCATTATTCGACATTTCTTTGGAGACTAGTAAGAGGACGCGCAAGGAAGTCTTAGTGTCCAAGGATT
TCGTGTTTACAGTGCTTAACCACATTCGAACATACCAGGCGAAAGCTCTTACATACGCAAATGTTTTGTC
CTTCGTCGAATCGATTCGATCGAGGGTAATCATTAACGGTGTGACAGCGAGGTCCGAATGGGATGTGGAC
AAATCTTTGTTACAATCCTTGTCCATGACGTTTTACCTGCATACTAAGCTTGCCGTTCTAAAGGATGACT
TACTGATTAGCAAGTTTAGTCTCGGTTCGAAAACGGTGTGCCAGCATGTGTGGGATGAGATTTCGCTGGC
GTTTGGGAACGCATTTCCCTCCGTGAAAGAGAGGCTCTTGAACAGGAAACTTATCAGAGTGGCAGGCGAC
GCATTAGAGATCAGGGTGCCTGATCTATATGTGACCTTCCACGACAGATTAGTGACTGAGTACAAGGCCT
CTGTGGACATGCCTGCGCTTGACATTAGGAAGAAGATGGAAGAAACGGAAGTGATGTACAATGCACTTTC
AGAATTATCGGTGTTAAGGGAGTCTGACAAATTCGATGTTGATGTTTTTTCCCAGATGTGCCAATCTTTG
GAAGTTGACCCAATGACGGCAGCGAAGGTTATAGTCGCGGTCATGAGCAATGAGAGCGGTCTGACTCTCA
CATTTGAACGACCTACTGAGGCGAATGTTGCGCTAGCTTTACAGGATCAAGAGAAGGCTTCAGAAGGTGC
ATTGGTAGTTACCTCAAGAGAAGTTGAAGAACCGTCCATGAAGGGTTCGATGGCCAGAGGAGAGTTACAA
TTAGCTGGTCTTGCTGGAGATCATCCGGAGTCGTCCTATTCTAAGAACGAGGAGATAGAGTCTTTAGAGC
AGTTTCATATGGCGACGGCAGATTCGTTAATTCGTAAGCAGATGAGCTCGATTGTGTACACGGGTCCGAT
TAAAGTTCAGCAAATGAAAAACTTTATCGATAGCCTGGTAGCATCACTATCTGCTGCGGTGTCGAATCTC
GTCAAGATCCTCAAAGATACAGCTGCTATTGACCTTGAAACCCGTCAAAAGTTTGGAGTCTTGGATGTTG
CATCTAGGAAGTGGTTAATCAAACCAACGGCCAAGAGTCATGCATGGGGTGTTGTTGAAACCCACGCGAG
GAAGTATCATGTGGCGCTTTTGGAATATGATGAGCAGGGTGTGGTGACATGCGATGATTGGAGAAGAGTA
GCTGTTAGCTCTGAGTCTGTTGTTTATTCCGACATGGCGAAACTCAGAACTCTGCGCAGACTGCTTCGAA
ACGGAGAACCGCATGTCAGTAGCGCAAAGGTTGTTCTTGTGGACGGAGTTCCGGGCTGTGGAAAAACCAA
AGAAATTCTTTCCAGGGTTAATTTTGATGAAGATCTAATTTTAGTACCTGGGAAGCAAGCCGCGGAAATG
ATCAGAAGACGTGCGAATTCCTCAGGGATTATTGTGGCCACGAAGGACAACGTTAAAACCGTTGATTCTT
TCATGATGAATTTTGGGAAAAGCACACGCTGTCAGTTCAAGAGGTTATTCATTGATGAAGGGTTGATGTT
GCATACTGGTTGTGTTAATTTTCTTGTGGCGATGTCATTGTGCGAAATTGCATATGTTTACGGAGACACA
CAGCAGATTCCATACATCAATAGAGTTTCAGGATTCCCGTACCCCGCCCATTTTGCCAAATTGGAAGTTG
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TGAGGGCTTTGTCATGAGCACTTCTTCGGTTAAAAAGTCTGTTTCGCAGGAGATGGTCGGCGGAGCCGCC
GTGATCAATCCGATCTCAAAACCCTTGCATGGCAAGATCCTGACTTTTACCCAATCGGATAAAGAAGCTC
TGCTTTCAAGAGGGTATTCAGATGTTCACACTGTGCATGAAGTGCAAGGCGAGACATACTCTGATGTTTC
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TCAAGGCACACCTGTTCGCTCAAGTACTACACTGTTGTTATGGATCCTTTAGTTAGTATCATTAGAGATC
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CTGCATCTTTGGTTGTAGATAAGTTTTTTGATAGTTATTTGCTTAAAGAAAAAAGAAAACCAAATAAAAA
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CTCGCAGATTTTGATTTTGTGGATTTGCCAGCAGTTGATCAGTACAGACACATGATTAAAGCACAACCCA
AACAAAAGTTGGACACTTCAATCCAAACGGAGTACCCGGCTTTGCAGACGATTGTGTACCATTCAAAAAA
GATCAATGCAATATTCGGCCCGTTGTTTAGTGAGCTTACTAGGCAATTACTGGACAGTGTTGATTCGAGC
AGATTTTTGTTTTTCACAAGAAAGACACCAGCGCAGATTGAGGATTTCTTCGGAGATCTCGACAGTCATG
TGCCGATGGATGTCTTGGAGCTGGATATATCAAAATACGACAAATCTCAGAATGAATTCCACTGTGCAGT
AGAATACGAGATCTGGCGAAGATTGGGTTTTGAAGACTTCTTGGGAGAAGTTTGGAAACAAGGGCATAGA
AAGACCACCCTCAAGGATTATACCGCAGGTATAAAAACTTGCATCTGGTATCAAAGAAAGAGCGGGGACG
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AATCAAAGGAGCCTTTTGCGGTGACGATAGTCTGCTGTACTTTCCAAAGGGTTGTGAGTTTCCGGATGTG
CAACACTCCGCAAATCTTATGTGGAATTTTGAAGCAAAACTGTTTAAAAAACAGTATGGATACTTTTGCG
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TGGTGCTAAACACATCAAGGATTGGGAACACTTGGAGGAGTTCAGAAGGTCTCTTTGTGATGTTGCTGTT
TCGTTGAACAATTGTGCGTATTACACACAGTTGGACGACGCTGTATGGGAGGTTCATAAGACCGCCCCTC
CAGGTTCGTTTGTTTATAAAAGTCTGGTGAAGTATTTGTCTGATAAAGTTCTTTTTAGAAGTTTGTTTAT
AGATGGCTCTAGTTGTTAAAGGAAAAGTGAATATCAATGAGTTTATCGACCTGTCAAAAATGGAGAAGAT
CTTACCGTCGATGTTTACCCCTGTAAAGAGTGTTATGTGTTCCAAAGTTGATAAAATAATGGTTCATGAG
AATGAGTCATTGTCAGAGGTGAACCTTCTTAAAGGAGTTAAGCTTATTGATAGTGGATACGTCTGTTTAG
CCGGTTTGGTCGTCACGGGCGAGTGGAACTTGCCTGACAATTGCAGAGGAGGTGTGAGCGTGTGTCTGGT
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TTTCAGTTCAAGGTCGTTCCCAATTATGCTATAACCACCCAGGACGCGATGAAAAACGTCTGGCAAGTTT
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TATTGTTTATAGAAATAATATAAAATTAGGTTTGAGAGAGAAGATTACAAACGTGAGAGACGGAGGGCCC
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TTCGATCTCGAACCGGAAAAAAGAGTGATGTCCGCAAAGGGAAAAATAGTAGTAGTGATCGGTCAGTGCC
GAACAAGAACTATAGAAATGTTAAGGATTTTGGAGGAATGAGTTTTAAAAAGAATAATTTAATCGATGAT
GATTCGGAGGCTACTGTCGCCGAATCGGATTCGTTTTAAATAGATCTTACAGTATCACTACTCCATCTCA
GTTCGTGTTCTTGTCATGGCTTTGTGGTACTTGTTCAATAAAAGGAGTCTTGGTGCAGCTGTCCTTATAC
TTGTTGGGCTGTTAATGTGCAATATTCAAATGACAGGAGCTAGTAACGGGATTGAGGCATCCCTGTTAA
CCGATCCGAAGGATGTCTCCGGGAGGACGGTAGACTACATAATTGCAGGAGGAGGACTGACAGGTCT
TACTACCGCAGCAAGGCTTACAGAAAACCCAAATATATCAGTGCTTGTAATTGAGTCTGGTAGTTAC
GAGTCCGACAGGGGGCCAATTATCGAGGATTTAAACGCATACGGGGATATTTTCGGTAGTTCAGTTG
ATCATGCTTATGAAACCGTTGAATTGGCCACCAACAACCGTACCGCCTTGATTAGATCAGGAAACGG
ATTGGGCGGATCTACGCTTGTTAATGGTGGAACATGGACCAGGCCCCATAAGGCTCAGGTAGATAGT
TGGGAGACTGTCTTTGGCAATGAAGGGTGGAACTGGGACAACGTAGCAGCTTATTCACTGCAGGCTG
AAAGGGCCCGTGCCCCGAACGCTAAACAAATTGCCGCAGGCCACTATTTCAATGCTTCCTGTCACGG
CACTAACGGTACTGTACACGCAGGCCCCCGAGACACAGGCGACGACTACTCTCCCATAGTAAAAGCA
CTTATGTCAGCAGTAGAAGACCGTGGGGTACCCACGAAAAAGGACTTCGGGTGTGGCGATCCACACG
GGGTTTCTATGTTCCCCAATACTCTCCACGAAGACCAAGTTCGTTCAGATGCCGCCCGAGAATGGCT
CCTGCCCAACTATCAACGACCAAATCTTCAAGTTCTTACTGGCCAGTACGTGGGCAAAGTTCTCCTC
TCACAGAATGGTACAACACCCCGTGCAGTAGGGGTCGAATTTGGTACACACAAGGGAAACACGCATA
ATGTTTACGCCGAACATGAAGTGTTGCTGGCCGCTGGATCAGCCGTTTCACCGACCATCTTAGAATA
TTCCGGAATAGGCATGAAAAGTATACTTGAGCCGTTGGGAATCGACACAGTGGTGGATTTACCGGTC
GGGCTCAACTTACAGGATCAAACGACAGCTACGGTTCGATCAAGGATAACAAGTGCCGGAGCCGGCC
AGGGTCAAGCAGCATGGTTTGCTACTTTCAATGAAACCTTCGGGGACTACAGTGAAAAGGCTCACGA
ATTGCTCAATACGAAACTTGAGCAGTGGGCCGAAGAAGCAGTTGCTAGGGGGGGCTTTCACAACACG
ACGGCCTTGCTCATCCAGTATGAAAACTATCGAGACTGGATAGTGAACCACAATGTGGCATACTCAG
AACTGTTTTTAGACACTGCAGGCGTAGCCAGTTTCGACGTGTGGGACTTACTTCCTTTCACGAGAGG
TTATGTACATATTCTTGACAAGGACCCCTATCTCCACCACTTCGCTTATGATCCCCAATATTTTCTT
AACGAACTGGATCTCTTAGGTCAAGCCGCTGCTACCCAACTCGCTCGAAATATCAGTAATAGCGGAG
CTATGCAAACATATTTCGCCGGTGAGACGATTCCGGGTGATAATCTGGCTTACGACGCAGATTTGTC
CGCCTGGACAGAGTACATTCCCGAGCACTTTAGGCCTAATTATCATGGAGTTGGAACGTGCTCAATG
ATGCCCAAGGAAATGGGAGGGGTCGTGGATAACGCTGCTAGAGTATATGGTGTTCAAGGTCTTCGTG
TAATAGATGGCTCCATCCCACCTATGCAGATGAGTAGCCACGTCATGACAGTTTTCTATGCCATGGC
ACTTAAGATCTCAGATGCTATATTGGAAGATTACGCATCAATGCAGTGAGGTCCTGCAACTTGAGGTA
GTCAAGATGCATAATAAATAACGGATTGTGTCCGTAATCACACGTGGTGCGTACGATAACGCATAGTGTT
TTTCCCTCCACTTAAATCGAAGGGTTGTGTCTTGGATCGCGCGGGTCAAATGTATATGGTTCATATACAT
CCGCAGGCACGTAATAAAGCGAGGGGTTCGAATCCCCCCGTTACCCCCGGTAGGGGCCCA

Table S1. PCR primers used for cloning purposes.

Primer	Orientation	Sequence	Product
D4377	Forward	5'ctcagttcgtgttcttgtcaTGGCTTTGTGGTACTTG TTCAATAAAA-3'	TMVΔCP-SP-GOX
D4556	Reverse	5'ctacctcaagttgcaggaccTCACTGCATTGATGCG TAATC-3'	

Table S2. LC-MS/MS identification of supernumerary proteins in apoplastic fluid of *N. benthamiana*.

Protein band	Estimated MW (KDa)	Uniprot accession	Description	Expected location	Expected MW (KDa)[a]
P1	54	P48709	Ribulose bisphosphate carboxylase large chain *Nicotiana debneyi*	Chloroplast	53
P2	34	Q9XIV8	Peroxidase N1 *Nicotiana tabacum*	Extracellular	33
P3	25	P17514	Acidic endochitinase Q *Nicotiana tabacum*	Extracellular	25
P4	22	P13046	Pathogenesis-related protein R major form *Nicotiana tabacum*	Vacuolar	22

[a]Calculated without signal peptide, when applicable.

General Discussion

General Discussion

Plants are fundamental organisms for human beings. They serve as an excellent repository of resources through their use as food, fibers or fuel, and indirectly as a source of high-value pharmaceutical or industrial products. The objective of plant molecular farming is to increase plant-derived resources converting these organisms into biofactories to obtain more sustainably pharmaceutical or industrially relevant products (Buyel, 2019; Kalia, 2018; Long et al., 2022; Shanmugaraj et al., 2020). Viruses possess biological properties that can be harnessed to develop genetic tools enabling transient expression while avoiding stable transformation of the plant, thus achieving the desired product in a matter of days and with high scalability (Y. Gleba et al., 2007; Molina-Hidalgo et al., 2021; Peyret & Lomonossoff, 2015). Since their discovery, plant viruses have become a powerful molecular tool for recombinant protein expressions. Viruses are obligatory parasites despite having tiny genomes, and upon hijacking the host cell machinery, they synthesize large quantities of their own proteins (Balke & Zeltins, 2019; Zahmanova et al., 2023). Taking advantage of this capability, plant viruses are modified in a way that allows to insert the desired heterologous sequences and transport them into the interior of the plant cell, triggering the necessary mechanisms for expression (Abrahamian et al., 2020; McGarry et al., 2017; Pasin et al., 2019; Sainsbury et al., 2010; M. Wang et al., 2020). While plant virus research has primarily focused on the devastating effects they cause, the generation of viral vectors for transiently expressing heterologous proteins of interest has opened new doors in plant virology and has changed the negative perception of plant viruses(Dawson et al., 2015; K. L. Hefferon, 2012; Pogue et al., 2002; Sainsbury & Lomonossoff, 2014; M. Wang et al., 2020). This thesis has investigated how to increase industrial and therapeutic relevant protein accumulation using plant viral vectors. The thesis has also explored the use of potyvirus proteases as biotechnological tools for in vivo processing of

recombinant proteins. This work has contributed to the concept of the tremendous versatility that plant viruses offer as tools for biotechnology.

Plant virus vectors have undergone a significant transformation and have become prominent molecular tools for expressing recombinant proteins. TMV-based vectors have been widely used to produce recombinant proteins in plants due to their ability to produce large quantities of their CP in infected plant cells (K. Hefferon, 2017; Zahmanova et al., 2023). Leveraging this capability, in TMV vectors mediated by *A. tumefaciens* used for recombinant protein production, a portion of the CP is deleted to incorporate a new ORF corresponding to the gene encoding the desired heterologous protein (Lindbo, 2007a; Shi et al., 2019).

In the first chapter of this thesis, an opportunity for improvement was sought to increase the accumulation of the inserted heterologous genes to produce the desired recombinant protein. The possibility of maintaining the 5' and 3' ends of the coding region of the TMV CP was explored by flanking the cDNA of the protein of interest. These untranslated regions can aid in accumulation as they contain essential elements for properly expressing the corresponding mRNA for the recombinant protein. It has been confirmed that this indeed occurs, and the results have shown that the 5' amino terminus is involved in increased expression. However, a concern arose that by adding the 5' end, the recombinant protein would have extra residues that could affect conformation or subsequent folding. This poses a problem in drug production as it could impact viability, in addition to regulatory issues.

The genus *Potyviru*s, one of the most important plant virus groups, consists of single-stranded positive-sense RNA viruses with a genome composed of a single ORF that translates into a large polyprotein. This polyprotein is processed by three viral proteases, generating different viral gene products (Revers & García,

2015). Most of the proteolytic processing is dependent on the activity of NIaPro, which recognizes a seven-amino-acid motif (-6/+1) around the different processing sites. The strict specificity of NIaPro for its substrate and its efficient catalytic activity has made this protease the subject of many biotechnological applications(Cesaratto et al., 2016; Xiao et al., 2022). Taking advantage of the cleavage specificity of NIaPro and proteases recognizing other substrates derived from different potyvirus species, the possibility of utilizing NIaPro's substrate specificity was investigated. A cleavage site was added between the 5' end flanking the heterologous gene and the gene itself, allowing *in vivo* processing and removing undesired residues from the recombinant protein. The specific 7-amino-acid cleavage sites from the NIaPro of TEV and TuMV were used. Each TMV construct was co-expressed with the corresponding protease for the cleavage site, resulting in a significantly positive effect on the accumulation of the three tested proteins: GFP, IFNα2a, and Nanobody72.

It is important to note that accumulation was not affected when NIaPro was co-expressed without a cleavage site between the NCP moiety and the protein of interest. This finding suggests that our observation is not the result of an indirect effect of NIaPro on the host cell but rather a process in which the NIaPro cleavage site is recognized and cleaved in the recombinant protein by the protease. This phenomenon was never described before, and we do not know precisely why this accumulation increase occurs. However, we observed that NCP-*tev*-IFNα2a, even in the absence of NIaPro, showed some degree of cleavage towards the mature form of IFNα2a. This suggests that endogenous plant proteases may be capable of recognizing the NIaPro cleavage site and partially cleaving the chimeric recombinant protein. These approaches open the possibility of incorporating this proteolytic processing to enhance the accumulation of recombinant proteins. It should be mentioned that potyvirus

NIaPro proteases are trypsin-like serine proteases and often have numerous cellular homologs (Cesaratto et al., 2016; Di Cera, 2009).

In this work, interesting therapeutic proteins have been produced, particularly Nanobody72, which is of great importance given the current context and could be used for developing vaccines against COVID-19. Furthermore, the ability of plants to produce monoclonal antibodies (mAbs) with quality and characteristics comparable to those produced in mammalian cells has already been demonstrated (Chen, 2022; England et al., 2023). Although plant-based vaccines represent a significant advancement due to cost reduction and rapid availability of the final product, certain issues still need to be addressed, such as batch-to-batch consistency, dose standardization, and product yields dependent on environmental factors. Plant-based vaccines are subject to strict regulatory requirements, mainly when produced in genetically modified plants. The use of transgenic technologies poses additional challenges that need to be addressed, including biosafety concerns and acceptance by people (Lee et al., 2023; Su et al., 2023b).

On the other hand, plants represent a widely underutilized source of bioactive compounds with a broad spectrum of antiviral activities that could be harnessed by seeking to increase their accumulation while expressing the desired recombinant protein (England et al., 2023). Molecular farming, when all the shortcomings are overcome, has the potential to become an important source of vaccines against viral diseases. We hope that as clinical trials provide more evidence of their safety and their efficacy is further tested, they will be better accepted, and regulations concerning them will change.

In the second chapter of the thesis, a viral vector derived from TMV was used, which had a deleted portion of the CP. This allowed the viral vectors to move from cell to cell but not systemically throughout the plant. Three constructions

were made using this vector, incorporating some improvements to explore its different capabilities for expressing a recombinant protein, specifically xylanase11 (Xyn11). This enzyme is of industrial interest as it is used, among other applications, in the paper industry and the hydrolysis of polysaccharides into simple sugars (G. K. Gupta et al., 2022; Walia et al., 2017). Of the three constructs, that in which an apoplast signal sequence was added at the amino-terminal end showed the highest accumulation. This also facilitated its subsequent purification, as a simple apoplastic extraction followed by heat treatment allowed the protein to be fully prepared for further use. Similar enzymatic properties were observed when comparing the Xyn11 produced by the viral vector in *N. benthamiana* and that made in *E. coli*. They exhibited similar activities at alkaline pH and high temperature, which are conditions required for various industrial applications. The yield of Xyn11 produced in *N. benthamiana* is comparable to that of *E. coli*, specifically 20 mg per liter of *E. coli* culture and 830 mg per kg (fresh weight) of *N. benthamiana* leaves. The results of the enzyme produced in plants are entirely comparable to those obtained in *E. coli*, making it an excellent alternative to traditional heterologous systems for recombinant protein production. In the literature, it is described that adding arabinogalactan at the carboxy-terminal end could have a positive effect, increasing the accumulated protein. This chapter briefly explored this possibility, but no positive effect was observed. However, it is possible to expand this study to determine if it could be another finding that would help improve the TMV viral expression vector by increasing the accumulation of recombinant proteins (Xu, J., 2007).

Finally, in the third chapter of the thesis, the viral vector derived from TMV (Shi et al., 2019) that yielded good results with Xyn11 was utilized for the expression of GOX. A high protein accumulation yield was obtained, indicating that the plants, specifically *N. benthamiana*, are a suitable host for GOX production. It is

important to highlight that the recombinant GOX is directed to the secretory pathway through fusion with an N-terminal peptide derived from *N. tabacum* (1-3)- β-endoglucanase, resulting in protein accumulation in the apoplastic space (Diego-Martin et al., 2020). This specific cellular localization, situated between the cytoplasmic membrane and the cell wall, allows for the production of a virtually purified protein since there is a low abundance of proteins in this cellular compartment. Moreover, the apoplastic content can be easily isolated from the rest of the plant tissues through simple buffer infiltration and subsequent tissue compression using a manual syringe, enabling the convenient recovery of a protein preparation enriched with the recombinant protein of interest. The antimicrobial activity of GOX is well-known (Bauer et al., 2022; F. Wang et al., 2022). Our results with the engineered GOX produced in *N. benthamiana* confirm its antimicrobial property, demonstrating a highly potent bactericidal effect against *E. coli* compared to *S. aureus*. This is evident from the reduction in viable *E. coli* cells observed in the cell suspensions treated with increasing amounts of the enzyme. Notably, the disk diffusion test shows a more pronounced inhibition of growth. With this, we have another example of recombinant proteins produced in plants that are active in their function and can be easily obtained on a large scale, ready for their final purpose in just a few days.

Chapters II and III have demonstrated the great potential of plants to produce industrially relevant enzymes in a fast and cost-effective manner. The door is wide open for further improvement of the TMV vector as a powerful tool for expressing heterologous proteins of interest. This work also suggests that we are getting closer to creating greener products with less environmental impact during production. Although there is still a long way to go, further research is needed to address the challenges in molecular farming and make it a more widely accepted and promising practice.

One of the problems encountered in the production of recombinant proteins is that endogenous proteases, which act redundantly and consecutively, can reduce the yield of the protein product. However, by expressing protease inhibitors, adjusting pH, or gene suppression, it is possible to increase the yield of recombinant proteins (Y. Wang & Demirer, 2023). All these aspects must be considered in new designs to continually improve and increase recombinant protein yield. Another challenge in therapeutic protein production lies in the differences in glycosylation pathways. Plant-specific glycans differ from those found in humans and most mammalian glycoproteins. Non-human glycophores, such as β 1,2-xylose and α1,3-fucose, can be perceived as foreign by the immune system, potentially causing immunogenicity and allergic responses. To address these limitations, various glycoengineering strategies have been developed, including "humanizing" plant N-glycans by eliminating genes responsible for plant-specific glycosylation using the CRISPR/Cas9 system, introducing humanized glycosylation pathways into plants, and coexpressing chaperones and mammalian folding enzymes compatible with the endogenous chaperone machinery of plants. These glycoengineering tools aim to modify plant glycosylation pathways and improve the quality of plant-derived pharmaceutical products in the future (Narayanan & Glick, 2023; Zahmanova et al., 2023).

Furthermore, work is being done to improve plants to increase their yield and make them resistant to various types of abiotic stress and a wide range of fungal and bacterial pathogens. Other improvements include glycosylation to achieve better pharmaceutical products and the ability to develop drugs entirely produced in plants, such as vaccines for different viral diseases, as well as enhancing their nutritional value. Another approach would be to utilize plants to deliver the drug produced within the plant. The goal is to express the therapeutic protein (antigen, antibody, etc.) in the selected plant. Once it has been produced, animal models are immunized using seeds, plant organs, or

lyophilized plants, either as biomass or encapsulated. Alternatively, at the clinical level, the objective is to safely immunize humans with that plants that express the therapeutic protein, which can result in an efficient antibody response to prevent and treat the targeted diseases (Fig. 1) (Bravo-Vázquez et al., 2023; Lee et al., 2023; Narayanan & Glick, 2023).

Although several products of molecular farming in plants have already been developed, technical, economic, and social challenges still need to be addressed before their full potential can be realized. What efforts are necessary to accelerate the process of translating laboratory discoveries into real-world applications? Is it possible to scale these synthetic biology approaches in plants at the field level? Can we reduce the high cost of downstream processing in plant molecular farms? Methods of scaling and production need to be improved, and concerns regarding biosafety and regulation must be addressed. We are currently in a flourishing stage of synthetic plant biology and molecular farming, and by addressing these outstanding challenges, we can substantially impact generating of sustainable food and biological products for human and planetary health (Y. Wang & Demirer, 2023).

Biotechnology is advancing by leaps and bounds, and there is no doubt that it will be an essential discipline for addressing challenges such as climate change and feeding the continuously growing global population. Tools are gradually emerging to produce foods with enhanced properties and improved health benefits, and research is also being conducted to develop pest-resistant crops to mitigate the significant financial losses caused by pests. However, it is also crucial to continue seeking tools that allow for the reduction of pesticides and agrochemicals that contaminate the environment and affect living organisms in the surrounding ecosystems (Abrahamian et al., 2020; Pasin et al., 2019; M. Wang et al., 2020).

Hopefully, with the advancement of this discipline, we can achieve these objectives and progress towards a greener and more ecological planet. However, one major challenge that still exists and needs to be addressed is the negative perception of products labelled as genetically modified organisms (GMOs). It is essential to open our minds to the fact that products derived from plants offer certain advantages over their synthetic counterparts (Arya et al., 2020; Chouhan et al., 2017). It is important to acknowledge that controversy and debates will persist since these products involve viruses, even if they are derived from plants and do not infect humans or animals.

The advancements described in this thesis are expected to contribute to the toolbox of biotechnology, specifically in producing high-value recombinant proteins in plants. This will lead to an increasing number of environmentally friendly products in the market and contribute to a more positive perception of the use of viral vectors for this purpose. As demonstrated in the three chapters of this thesis, plant viruses, apart from being responsible for nearly half of plant diseases and causing significant economic losses in agriculture, can also be our allies and be utilized for beneficial applications (Abrahamian et al., 2020; Pasin et al., 2019; M. Wang et al., 2020). Plant viruses have the potential to become our greatest allies, transforming into valuable tools for biotechnology.

Fig. 1. Future directions or the developments of plant-based recombinant proteins. One of the main advantages of plants is that they can serve as a delivery vehicle for pharmaceuticals, so this property must be taken as an advantage and improved. Humanization of glycosylation pathways in plants can improve the quality of pharmaceutical products. Additionally, improvements can be made to the viral expression vectors, such as TMV, to enhance its capabilities. Lastly, several biotechnological techniques can be utilized to produce recombinant proteins in plants efficiently.

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Conclusions

Conclusions

1. The production of some recombinant proteins in plants using tobacco mosaic virus (TMV)-derived vectors can be enhanced by incorporating a translational fusion of the amino-terminal fragment of the TMV coat protein (CP) into the target recombinant protein, increasing its accumulation in plant tissues.

2. The accumulation of some recombinant proteins can even be improved by inserting a tobacco etch virus (TEV) or turnip mosaic virus (TuMV) nuclear inclusion *a* protease (NIaPro) cleavage site between the TMV CP amino-terminal moiety and the recombinant protein, when the cognate NIaPro is co-expressed. The TMV CP amino-terminal tag undergoes significant cleavage, resulting in an almost mature protein, but a single amino acid imposed by the residue of the NIaPro cleavage site.

3. The thermophilic xylanase Xyn11 from *Pseudothermotoga thermarum* DSM 5069 can be efficiently produced in *Nicotiana benthamiana* plants using a TMV expression, reaching a yield that can be considered comparable to that obtained in *Escherichia coli*. Targeting the recombinant protein to the apoplast of *N. benthamina* facilitated the purification process.

4. The plant-derived Xyn11 exhibits excellent catalytic activity under alkaline pH and high-temperature conditions, comparably to that produced in *E. coli*. Furthermore, the plant-derived enzyme surpasses its bacterial counterpart in terms of catalytic activity at 90°C and pH 9.0, which are favorable reaction conditions for industrial enzymatic xylan hydrolysis.

5. An engineered variant of glucose oxidase (GOX) was efficiently produced in *N. benthamiana* using a TMV vector. The plant-made recombinant protein

exhibits a potent bactericidal action against *Staphylococcus aureus* and *E. coli*.

6. The TMV vector holds a wells-knonpotential to produce recombinant proteins of industrial and pharmaceutical interest in biofactory plants. Remarkably, investigation on this vector still allows optimization of the production process.

Acknowledgements / Agradecimientos

Quiero aprovechar estas páginas para agradecer a toda la gente que, de una u otra manera, me habéis acompañado durante esta etapa de la tesis. Sin vuestra ayuda y apoyo, llegar hasta aquí no hubiese sido igual.

En primer lugar, a mi director José Antonio, por haber confiado en mí a pesar de venir de un mundo totalmente distinto de las plantas. Me has formado y orientado durante la etapa predoctoral, aconsejándome y haciendo que mi entusiasmo por el mundo de las plantas creciese. Gracias por los buenos consejos, la paciencia y la dedicación. También a mi tutor académico Carmelo, siempre tan atento para solucionar cualquier duda con trámites de estancias y escuela de doctorado.

A Renier Van der Hoorn mi director durante la estancia en Oxford por permitirme vivir la experiencia de aprender en un lugar tan entrañable y a todos mis compañeros de laboratorio por convertirlo en una experiencia increíble y hacerme sentir una más desde el principio.

A mis compañeros del laboratorio del 1.04, MariCarmen i Beltran els meus imprescindibles i el meu TOT EL DIA sense vosaltres res haguera sigut possible. Gràcies per estar al meu costat incansablement per ajudar-me en tot i per escoltar-me sempre sempre sempre, gràcies per guiar-me pel millor camí per arribar fins aci. Mai oblidaré tot el que em viscut en aquesta etapa junts, sou enormes. A Vero la que siempre está y siempre tiene una solución para todo, gracias por tus super consejos y trucos, echaré de menos nuestros cafés y comidas. A Mireia la que nunca cesaba su energía fuesen las 8 de la mañana o las 18 de la tarde y que a pesar de todo siempre tenía buenos consejos. A Arcadio que siempre tiene una sonrisa y una buena palabra espero que disfrutes mucho de esta etapa y que fluyas un poco más. A Fakhri, Alessandra, Javi,

Fernando, Enrique, Lucia, Marie, Diego, Thais, Marcelo gracias por aportar vuestro granito de arena haciendo todo mejor en esta etapa. A Miryam que, aunque no sea del 1.04 siempre ha sido una más gracias por estar siempre con tu alegría y tu energía.

A mis amigos del IBMCP, Bea, Maria Jesús, Rubén, Buri, Maria, Salva gracias por todas las charlas de desahogo y por todos los momentos que hemos vivido espero poder seguir contando con vosotros y seguir sumando vivencias i festivales.

I com no als meus incondicionals, els meus pares i familia. Gràcies per aguantar-me quan ni jo mateixa podia, per soportar les meues ploreres infinites i per a pesar de tot estar sempre al meu costat incansables animan-me a seguir endavant contagian-me la vostra alegría i força. Gràcies papa i mama per confiar en mi i desviures per mi en cada moment i etapa de la meua vida esta tesis també es un poquet vostra perque sense vosaltres no haguera arribat on estic. A la meua estrella que sé que des d'alli dalt estaràs ben orgullosa de vorer tot el que estem aconseguint. VOS ESTIME.

¡Para dejar por escrito que no voy a abandonar y que todo lo que importa está en el aire!

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