Towards nutritionally complete crops:
The creation of FoliFerA potato

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Preface

The work presented in this thesis was performed at the Functional Plant Biology Laboratory in the Department of Biology from the Faculty of Sciences of Ghent University (Ghent, Belgium), during the period February-September 2019, under the supervision of Prof. Dr. Dominique Van Der Straeten, and within the frame of the Erasmus programme. The thesis was co-supervised at Instituto Superior Técnico by Prof. Dr. Isabel Maria De Sá Correia Leite de Almeida.

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa**.**

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Abstract

Micronutrient malnutrition, a condition named “The hidden hunger”, affects mainly pregnant and lactating women and children. Although clinical symptoms are not always evident, lifelong health complications arrive from this. Different health improvement programs have been established to tackle this problem, including supplementation and fortification strategies. Biofortification programs have gained increasing attention since they represent a cost-effective and sustainable initiative to complement low-nutritional value diets, requiring little cultural adaptation and being easily introduced in regular diets. Different approaches for biofortification exist, however transgenic methods are the most promising as they are highly flexible and relatively rapid to obtain. In this work the expression vectors for the creation of multi-biofortified potato tubers were designed and constructed. This relied on the utilization of a novel cloning strategy, named Golden Braid, which allows a modular and iterative vector construction process. In this way, fifty-nine different vectors were produced, that can be used in the future for plant transformation, aiming for the enhancement of provitamin A (beta-carotene), iron and folate (vitamin B9). Additionally, in search of appropriate promoters to use in this project, the potential of a *Solanum tuberosum* promoter in driving root specific expression was revisited, showing that there is a need for updating DNA sequences repositories with newly curated information.

Key words: Micronutrient deficiency, Biofortification, Metabolic engineering, Folate, Vitamin A, Iron

Resumo

A deficiência de micronutrientes, uma condição popularizada como “A fome oculta”, afeta uma larga percentagem de mulheres em idade reprodutiva, bem como crianças, podendo conduzir ao desenvolvimento de diversas patologias, que nem sempre são acompanhadas por sintomas clínicos evidentes. De modo a erradicar este tipo de condições, vários programas de saúde pública foram já desenvolvidos, incluindo programas de suplementação e fortificação. A biofortificação tem ganho cada vez mais relevo, uma vez que este tipo de intervenção tem um elevado custo-benefício e é uma estratégia sustentável, que permite complementar dietas com baixo valor nutricional. Apesar de existirem diferentes tipos de estratégias para biofortificar alimentos, os organismos transgénicos apresentam maior promessa, uma vez que há maior flexibilidade de produção e podem ser obtidos em curto-prazo. Este projeto visa a conceptualização e construção de plasmídeos para a transformação de *Solanum tuberosum*, de modo a obter tubérculos biofortificados em vários micronutrientes. Este objetivo foi atingido através da utilização de uma nova metodologia de clonagem, denominada Golden Braid, que permite a construção de plasmídeos de um modo iterativo e modular. Deste modo, foram construídos cinquenta e nove plasmídeos distintos, que serão futuramente utilizados para a transformação de batata, visando aumentar o conteúdo de provitamina A (betacaroteno), ferro e folato (vitamina B9) no tubérculo. Adicionalmente, com o objetivo de encontrar promotores adequados a este projeto, foi analisado o padrão de expressão de uma família de genes proveniente de *Solanum tuberosum*. Esta análise preliminar reforçou a necessidade de atualizar repositórios de sequências de DNA com informação atualizada.

Palavras-chave: Deficiências em micronutrientes, Biofortificação, Engenharia Metabólica, Folato, Vitamina A, Ferro

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List of Abbreviations

MND - Micronutrient deficiency;

RDA - Recommended daily allowance;

RDI - Recommended daily intake;

ABA - Abcsisic acid;

RA - Retinoic acid;

VAD - Vitamin A deficiency;

GA3P - glyceraldehyde 3-phosphate;

IPP - isopentenyl diphosphate;

DMAPP - dimethylallyl diphosphate;

GGPP - geranylgeranyl diphosphate.

DXS - 1-deoxy-d-xylulose 5-phosphate synthase;

DXR - 1-deoxy-d-xylulose 5-phosphate reductoisomerase;

GGPPS - geranylgeranyl pyrophosphate synthase;

PSY - phytoene synthase;

PDS - phytoene desaturase;

Z-ISO - z-carotene isomerase;

CrtISO - carotene isomerase;

LycB - lycopene β-cyclase;

LycE - lycopene α-cyclase;

BCH - β-carotene hydrolase;

ZEP - zeaxanthin epoxidase;

VDE - violaxanthin de-epoxidase;

NXS - neoxanthin synthase;

Crt B - Bacterial phytoene synthase;

Crt I - Bacterial phytoene desaturase;

Crt Y - Bacterial β-cyclase;

Or – Orange;

CCD - carotenoid cleavage dioxygenases;

SNP - single nucleotide polymorphism;

DHF – dihydrofolate;

THF - tetrahydrofolate;

SAM - S-adenosylmethionine;

NTD - neural tube defect;

ADC - aminodeoxychorismate;

p-ABA - para-aminobenzoate;

DHN-P3 - dihydroneopterin triphosphate;

DHN-P - dihydroneopterin monophosphate;

DHN - dihydroneopterin;

HMDHP - 6-hydroxymethyldihydropterin;

HMDHP-P2 - HMDHP pyrophosphate;

DHP - dihydropteroate;

Glu - glutamate;

GTP - Guanosine-5'-triphosphate;

ADCS - ADC synthase;

ADCL - ADC lyase;

GTPCHI - GTP cyclohydrolase I;

DHNTPPH - dihydroneopterin triphosphate pyrophophohydrolase;

NSP - non-specific phosphatase;

DHNA - DHN aldolase;

HPPK - HMDHP pyrophosphokinase;

DHPS - DHP synthase;

DHFS - DHF synthetase;

DHFR - DHF reductase;

FPGS - folylpolyglutamate synthetase;

FBP - folate binding protein;

PS – phytosiderophores;

FRO2 - ferric reductase oxidase 2;

IRT1 - iron-regulated transporter 1;

NA - nicotianamine;

NAAT - NA aminotransferase;

NAS - NA synthase;

PS - phytosiderophore;

SAM - S-adenosyl methionine;

YS1 - yellow stripe 1;

NA – nicotinamide;

NAS - nicotinamide synthase;

DMA - deoxymugineic acid;

NAAT - nicotinamide aminotransferase;

IDA - Iron-deficiency anaemia;

VIT - Vacuolar iron transporter;

lpa - low phytic acid;

CIP - International Potato Centre;

GB - golden braid;

TsP - tuber specific promoter;

RsP - root specific promoter;

IP - inducible promoter;

CP - constitutive promoter;

T – terminator;

CDS - coding sequence ;

RS - recombination site ;

TU - transcription unit ;

Nos - nopaline synthase ;

OCS - octopine synthase ;

GFP - green fluorescent protein.

1- Introduction

1.1- The Hidden hunger

Two billion people worldwide suffer from micronutrient deficiencies (MND), collectively known as “The Hidden Hunger”. This deficiency state is linked with different circumstances, such as, poor diets, increased needs due to metabolic adjustments linked with age, or specific health conditions (Von Grebmer et al., 2014). The major risk groups for micronutrient deficiencies are pregnant and women of reproductive age, young children and the elderly, especially in low-income countries (Muthayya et al., 2013). Nevertheless, MNDs can affect human health at any life stage, impairing proper cognitive development and optimal performance (Von Grebmer et al., 2014).

Micronutrients are a broad category of molecules, including vitamins (like vitamin A, vitamin B9 or vitamin B12) and minerals (such as zinc, iron and iodine). These compounds are key to optimal nutrition (Bailey et al., 2015), since they are known to participate in a number of biochemical processes, required for normal cellular functions. The recommendations for their intake depend on each country’s policy, but they can be defined through an RDA (recommended daily intake), that is an average daily intake for each nutrient that meets the requirements of 97% of a healthy population (“Nutrient Recommendations,” 2015). However, it can be generally stated that they will a have a value for which a clinical deficiency will occur, if the micronutrient is in a concentration below it (Shenkin, 2006).

Although it is difficult to pinpoint the exact factors that lead to the appearance of an MND, low nutritional-value diets are a very common cause, particularly in low-income countries (Virot, 2007). These monotonous diets are mainly based on starchy crops, like wheat, rice, cassava and potato, which deliver high caloric intakes, however lack sufficient levels of minerals and vitamins. Because these populations lack sufficient access to micronutrient-dense foods, such as fruits and vegetables, they are commonly victims of deficiencies in multiple micronutrients (Brinch-Pedersen et al., 2007).

In terms of worldwide prevalence, the most common mineral deficiencies are iron, zinc and iodine deficiency. In respect to vitamins, vitamin A, vitamin B9 (folate) and vitamin B12 (cobalamin) show to have the highest incidence of the corresponding deficiency (Blancquaert et al., 2017). Table 1 contains an overview of these important micronutrients, with their natural dietary sources and major health consequences associated with their deficiency.

Table 1 - Assessment of the dietary sources and deficiency-associated health consequences (pathophysiology) of the micronutrients for which the deficiency is common (Allen, 2006; Tulchinsky and Varavikova, 2014; Gilbert et al., 2019; Zimmermann and Hurrell, 2002)

Micronutrient	Natural food sources	Health consequences derived from deficiency
Iron	Animal-derived foods, legumes, peaches, apples, raisins, prunes, molasses	Iron-deficiency anemia; Fatigue; Poor linear growth in infants; Psychomotor deficiency affecting school and work performance.
Zinc	Animal-derived foods, legumes, peaches, apples, raisins, prunes, molasses	Stunting; Increased susceptibility to infectious diseases; Retarded growth, diarrhea, mental disturbance, delayed sexual maturation and/or recurrent infections, dermatitis.
Iodine	Iodized salt, seafood	Stunting; retardation; cretinism; goitre.
Vitamin A	Yellow/Orange coloured vegetables, animal-derived products	Night blindness; Loss of color vision, corneal dryness, ulceration and scarring, blindness; Poor bone and tooth formation; Susceptibility to infection and poor survival rates from infectious diseases.
Vitamin B9 (Folate)	Whole-grain cereals, legumes, leafy vegetables, meat, and dairy foods	Megaloblastic anaemia of pregnancy; Neural tube defects; Elevated homocysteine (possible links to coronary heart disease and mental deterioration with aging).

1.2- Strategies for eliminating micronutrient deficiencies

Diets containing a high food diversity should be sufficient to satisfy all micronutrient and energetic requirements of an individual. However, diet-diversification is not a realistic possibility for a lot of people living in low-income/rural areas (Yadava et al., 2018). As such, because they rely on starchy energy-rich crops, that are poor suppliers of micronutrients (table 2 and table 3), food fortification and supplementation constitute relevant tools to complement these diets. Additionally, if these strategies are used in cohort with educational efforts on food quality and nutritional value, longer-lasting effects on population health might be achieved (Mannar, 2006).

Table 2 - Nutritional composition for iron, zinc, folate and vitamin A, in terms of mg or µg per 100 g of fresh weight (FW) of the most consumed food crops worldwide (Data collected from the USDA National Nutrient Database for Standard Reference Legacy Release)

Micronutrient (in 100g FW)	Wheat (Grain, raw)	Maize (Sweet, yellow)	Rice (White, raw)	Potato (Skin and flesh, raw)	Soybean (Green, raw)	Cassava (Root, raw)	Sweet Potato (Root, raw)	Yam (Root, raw)	Sorghum (Raw, grain)	Plantain (Fruit, raw)	Millet (Grain, raw)
Iron (mg)	3.27	0.52	0.8	0.81	3.55	0.27	0.72	0.52	3.36	0.58	3
Zinc (mg)	2.73	0.46	1.16	0.3	0.99	0.34	0.2	0.2	1.67	0.13	1,68
Vitamin A (µg)	2.7	9	0	0.6	9	3.9	787	6	0	338.1	0
Vitamin B9 (µg)	44	42	8	15	165	27	6	16	20	22	85

Table 3 - Nutritional daily requirements for iron, zinc, folate and vitamin A (in terms of mg or µg), according to the life stage (Data collected from the USDA National Nutrient Database for Standard Reference Legacy Release).

Life Stage	Iron (mg/day)	Zinc (mg/day)	Vitamin A (µg/day)	Vitamin B9 (µg/day)
Infants (6-12 months)	11	3	500	80
Children (4-8 years)	10	5	400	200
Adult Males (19-70 years)	8	11	900	400
Adult Females (19-70 years)	8-18	8	700	400
Pregnant Females	27	12	770	600
Lactating Females	10	13	1300	500

The artificial delivery of micronutrients, either through the form of pills, capsules or syrups – supplementation -, or by adding them to a given processed food – fortification – has proved to alleviate micronutrient deficiency in various situations (Allen et al., 2006). The most successful example is iodine fortified salt, which has, since its introduction in Switzerland and the USA in the 1920s, shown to be successful in reducing the prevalence of goitre and cretinism, two diseases known to be causally linked to iodine deficiency (Mannar, 2006). Also, vitamin A supplementation programmes have proven their ability to significantly reduce child mortality, in children aged from 6 months-old to 5 years-old, in various at-risk countries (Mayo-Wilson et al., 2011).

However, both supplementation and fortification rely on good delivery systems that offer controlled dosage and quality control; cooperation between the food industry and policy makers and specialized technicians and infrastructures, which are limited in most developing countries and are of even more difficult access for rural communities, making its implementation and effectiveness, on the long-term, difficult (Bailey et al., 2015; Harrison, 2010; Van Der Straeten et al., 2017). In response to these

concerns, biofortified foods have gained increasing attention for their potential role in tackling malnutrition, as this strategy aims for the generation of crops with enhanced micronutrient content, that could reach rural and poorer areas and would not depend on efficient delivery systems (Garcia-Casal et al., 2017).

Biofortification technology is used to alter the micronutrient content of an existing crop through one of the following methods: agronomical interventions, conventional plant breeding or genetic engineering (Zhu et al., 2007).

Agronomical approaches typically involve the application of micronutrient-dense fertilizers is the least practised, once it is limited by the application method, soil composition, mineral mobility and accumulation within the target plant. Also, this type of strategy becomes costly as it requires multiple applications, and it can have serious environmental implications since mineral mobility in soil might lead to water contamination (Cakmak, 2002).

Plant breeding is currently the technology with the most public acceptance, as it relies on intrinsic genetic characteristics of the target crop. Essentially, it is based in the crossing of sexually compatible plants with different genetic backgrounds to improve micronutrient levels in progeny. The development of the nutritional genomics field and efficient molecular markers have aided the breeding process, as they allow the evaluation of complex traits throughout development (Mayer et al., 2008). Its main disadvantages are the fact that genetic variation in some plants may be limited in the available germplasm collections, or in a worst-case scenario, the desired trait may be unavailable, which restricts the introduction/selection of interesting traits Also, there is a large timescale for breeding programs, since crossing compatible individuals, selecting interesting progeny and propagating them is a lengthy process (Bouis et al., 2011).

Recombinant DNA approaches present the greatest promise in terms of flexibility, given that they are not constrained by the host endogenous gene pool and allow organ-specific targeting (Mayer et al., 2008). Their aim is to modify endogenous metabolic pathways, or introduce new pathways, that ultimately result in an enhancement of the target product biosynthesis, lower concentrations of an unwanted molecule, or higher target product accumulation (Blancquaert et al., 2017).

To achieve this, there are various possibilities (figure 1), including, increasing the biosynthetic rate by introducing or enhancing expression of one or several biosynthesis genes (‘push’ strategy); decreasing metabolic flux downstream of the target product, either by avoiding product degradation or elimination of a competing metabolic branch (‘block’ strategy); creating an accumulation compartment for the target product, or increasing the sink capacity of an already existing sub-cellular compartment (‘metabolic sink’ strategy); promoting the over-activity of a specific enzyme, causing a depletion of upstream intermediaries, which forces the flux towards a specific metabolic branch (‘pull’ strategy); and increasing long-term stability by the addition of binding agents that prevent degradation, or enhance stability (‘post-harvest stability’ strategy) (Capell and Christou, 2004; Zhu et al., 2007).

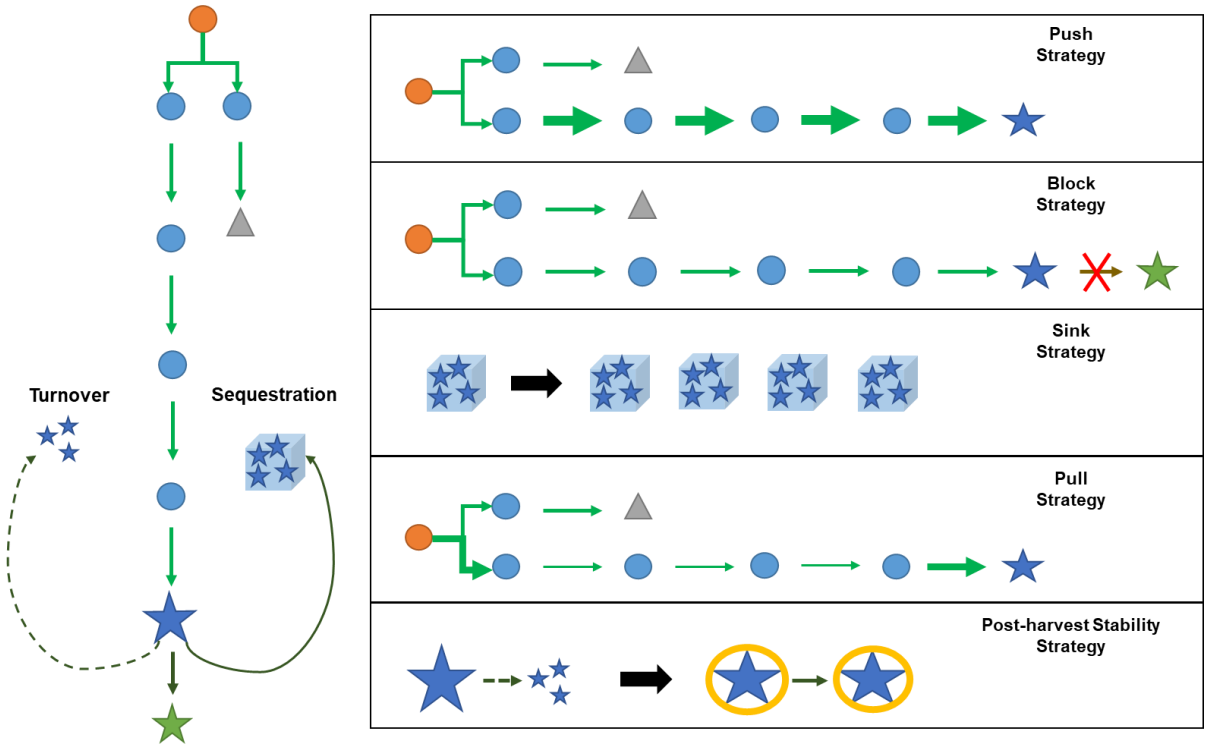


Figure 1 - Schematic representation of the different approaches in a metabolic engineering project, namely, the 'push' strategy, in which which the biosynthetic rate of the metabolic branch of interest is enhanced, amounting in more target molecule of interest; the 'block' strategy, that prevents the degradation and/or conversion of the molecule of interest through the elimination of that/those reaction(s); the 'sink' strategy, that either increases an already existing accumulation or promotes this by creating a compartment for it; the 'pull' strategy, where the flux of a specific branch of the pathway is enhanced through the over-activity of a specific enzyme; and, finally, the 'post-harvest stability' strategy, in which the degradation of the molecule of interest is prevented, or its stability increased, through the addition of a binding agent. In the left panel, a hypothetical metabolic pathway is represented, in which, the precursor molecule is shown by an orange circle; final molecule of interest is represented by a blue star; the pathway intermediaries are represented through the blue circles; and an alternative product is depicted with a grey triangle. Additionally, three different fates of the molecule of interest (blue triangle) are shown, namely, turnover (small blue triangles); sequestration into a subcellular compartment (blue triangles within a blue cube); and conversion into a different metabolite (green star).

All these metabolic engineering approaches must not impair crop yield, development and important organoleptic properties, whilst aiming to increase micronutrient content. Therefore, it is crucial to possess a detailed knowledge on plant metabolism for the biofortification to be successful (Blancquaert et al., 2017).

The main staple crops, such as wheat, rice and potato, are generally insufficient providers of different micronutrients, and it is known that complicated and unstable socio-economical situations promote a higher consumption of these foods by large percentages of the population. As such, these diets often result in the occurrence of multiple MNDs in a more frequent way, than isolated MNDs (Bailey et al., 2015). To obtain plant crops that are capable of addressing the full scope of MND, multi-biofortification appears to be the most prominent approach, since it could tackle multiple deficiencies at once, for which breeding would be more difficult to achieve. This will rely on multigene engineering strategies, that can be achieved through recombinant DNA technology (Naqvi et al., 2009).

So far, there are few examples of multigene metabolic engineering projects. In Naqvi et al., a white corn variety was transformed with 5 distinct constructs, aiming to ectopically overexpress genes involved in folate, ascorbic acid and β-carotene (provitamin A) biosynthesis. This resulted in a significant enhancement of each of the target nutrients, with a single portion of these grains providing the β-

carotene recommended daily intake (RDI), a sufficient amount of folate and 20% of the RDI for ascorbic acid (Naqvi et al., 2009). In Lee et al., the overexpression of the nicotinamide synthase gene, a key intervenient in plant metal assimilation and homeostasis, was performed in rice, resulting in an enhanced iron and zinc concentration in both leaves and seeds (Lee et al., 2009). Also, in transgenic rice overexpressing three nicotinamide synthase genes simultaneously, up to 4-fold increase in iron concentration and 2-fold increase in zinc was observed (Johnson et al., 2011).

The success of any future multi micronutrient (or single micronutrient) metabolic engineering strategies relies on a good fundamental knowledge into each nutrient metabolism, as well as its function in physiology. Additionally, as it has been previously mentioned the crop utilized for the strategy will also, ultimately, dictate its success within the target population. With this in mind, the next sections constitute a summary on the metabolism, pathophysiology and previous engineering approaches regarding the micronutrients of interest. Moreover, the potential of potato tubers in alleviating micronutrient deficiencies is also addressed.

1.3- Micronutrients of interest

1.3.1- Vitamin A

Vitamin A comprises a set of fat-soluble vitamers that can be structurally divided into two main components, a β-ionone ring and an isoprenoid chain (Berdanier, 1995). Its three major isoforms are retinal (an aldehyde), retinol (an alcohol) and retinoic acid (an irreversibly oxidized format of retinol) (Oruch and Pryme, 2012) (figure 2), which arise from modifications in the isoprenoid chain.

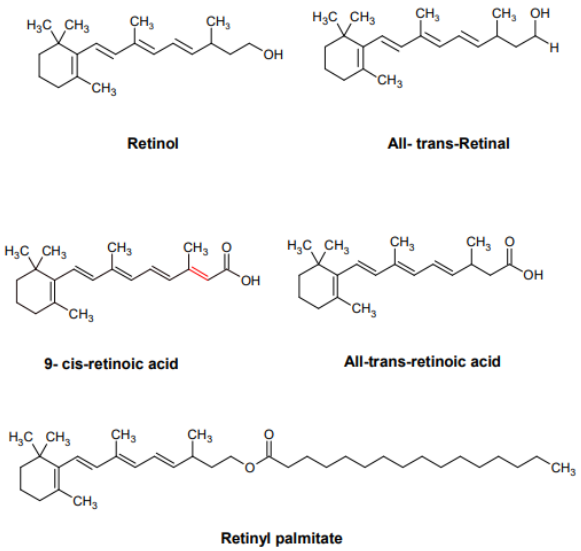


Figure 2 - Representation of the chemical structure of different conformers of vitamin A. Retinol represents the alcohol isoform; retinal represents the aldehyde form; retinoic acid is an oxidized format of retinol; retinyl palmitate is the ester isoform of retinol. (Accessed from Oruch and Pryme, 2012: figure 1 pp.2)

These vitamers result from the conversion of carotenoids, a family of C40 isoprenoid pigments, associated with yellow/red/orange colours in different fruits and vegetables (Giuliano, 2017a), among them β-carotene constitutes the major vitamin A precursor, so it is commonly mentioned as provitamin A (Oruch and Pryme, 2012). The principle dietary sources of this nutrient are animal products as retinol

or retinyl esters (retinyl palmitate) and fruits and vegetables as carotenoids, however in the major staple foods, carotenoids are present at very low concentrations (tables 2 and 3), which causes massive vitamin A deprivation in poor populations, as they largely depend on these cheap, calorie-rich food sources. (World Health Organization and Food and Agriculture Organization of the United Nations, 2004). Additionally, absorption will depend on the lipids that are ingested simultaneously, which might impact the number of carotenoids that will be converted to provitamin A (Oruch and Pryme, 2012). So, a higher amount of provitamin A carotenoids need to be consumed to achieve the daily requirements (Institute of Medicine (U.S.) and Panel on Micronutrients, 2002). Since vitamin A is fat-soluble, it can be stored, which can promote toxic effects when consumed in excess, such as liver damage and bone abnormalities (World Health Organization and Food and Agriculture Organization of the United Nations, 2004), so the RDA is 700 and 900 µg/day for female and male adults, respectively, and the maximum intake per day is 3000 µg (Oruch and Pryme, 2012).

1.3.1.1- Vitamin A role in metabolism and pathophysiology

In plants, there are three main functions that carotenoids and its derivatives possess. First, carotenoids are involved in light capture, acting as photo protective compounds, due to their antioxidant potential. Second, carotenoids are required for correct assembly of the photosystems, as they transfer light to chlorophylls. In this regard, they produce powerful colours in flowers, which influences the attraction of animals, aiding in pollination and plant reproduction. Third, carotenoid derivatives act as precursors to abscisic acid (ABA) and stringolactones, which are key phytohormones in the response to abiotic stress and root development and fungal symbiosis (Cazzonelli, 2011).

Vitamin A has different functions in humans, being critical for vision, immune function, growth and development, reproduction, maintenance of epithelial cells and erythrocyte development (World Health Organization and Food and Agriculture Organization of the United Nations, 2004).

Retinal is the vitamer involved in the vision cycle, participating in a photo isomerization reaction that is key for the triggering of nervous signalling from low-light environments (Zhong et al., 2012). Retinol and retinoic acid bind to specific receptors in epithelial cells, regulating the expression of genes encoding structural proteins, enzymes, extracellular matrix proteins and retinol binding proteins and receptors (Institute of Medicine (U.S.) and Panel on Micronutrients, 2002). Also, retinoic acid (RA) is a vital intervenient during embryogenesis, as it has been identified in temporal specific patterns involved in the vertebrae and spinal cord development, as well as the limbs, heart, eyes and ears (Dickman and Smith, 1996, Kam et al., 2012). Finally, retinol and RA are required to maintain the immune system, with retinoic acid being involved in the regulation of the number of circulating natural killer cells (Zhao and Ross, 1995), phagocytic activity in murine macrophages (Katz et al., 1987) and production of cytokines, and retinol being required for B lymphocyte development and activation (Blomhoff et al., 1992).

Vitamin A has been described to aid iron metabolism and utilization, by facilitating its integration into haemoglobin, a key step in the development of red blood cells. It is also a known antioxidant, scavenging reactive oxygen species that might have detrimental effects in cells (Oruch and Pryme, 2012). Within the scope of biological functions attributed to vitamin A isoforms, gene expression regulation is one of

the most relevant as it may be a regulatory mechanism for the functions aforementioned in iron metabolism, as well as oxidative stress response. Through the activation and/or repression of specific retinoid-controlled genes, vitamin A plays a modulatory role in spermatogenesis and in the expression of the human growth hormone (Oruch and Pryme, 2012).

The deficiency (VAD) can occur in two separate regimes, namely, as a result from insufficient dietary sources for (pro)vitamin A, or due to an impaired lipid absorption that leads to inadequate vitamin A levels (Oruch and Pryme, 2012). This is one of the biggest global health problems, with a higher incidence in developing countries and low-resource regions (Shrivastava et al., 2014).

VAD is the world's most prevalent preventable cause of blindness in children, with 250 to 500 thousand cases worldwide, with nearly half of these children perishing within a year after becoming blind (Allen et al., 2006). It has also been linked with a higher rate of infections and childhood mortality, especially from diarrhoea and measles, with a higher occurrence in Africa and Southeast Asia (Bailey et al., 2015). In addition, VAD is known to contribute to maternal mortality and health problems, for both the mother and child, during pregnancy and lactation (Oruch and Pryme, 2012). Its role in iron mobilization and metabolism means that suboptimal values of vitamin A may contribute to the development of iron-deficiency related disorders, such as anaemia (Michelazzo et al., 2013).

1.3.1.2- Vitamin A biofortification strategies

Vitamin A is critical in the human diet as it participates in various mechanisms important for good health. Unfortunately, the majorly consumed plant foods have low levels of its precursors, which aggravates the low vitamin A status in a significant proportion of people. This has motivated a lot of investigation on carotenoid metabolic engineering of plants (Giuliano, 2017a). Biotechnology-based approaches have made use of knowledge regarding carotenoid biosynthesis and metabolism (figure 3).

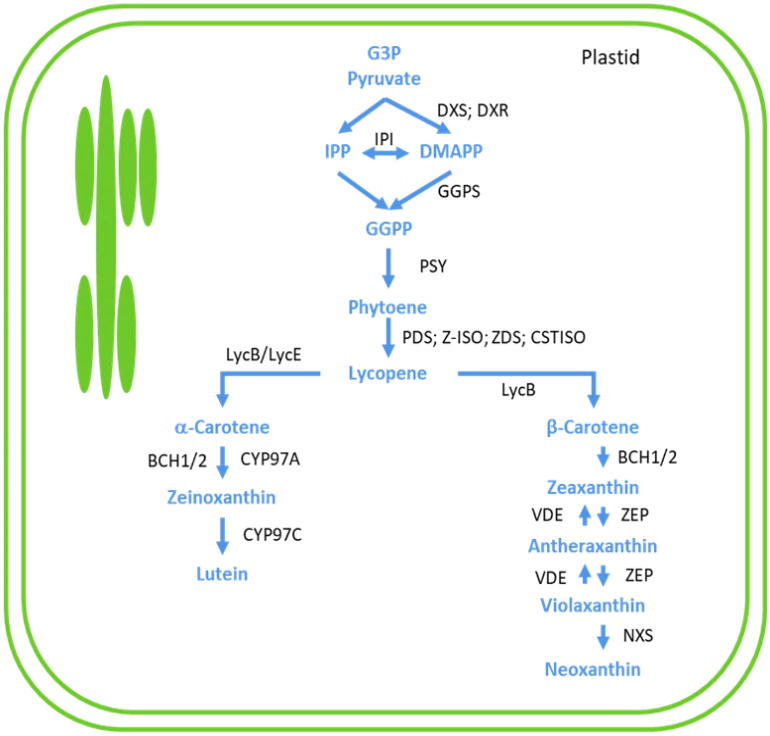


Figure 3 - Carotenoid biosynthesis pathway in plants (Adapted from Sun et al., 2018: figure 1 pp.59). Biosynthesis pathway is shown in blue and enzymes in black. Products: GA3P, glyceraldehyde 3-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate. Enzymes: DXS, 1-deoxy-d-xylulose 5-phosphate synthase; DXR, 1-deoxy-d-xylulose 5-phosphate reductoisomerase; GGPPS, geranylgeranyl pyrophosphate synthase; PSY, phytoene synthase; PDS, phytoene desaturase; Z-ISO, z-carotene isomerase; CrtISO, carotene isomerase; LycB, lycopene b-cyclase; LycE, lycopene 3-cyclase; BCH, b-carotene hydrolase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NXS, neoxanthin synthase.

Carotenoid synthesis occurs in the plastids and begins with the formation of isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMAPP), which will be condensed into one C20 molecule of Geranyl-geranyl diphosphate (GGPP). This is followed by the conversion of 2 GGPP molecules into phytoene by phytoene synthase (PSY), being this the first committed step in carotenoid biosynthesis. Phytoene will undergo four sequential reactions to originate lycopene, after which the pathway can follow two separate branches depending on the cyclization type. Cyclization by both lycopene ε-cyclase and lycopene β-cyclase, in a two-step mechanism, yields α-carotene, whilst cyclization by only lycopene β-cyclase leads to β-carotene synthesis. Further modifications of the carotenes produced in each branch allow the synthesis of xanthophylls and other species-specific carotenoids (Sun et al., 2018).

The most known biotechnology strategy in increasing provitamin A content is the 'Golden Rice' project, in which a complete β-carotene biosynthetic pathway was introduced into rice endosperm (Ye, 2000). The best varieties (Golden Rice II) would contain up to 37 µg of β-carotene per g of rice, which fuelled concerns regarding the amount of plants that would have to be eaten in order to fulfil daily requirements (Zhu et al., 2007). However, subsequent studies eliminated this question, as it was found that the conversion of carotenoids into vitamin A was sufficient to provide 25% of RDA, with only 16g of rice (Giuliano, 2017b).

Other provitamin A-biofortified crops have been developed, the most common approach is to increase the metabolic flux by overexpressing specific genes in the biosynthetic pathway ("push" strategy) (Sun et al., 2018). Analysing the metabolism of carotenoids, the PSY enzyme appears as an optimal candidate as it catalyses the rate-limiting step (Sun et al., 2018). Also, the subsequent desaturation reactions that lead to the formation of lycopene (catalysed by four different enzymes) have also been targeted for enhancing carotenoid production (Sun et al., 2018). Overexpressing the plant phytoene synthase gene (*PSY*) through the addition of its bacterial homologue (C*rtB*) has proved to successfully enhance carotenoid concentration in canola seeds, obtaining between 1000-1500 µg total carotenoids gFW^{-1}, which is higher than what is observed in high carotenoid containing vegetables, like carrots (50-200 µg gFW^{-1}) (Shewmaker et al., 1999). Transgenic wheat containing two bacterial carotenoid biosynthesis genes, *CrtB* and *CrtI*, involved in phytoene and lycopene production, respectively, displayed a higher provitamin A content in comparison with wild-type cultivars (Wang et al., 2014). Also, potato tubers transformed with a set of bacterial genes that allowed the synthesis of β-carotene from GGPP, namely, *CrtB*, *CrtI* and *CrtY* (a lycopene β-cyclase), led to the 20-fold increase in total carotenoids and 3600-fold increase in β-carotene (Diretto et al., 2007a).

The total carotenoid pool is also greatly impacted by their turnover. Therefore, reducing this activity ("block" strategy) is a potential target for carotenoid metabolic engineering (Sun et al., 2018). In this sense, silencing the plant carotenoid cleavage dioxygenases enzymes (CCD1, CCD2 and CCD4) would allow higher accumulation of carotenoids, which was observed in potato tubers with a down-regulated

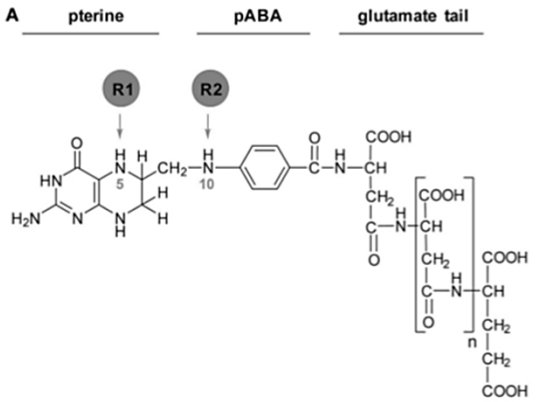
CCD4 gene that had a 2- to 5-fold higher carotenoid concentration (Campbell et al., 2010). Other "block" strategies have a higher emphasis in increasing upstream β-carotene (provitamin A) content, instead of focusing in total carotenoids. This was performed in potato tubers by either removing the α-branch of carotenoid synthesis (silencing lycopene ε-cyclase) or eliminating the β-carotene hydroxylase, resulting in a maximum 14-fold and 38-fold increase in β-carotene, respectively (Diretto et al., 2006; Diretto et al., 2007b).

Carotenoids are synthesized and stored in plastids (with the exception of proplasts), thus engineering their ability to sequester carotenoids is a suitable approach ("sink" strategy) (Sun et al., 2018). For this, the most prominent candidate is the *Or* gene since it exhibits a dual action, exerting a post-transcription regulation on PSY protein level and allowing chromoplasts to differentiate from chloroplasts (Chayut et al., 2017). Transgenic potato tubers expressing the orange cauliflower *Or* gene were found to have enhanced β-carotene accumulation, that permitted stable plant storage and promoted a higher abundance of the phytoene synthase enzyme (Li et al., 2012). Recently, the overexpression of an Arabidopsis *Or* gene, with a golden SNP, meaning the SNP that occurs in orange cauliflower, in tomato fruit proved to promote chromoplast formation and carotenoid accumulation, as well as early flowering and seed production (Yazdani et al., 2019).

Finally, aiming for carotenoid post-harvest stability is a crucial step in ensuring that the target population obtains the nutrients that are lacking (Giuliano, 2017a). So far, one metabolic engineering strategy has been proposed, in which the biosynthesis of vitamin E was enhanced, together with β-carotene biosynthesis, in sorghum, minimizing oxidative degradation of β-carotene during storage (Che et al., 2016).

1.3.2- Vitamin B9

Vitamin B9 or folate represents a group of water-soluble vitamers which can be structurally divided into three moieties, a pteridine ring, a *para*-aminobenzoate (*p*-ABA) ring and a tail of one or more L-glutamates (figure 4) (Blancquaert et al., 2010).



Folate species	R1 (N5)	R2 (N10)
Tetrahydrofolate (THF)	-H	-H
5-methyl THF	$-CH_3$	-H
5,10-methylene THF	$-CH_2-$	$-CH_2$
5,10-methenyl THF	$-CH=N^+$	$-CH=N^+$
5-formyl THF	-CHO	-H
10-formyl THF	-H	-CHO
10-formimino THF	-H	-CHNH

Figure 4 - Representation of tetrahydrofolate (A) and other B9 vitamers (B). (Accessed from Gorelova et al., 2017: figure 1 pp.2)

The oxidation state will directly impact stability, being that the most oxidized conformers are the most stable. The glutamate tail can vary from one to fourteen molecules and it is thought to aid in *in vivo* stability, as it ensures cellular retention and association with folate-dependent proteins (Blancquert et

al., 2010). The C1-substituents determine the properties and biological function of the vitamers (Strobbe and Van Der Straeten, 2018). The commercially available, and commonly referred, folic acid is a synthetic format that is fully oxidized, and upon ingestion, the gut mucosa transform it into DHF (dihydrofolate) and THF (tetrahydrofolate), the latter of which being the metabolically active form (Blancquaert et al., 2010).

Folate can be found in a variety of food sources, but much like other minerals and vitamins, its content in the main staple foods is inadequate (tables 2 and 3), being much below the RDA of 400 μg/day (for adult males). This problem is aggravated by the fact that around 50% of folate content is lost during cooking and post-harvesting techniques, which further intensifies the inadequacy of these foods (World Health Organization and Food and Agriculture Organization of the United Nations, 2004).

1.3.2.1- Vitamin B9 role in metabolism and pathophysiology

Folate are key to the central carbon metabolism, since their structure allows them to be C1 carriers, functioning both as acceptors and donors (Gorelova et al., 2017). Their involvement has been reported in the biosynthesis of purines, thymidylate (or thymine), pantothenate (or vitamin B5, a precursor to co-enzyme A) and formyl methionyl tRNA, as well as in histidine catabolism, serine and glycine interconversion and assembly of iron-sulphur cluster enzymes (Strobbe and Van Der Straeten, 2018). Perhaps the most noteworthy role of folate is their role in the methyl cycle, since 5-methyl-THF functions as a methyl donor for the conversion of homocysteine to methionine. This is a necessary step in the renewal of the SAM (S-adenosylmethionine) -pool. SAM is a methyl storage that supplies this substituent to different types of methyltransferases, including DNA methyltransferases (Strobbe and Van Der Straeten, 2018). For these reasons, damage to the cellular epigenetic system may be caused by folate deficiency, due to the impairment of the methyl cycle.

In plants, folate synthesis is initiated very rapidly after germination, but also upon light exposure, which points to its pivotal role in plant development. Indeed, it has been shown that chlorophyll synthesis is dependent on folate. Also, different plant stress responses appear to modulate the biosynthesis of folate. On one hand, in Arabidopsis plants are subjected to salt stress and rice plants under cold stress, the biosynthesis of folate is down-regulated (Storozhenko et al., 2007; Neilson et al., 2011). However, in heterotrophic Arabidopsis cultures subjected to oxidative stress, a metabolic re-programming led to an up regulation of folate biosynthesis genes (Baxter et al., 2007). Nevertheless, it is its role in maintaining genome stability, as a C1 carrier and key intervenient in epigenetic regulation that indicate the systematic effect that folate have in essentially all cell types and living organism (Gorelova et al., 2017).

All this foretells the impact of folate deficiency in human health. While a causal relationship was established with the occurrence of megaloblastic anaemia and neural tube defects (NTDs), neurodegenerative and cardiovascular diseases, stroke and a number of cancers (colorectal, leukaemia, breast, cervical, pancreatic and bronchial) have all been implied to be correlated with folate deficiency (Blancquaert et al., 2010). Within these, the most widespread pathologies are megaloblastic anaemia, vascular disease and neural tube defects (Strobbe and Van Der Straeten, 2018).

The major risk group for folate deficiency are pregnant and lactating women, as during these phases an increase in RDA is needed (from 500 µg to 600 µg per day). Consequently, young children are also greatly impacted (Strobbe and Van Der Straeten, 2018). This MND is a global problem, as records of its adverse health implications exist worldwide, with a higher predominance in developing countries. However, it is difficult to pinpoint specific areas for urgent intervention, as comprehensive data collection programs are scarce (Blancquaert et al., 2010).

1.3.2.2- Vitamin B9 biofortification strategies

Metabolic engineering strategies aiming to increase folate content in food plants have had an increasing amount of success, which can be mainly attributed to a greater knowledge on the biosynthesis of folate (figure 5), as well as the extrapolation of successful engineering strategies from one crop to the other.

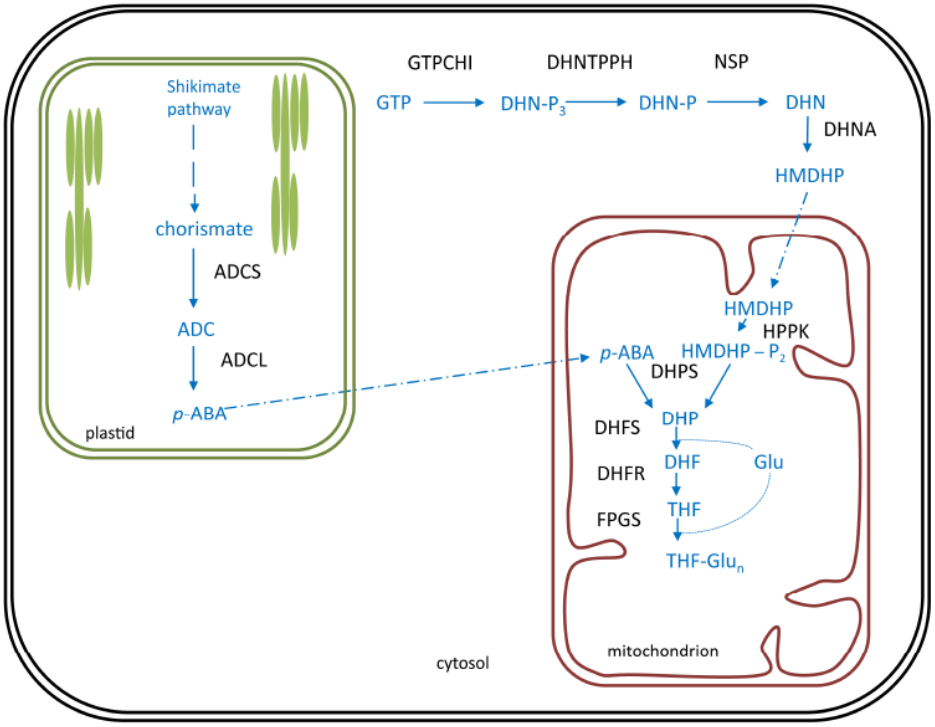


Figure 5 - Folate biosynthetic pathway in plants (Accessed from Strobbe and Van der Straeten, 2018: figure 5 pp 10). Biosynthesis pathway is shown in blue and enzymes in black. Polyglutamylated folate are considered the end product of folate biosynthesis. Products: ADC, aminodeoxychorismate; p-ABA, para-aminobenzoate; DHN-P3, dihydroneopterin triphosphate; DHN-P, dihydroneopterin monophosphate; DHN, dihydroneopterin; HMDHP, 6-hydroxymethyldihydropterin; HMDHP-P2, HMDHP pyrophosphate; DHP, dihydropteroate; DHF, dihydrofolate; Glu, glutamate; THF, tetrahydrofolate. Enzymes: ADCS, ADC synthase; ADCL, ADC lyase; GTPCHI, GTP cyclohydrolase I; DHNTPPH, dihydroneopterin triphosphate pyrophophohydrolase; NSP, non-specific phosphatase; DHNA, DHN aldolase; HPPK, HMDHP pyrophosphokinase; DHPS, DHP synthase; DHFS, DHF synthetase; DHFR, DHF reductase; FPGS, folylpolyglutamate synthetase.

Folate synthesis occurs in three different subcellular localizations, the chloroplast, mitochondria and cytosol. It begins with the production of the *p*-ABA and pterin moieties in the chloroplast and cytosol, respectively. The conversion of GTP into dihydroneopterin triphosphate (DHN-P_3) is the first step in the synthesis of the pteridine ring and it is a rate-limiting reaction, controlled by a GTP cyclohydrolase I (GTPCHI). This branch of the pathway yields glycol aldehyde and 6-hydroxymethyldihydropterin (HMDHP), the latter being transported via (yet unknown) carrier(s) to the mitochondria. The *para*-aminobenzoate moiety is synthesized in a two-step mechanism that takes place in the chloroplast and uses chorismate (derived from the shikimate pathway, a biosynthetic mechanism for producing aromatic

amino acids) and glutamine as initial substrates. *p*-ABA is an amphiphilic molecule, so it will arrive to other subcellular compartments by membrane diffusion. After HMDHP enters the mitochondria, it will be pyrophosphorylated and combined with the p-ABA moiety, forming dihydropteroate (DHP), these two subsequent reactions are catalysed by the dual enzyme HMDHP pyrophosphokinase and dihydropteroate synthase (HPPK/DHPS). This molecule will be further reduced and finally, the tetrahydrofolate (THF) are attached to glutamate residues(s), through the activity of FPGS (folylpolyglutamate synthetase). The polyglutamylated tetrahydrofolate are accumulated in three separate intracellular compartments, plastids, mitochondria and cytosol, which overlaps with the activity of FPGS in all these (Gorelova et al., 2017).

Based on this knowledge, some crop engineering strategies have been designed, focusing on stimulating enzymes that currently limit the pathway productivity, or increasing folate storage stability (Bekaert et al., 2008).

Earlier studies aimed at increasing the activity of the GTPCHI enzyme, as it catalyses a bottleneck reaction that was considered to be the rate limiting step in folate biosynthesis. In tomato, a mammalian-based *GTPCHI* gene, thereby escaping the plant regulatory mechanism, was introduced, resulting in a 140-fold increase in pterin moieties and 2-fold increase in folate. However, the authors identified a huge reduction in the plants *p*-ABA pools, which could be restored if an exogenous source was provided to these transgenic lines, leading to a 10-fold increase in folate content (de la Garza et al., 2004). The same approach was tested in *A. thaliana*, introducing a bacterial gene, which accounted for a 3.3-fold increase in total folate content (Hossain et al., 2004). With this knowledge at hand, bigenic procedures were attempted, in which both the pterin and *p*-ABA moieties were targeted. In tomato and in rice, the tissue-specific overexpression of both GTPCHI and ADCS enzymes led to an increase in folate content up to 25-fold and 100-fold, respectively (e la Garza et al., 2004; Storozhenko et al., 2007). When this strategy was applied to other plants (potato tubers and *A. thaliana*), the strategy (GA-strategy) appeared less effective, which points to the fact that this approach might be limited in its applications (Blancquaert et al., 2013). However, when this GA-strategy was applied in wheat and corn, under the control of endosperm specific promoters, an enhancement of folate was observed, between 2 and 4-fold, also when a wheat endosperm-specific promoter was used to control expression, there was an increase of 5.6-fold in wheat grains (Liang et al., 2019).

Folate stability is also a major concern due to its susceptibility to degradation, so increasing its storage stability has also been addressed. This was performed by the introduction of folate binding proteins (FBP) from mammalian origin and by overexpressing FPGS (folylpolyglutamate synthetase), since the glutamate tail promotes cellular retention and association with folate-dependent enzymes (Blancquaert et al., 2015). Both strategies allowed a higher folate stability in storage, with the FBP method allowing the highest folate accumulation.

Recently, the tuber-specific introduction of four folate biosynthesis genes was assessed in potato, including *ADCS*, *GTPCHI*, *HPPK/DHPS* and *FPGS*. This was performed to investigate whether the mitochondrial biosynthesis branch is limiting folate production. It amounted to a 12-fold increase in total folate as well as, an increase in stability during long-term storage of the tubers. These findings point to

the fact that the whole pathway should be taken into consideration, and not only the main rate-limiting steps (De Lepeleire et al., 2018).

1.3.3- Iron

Living organisms cannot synthesize minerals *de novo* they must absorb them from the soil. Therefore, the relative abundance of the specific minerals in the soil will impact the nutritional composition of the crops grown there. Iron is widespread in earth, however variations in soil pH and redox state lead to the formation of various non-soluble formats, the uptake of which proves to be challenging. As such, different species have developed intricate mechanisms for iron absorption from soil. Plants can be divided in two main groups regarding iron uptake, strategy I (non-grasses), which rely in a reduction based strategy to convert iron into a Fe^{+2} oxidation state, and strategy II (grasses), who secrete phytosiderophores (PS), forming complexes with Fe^{3+}, so that it can be transported up the roots, constituting a chelating strategy (Morrissey and Guerinot, 2009) (figure 6).

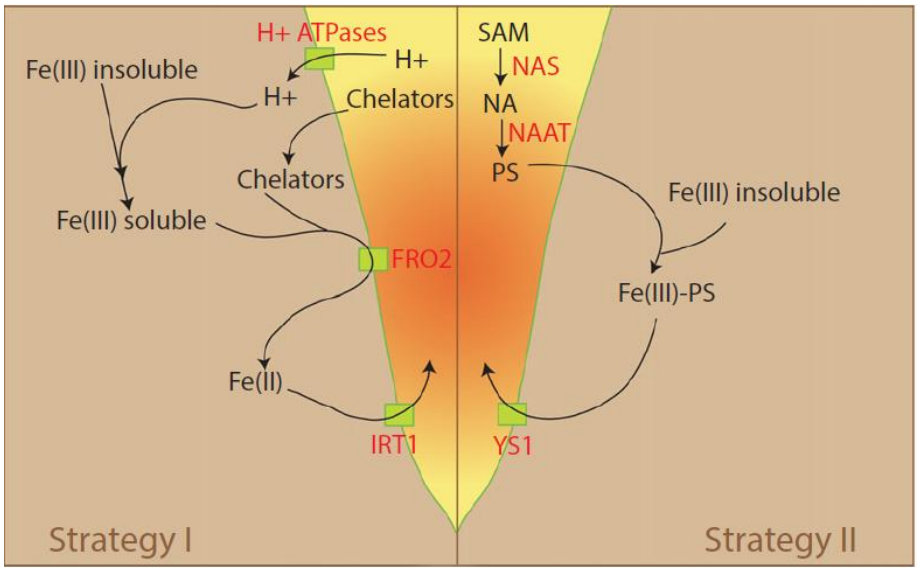


Figure 6 - Iron uptake mechanism in plant roots (Accessed from Blancquaert et al., 2017: pp. 66). Enzymes are in red and intermediates in black. FRO2, ferric reductase oxidase 2; IRT1, iron-regulated transporter 1; NA, nicotianamine; NAAT, NA aminotransferase; NAS, NA synthase; PS, phytosiderophore; SAM, S-adenosyl methionine; YS1, yellow stripe 1.

The reduction-based strategy begins with a release of protons into the rhizosphere by H^+-ATPases present at the root membrane, which promotes an acidification of soil, increasing Fe solubility. Then, the soluble Fe^{3+} is reduced to Fe^{2+} by FRO2, a NADPH-dependent ferric chelate reductase and finally, the IRT1 transporter ensures uptake of Fe^{2+} into the root epidermal cells (Morrissey and Guerinot, 2009). Although there are other metal ion transporters, IRT1 is the major high-affinity iron transporter at the root level. However, activity of IRT1 in iron limiting conditions leads to the accumulation of other minerals, like cadmium and zinc, which may be problematic due to their toxic effect upon present in very high concentrations (Connolly et al., 2002). The chelation-based strategy, on the other hand, depends on the release of phytosiderophores (PS) into the rhizosphere. This strategy II begins with the condensation of 3 molecules of *S*-adenosyl methionine (SAM) and the resulting formation of nicotinamide (NA) by nicotinamide synthase (NAS). The subsequent step involves the production of deoxymugineic acid (DMA) through the action of nicotinamide aminotransferase (NAAT), which is

thereafter converted into mugineic acid and derivatives, which will function as the Fe^{3+} chelating molecules. These complexes (Fe^{3+}-PS) are imported into epidermal root cells through specialized high affinity transporters (YS1) (Blancquaert et al., 2017). As an exception to this division in higher plants, rice, which is a grass, possesses a mechanism of uptake which is a combination of both strategies, as it possesses an IRT1 transporter in addition to all the strategy II components. This allows the plant to take up Fe^{2+} in paddy fields (anaerobic conditions), where it is highly abundant (Kobayashi and Nishizawa, 2012).

In humans, there is no regulated mechanism to excrete iron, so all metabolic regulation is executed at the level of iron uptake and absorption, from the duodenum mucosal cells into plasma. This uptake takes place in the intestine, where both haem (animal source) and non-haem iron (plant and animal source) are transported to enterocytes, followed by delivery to the different tissues by transferrin, which present in plasma (Abbaspour et al., 2014). The absorption of non-haem iron varies greatly according to the food matrix composition, influenced by the amount and type of certain inhibitors as well as enhancers. Inhibitors include phytate, polyphenols, calcium and specific animal proteins, such as albumin and milk proteins, whilst the two major enhancers of iron bioavailability are citric acid and animal muscle tissue (Hurrell and Egli, 2010; Zimmermann and Hurrell, 2007). This constitutes a problem in the sense that populations relying predominantly on starch-dense crops have a higher chance of ingesting bigger ratio of low-bioavailability iron (Institute of Medicine (U.S.) and Panel on Micronutrients, 2002) Additionally, iron intake has to be increased in the case of recurrent bleeding, due to the drastic loss that it causes in the total haemoglobin pool, so pre-menopausal low-income women are at very high risk for iron deficiency (Institute of Medicine (U.S.) and Panel on Micronutrients, 2002; Zimmermann and Hurrell, 2007). Also, growth spurts cause big depletions in iron stocks, due to high oxygen demands, which means that children and adolescents are also vulnerable to mineral deficiency (Zimmermann and Hurrell, 2007).

1.3.3.1- Iron role in metabolism and pathophysiology

Iron is vital to oxygen transport in mammals, mainly acting as a component of haemoglobin and myoglobin, but also as a cofactor for many enzymes involved in electron transfer and redox reactions (Abbaspour et al., 2014). Iron-containing proteins can therefore be divided into four major classes: haem proteins (oxygen binding); iron-sulphur proteins (flavoproteins); iron storage and transport proteins (ferritin, transferrin, lactoferrin, etc.) and other specific iron-containing enzymes (Non-haem or sulphur proteins) (Institute of Medicine (U.S.) and Panel on Micronutrients, 2002).

In plants, iron plays a key role as well, being that iron-sulphur clusters are necessary for chlorophyll synthesis (Hu et al., 2017) and maintenance of chloroplast integrity as well as being required for cytochrome biosynthesis and many iron-sulphur clusters (Briat et al., 1995; Willows et al., 2006). Therefore, iron is a vital micronutrient to photosynthetic capacity, which is nicely reflected in the observation that iron deficient plants tend to develop chlorosis in their leaves. Additionally, it is a cofactor to the biosynthesis of different phytohormones, including ethylene (Bouzayen et al., 1991) and the precursor for jasmonic acid, lipoxygenase (Vahedi-Faridi et al., 2004). Furthermore, it constitutes a major component of redox systems (Briat et al., 1995).

There is a multitude of biological processes that require iron as a cofactor, including nucleic acid and protein synthesis as well as electron transport and cellular respiration, making it pivotal for cellular development and differentiation (Lieu et al., 2001). Additionally, iron is involved in neuronal maturation and myelin production. As such, it is a key micronutrient for proper cognitive development, particularly, learning and memorizing skills (Bastian et al., 2016). The most notable disease resulting from iron deficiency is IDA (iron-deficiency anaemia), which occurs due to a reduction in red blood cells, amounting in an inefficient oxygen transport in the body (Murgia et al., 2012). Common symptoms of IDA are weakness and fatigue. However, in extreme long-term deficiency situations, the condition may lead to maternal and child mortality (Lynch et al., 2018). IDA affects 50% of women and 40% of children in developing countries (Murgia et al., 2012). However, despite this being the most described iron deficiency associated condition, the disruption of many neural functions by iron deficiency, has been shown to result in more complicated health problems and development impairments (Lynch et al., 2018). Because iron metabolism is highly dependent on proper uptake, once an insufficient intake overlaps with an increased demand, a negative iron balance occurs, which promotes limitations in learning and memory development. This is particularly severe in case it occurs in early childhood or foetal growth. Additionally, these problems often persist in the individual, long after restoring the iron balance (Fretham et al., 2011). Finally, there is some evidence on the correlation between susceptibility to certain infectious diseases and low iron status in anaemic individuals, however the scarcity of clinical studies makes this correlation still unclear (Lynch, 2011; Tansarli et al., 2013).

1.3.3.2- Iron biofortification strategies

Iron deficiency is one of the most prevalent MNDs worldwide, so there have been numerous attempts to increase its levels in various staple crops. However, the problem is that enhancing mineral content *in planta* requires intervention at different levels, since they are not produced by the plant. As such, there are four main areas of intervention for increasing iron concentration, namely, uptake, transport, storage and bioavailability (Connorton and Balk, 2019).

In terms of iron uptake, the strategy usually relies in overexpressing root-level transporters For this strategy, there are multiple genes with potential, although the most common methodology is to overexpress the IRT1 transporter, which proved successful in cassava roots transgenic lines, engineered for both IRT1 and Ferritin, amounting to a 5.5-fold enhancement of root iron levels(Narayanan et al., 2019). A similar strategy in rice grains, in which IRT1, ferritin and nicotinamide synthase were tissue-specifically introduced (using a single locus construct) resulted in a 4-fold increase of iron content of polished rice (Boonyaves et al., 2017).

Iron is transported, in the form of complexes, from the root epithelial cells through the xylem and phloem to the various plant organs Therefore, whenever this section of metabolism is targeted, the objective is to facilitate the distribution of these complexes (Connorton and Balk, 2019). One of the most common chelators is nicotinamide (NA), which, once chelated with iron as Fe-NA complexes, are transported through YSL membrane transporters (Conte and Walker, 2012). As such, overexpressing nicotinamide synthase (NAS) under the control of constitutive promoters has been achieved in rice, amounting to a 10-fold increase of iron in leaves, and a very modest 2-fold increase in the seeds (Lee et al., 2009).

Nicotinamide is converted to DMA by nicotinamide aminotransferase (NAAT) and DMA synthase (DMAS) in grasses (Banakar et al., 2017), and DMA is another molecule that facilitates Fe^{3+} uptake, occurring also in leaves. In polished rice, the overexpression of both *NAS* and *NAAT* amounted to a 29-fold increase in DMA and 4-fold increase in iron. Finally, iron transport from the phloem into the developing seed has also been targeted through the overexpression of YSL2 in rice, under a sucrose transporter promoter, resulting in a 4.4-fold increase of iron in polished rice grains (Ishimaru et al., 2010).

In plants, iron homeostasis is needs to be very tightly controlled, as drastic physiological effects could occur upon both deficiency and excess status. In this respect, there are numerous proteins and small molecules involved in iron storing and buffering (homeostasis), of which ferritin is the most relevant in the light of potential biofortification approaches (Briat et al., 2010). Indeed, there have been some attempts with modest results. In rice seeds, transgenic expression of soybean ferritin led to a 3-fold increase in seeds (Goto et al., 1999), whilst this approach only amounted to a 1.5-fold increase in iron content of wheat grains(Borg et al., 2012; Neal et al., 2013). Iron and other mineral metabolites are also stored in plant vacuoles. Several observation have led to the consideration that this subcellular sequestration contributes to long-distance transport of metals in plants (Peng and Gong, 2014), so overexpressing vacuolar iron transporters is another strategy to improve iron storage. In cassava roots, the overexpression of an Arabidopsis vacuolar transporter (VIT1) raised iron content up to 4-fold (Narayanan et al., 2015) However, these transgenic plants also depicted signs of chlorosis in leaves. Another study in wheat showed evidence that the utilization of a wheat vacuolar transporter enhanced white flour iron content by 2-fold, and that these fractions of flour possessed bioavailable iron (Connorton et al., 2017).

Finally, bioavailability of iron, despite being often neglected, is also an area where intervention is necessary, especially since the majority of plant-based iron is not absorbed by the human gut. Therefore, it would be of high relevance to decrease the amount of anti-nutrients (inhibitors of uptake) or increase the amount of uptake enhancers (Murgia et al., 2012) However it is important to note that this is not a straightforward parameter, as bioavailability is also influenced by age, gender, nutrient status, genetic disorders and life stage, as referred previously. An obvious candidate is phytate (phytic acid) due its role in bioavailability reduction. As phytate represents 50-85% of total phosphorous in plants (Ravindran et al., 1994), reducing its quantity may pose problems in plant physiology and yield. A good approach would the utilization of *low phytic acid* mutants (lpa), which have been identified in corn, barley, rice and soybean. This mutation allows seeds to accumulate phosphate, albeit with a low production of phytate, so it could be used to breed new crop varieties with a better phytate ratio (Gupta et al., 2015). Indeed, upon investigating maize *lpa* mutants, it was verified that they are defective in a specific ABC transporter, and when this was replicated in transgenic maize and barley, a lower phytate level was obtained, without compromising germination or yield (Shi et al., 2007). In the opposite spectrum, enhancing the amount of citric acid in common food crops could also yield a positive effect in mineral bioavailability, as it has a strong oxidation power which promotes the transition of Fe^{3+} to Fe^{2+}as well as being an effective chelator of Fe^{3+}, promoting higher absorption (Teucher et al., 2004). Also, when discussing bioavailability, it is important to consider nutrient-nutrient interactions, since they have intertwining metabolic pathways, both in plants and human physiology, as such it makes sense to refer

two commonly described iron interactions, namely with zinc and with vitamin A (Lynch et al., 2018). It appears that vitamin A affects iron mobilization in tissues but doesn't impact intestine absorption (Citelli et al., 2012). Furthermore, it has also been shown that in vitamin A-deficient rats, iron incorporation in red blood cells is lowered (Roodenburg et al., 2000). Mineral interactions are more complicated, particularly between iron and zinc, since they have many similarities in terms of absorption and transport. Nevertheless, there have been reports of decreased bioavailability of either one, whenever one is in excess, but it is not clear how this impacts public health and the dimension of the problem (Sandström, 2001).

1.4- Potatoes are a good candidate for biofortification

Biofortified foods require a one-time investment for the crop development, after which the seeds can directly be provided to farmers around the world, eliminating pricy infrastructure and specialized technicians. This strategy is most cost-effective when crops that will have a high consumption rate in the region of interest are selected (Saltzman et al., 2013), hence the utilization of cereal, tuber and pulse crops, as they are heavily consumed worldwide (table 4) (Kearney, 2010).

Table 4- List of the most produced food crops worldwide (ordered from the least to the most produced), including their yearly consumption, as well as, principle consumer countries (Source: FAOSTAT).

Major food crops	Worldwide production (in 2013)	Major worldwide consumers (per capita, 2013)	Worldwide consumption (in 2013/2014)
Millet	26.4 million tonnes	Niger, Mali, India, China and Sudan	23 million tonnes
Plantain	39 million tonnes	Ghana, Gabon, Uganda	22 million tonnes
Sorghum	57 million tonnes	Sudan, Burkina Faso, Chad	24 million tonnes
Yams	73 million tonnes	Ivory Coast, Ghana, Benin	32 million tonnes
Sweet Potato	112 million tonnes	Solomon Islands, Rwanda, Angola	57 million tonnes
Cassava	291 million tonnes	Ghana, Thailand, Nigeria	151 million tonnes
Soybean	352 millions tonnes	Taiwan, Korea, Japan	11 million tonnes
Potato	388 million tonnes	Belarus, Ukraine, Malawi	230 million tonnes
Rice	770 million tonnes	Bangladesh, Lao, Cambodia, Vietnam	548 million tonnes
Wheat	772 million tones	Azerbaijan, Tunisia, Turkmenistan	447 million tonnes
Maize	1135 million tonnes	Lesotho, Malawi, Zambia	118 million tonnes

The selection of the food vehicle for biofortification is vital in determining the success of the intervention. As such, it is important to select crops that are already regularly consumed by the target population,

respecting their cultural preferences, in this way, there will be higher chances of success without requiring drastic dietary and/or cultural adaptations.

Potatoes represent an optimal candidate for biofortification interventions, since they are already the 3rd most consumed crop, and 4th most produced, in the world, and many poor and undernourished homes heavily depend on rely on a fairly monotonous diet with high potato consumption (Wijesinha-Bettoni and Mouillé, 2019). In fact, while this crop has been traditionally associated with western diets, there has been a constant decrease in European consumption, accompanied by a steady increase by African and Asian households (Wijesinha-Bettoni and Mouillé, 2019). For instance, in Bangladesh, a country associated with the highest per capita consumption of rice (table 3), potatoes represent the most consumed vegetable and there have been multiple government interventions to promote the switch from rice to potato consumption, as a primary staple, resulting in a thriving industry that now constitutes the seventh largest potato production capacity worldwide (Singha and Maezawa, 2019). Additionally, an aggregation study conducted in 2010, concluded that the following regions showed a higher need for potato research and improvement (figure 7), based in population consumption and poor livelihood indicators:

- High altitude regions of Sub-Saharan Africa – Ethiopia, Cameroon, Kenya, Rwanda, Uganda, Tanzania, Malawi, Angola, Mozambique and Madagascar
- Andean South America – Bolivia, Peru, Ecuador and Colombia
- Indo-Gangetic region of South Asia – Bangladesh, India, Nepal and Pakistan
- Rural China
- Central and Western Asia – Tajikistan and Kyrgyzstan
- Caucasus region – Armenia and Azerbaijan

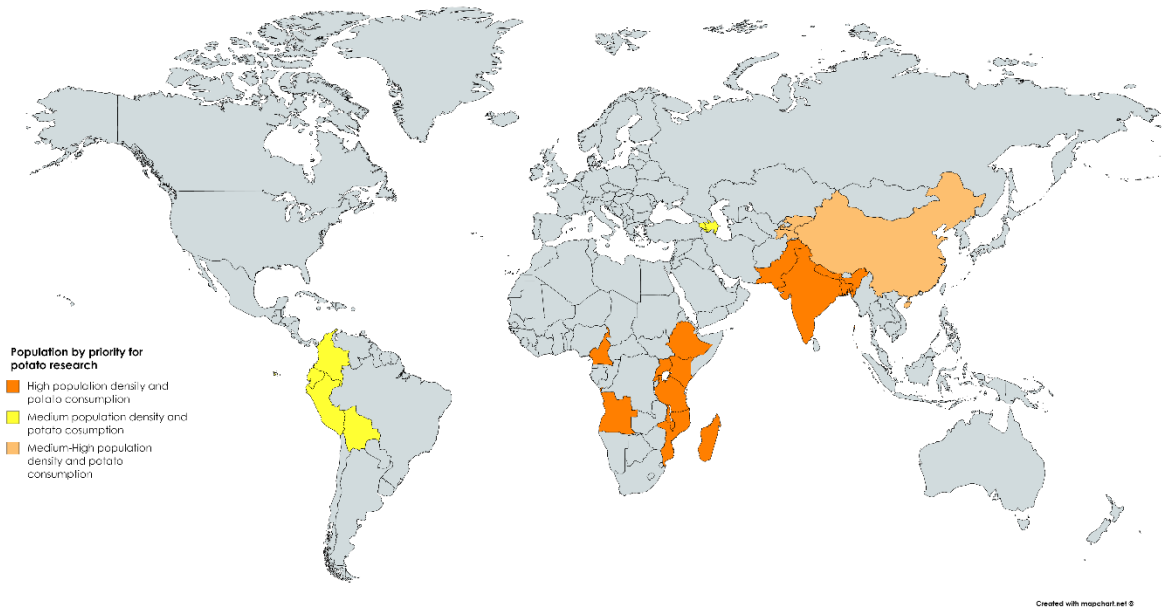


Figure 7- World map representation of the populations that have a higher priority for the development of potato research.

The authors state that potato contributes to food security in two separate ways, namely, by reducing hunger as well as poverty. Also, they warn about the need for a more detailed look into defined regions in different countries, in the sense that this could help reveal data obscured by the country-level searches, allowing a more thorough analysis of the real need of potato research and development (Thiele et al., 2010).

Potatoes are suitable for production in a wide variety of climate conditions and yield more edible food than any other of the 3 most consumed crops (wheat, rice and maize), since around 85% of the plant is safe for human consumption (Lutaladio and Castaldi, 2009). Nutrition-wise, potatoes constitute a low-fat staple with an already good level of certain micronutrients, such as vitamin B6, potassium and vitamin C (figure 8). The naturally high levels of ascorbic acid (vitamin C) in potato tubers, in combination with a natural lack of phytate, increases the bioavailability and absorption of many other vitamins and minerals, of which iron is the most notable. Despite the low iron content of potato tubers, absorption is adequate compared to other plant-derived food sources (King and Slavin, 2013). Also, they represent a good source of antioxidants, such as polyphenols, which do not have a specific daily recommendation for consumption, although having been established as key components of health-promoting human diets (Navarre et al., 2019).

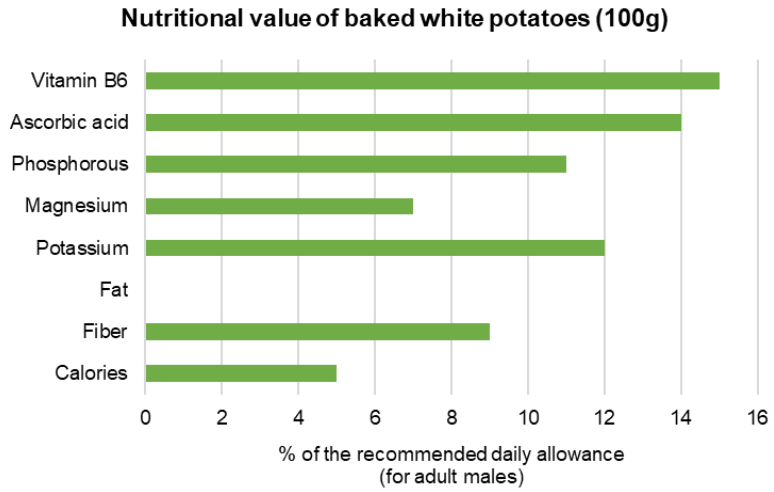


Figure 8 - Percentage of the recommended daily allowance (RDA) that could be met with 100 g of common white potatoes, in terms of vitamin B6, ascorbic acid, phosphorous, magnesium, potassium, fat, fibre and calories.

All in all, the high yield returns, good nutritional value and cultivation easiness have made this crop to be increasingly popular in developing countries (Kennedy et al., 2018), thereby making it a prime candidate to deliver micronutrients to the populations in need by means of a biofortified crop product.

A good example of the utilization of potato in biofortification is the iron and zinc-rich potato varieties developed by the International Potato Centre (CIP), in 2016. These clones were obtained through a 15-year breeding programme, which allowed the production of potatoes with 40 to 80% more zinc and iron than the traditionally consumed varieties. Consumer trials were allowed in Peru's Andean Highlands, where the seeds were provided to farmers and the population received nutritional education and incentives to include a higher variety of vegetables and animal-derived products (like eggs) in their diet.

This integrated approach resulted in a general health improvement, in a population that was previously marked by a 40% anaemia incidence in children bellow five years and high rates of malnutrition in children and women of reproductive age. The multi-disciplinary team involved also attribute the success of the intervention to the selection of locally grown and appreciated species for the biofortification project and in the involvement of different qualified professionals (CPAD, 2018). Trials like this are envisioned also for rural regions in Ethiopia and Rwanda, but don't have yet a defined date.

"Food security exists when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food to meet their dietary needs and food preferences for an active and healthy life". (Declaration from the 1996 World Food Summit). At the moment, food security is not a reality for a large proportion of the world population. Potato tubers hold the potential to help in the alleviation of these concerns, as they constitute a high value crop, that already is heavily consumed by many affected populations. Also, their natural nutritional value makes them an ideal vehicle, as little addition of specific micronutrients is necessary, in order to make it a complete crop. In this sense, investigation into meliorating its nutritional status is of outmost importance.

2- Main objectives

Eradicating micronutrient deficiencies is a complex problem. In larger-scale interventions, however, multi-biofortification strategies present the most promise, as they require little more investment than single nutrient biofortification as well as having a broader spectrum of action. There have been extensive research efforts into developing single-micronutrient enhanced food crops, with variable degrees of success in terms of fold enhancement. However, most of the projects focus on rate-limiting steps of the metabolic pathway, which may not always be best choice for improvements, as it is known that the modification of key metabolic reactions promotes the development of compensation mechanisms for the loss of regulation (Martin, 1996). Moreover it is important to target the enhanced nutrient to an edible portion of the plant, so that the impact on overall plant growth and development is limited, which requires the utilization of specific promoters for targeted expression (Halpin et al., 1999). This means that a good knowledge of the target genes and relevant regulatory sequences, i.e. promoters and terminators, is pivotal for the strategy to be successful.

In this sense, the main objective of this thesis is to develop the molecular tools needed for the multi-biofortification of *Solanum tuberosum* cv. Desiree with folate, β-carotene and iron. This is to be achieved with particular emphasis in the promoters and terminators that regulate the expression of the various chosen genes, in order to maximize expression in an organ-specific manner (Diamos and Mason, 2018; Dutt et al., 2014; Manavella and Chan, 2006; Potenza et al., 2004).

3- Materials and methods

3.1- Vector construction

3.1-1. Genomic DNA extractions and cDNA preparation

Five adult plants were selected for genomic DNA extraction, namely, *Nicotiana benthamiana*, *Solanum tuberosum*, *Solanum lycopersium*, *Arabidopsis thaliana* and *Pisum sativum*. For all but *P. sativum*, for which only seeds were available, leaf tissue was collected and used for the extractions with the Invisorb Spin Plant Mini Kit (Invitek Molecular). The synthesis of cDNA from *A. thaliana* was initiated with mRNA extraction using the GeneJET plant RNA purification kit (Thermo Fischer Scientific) First, the retrieved RNA was deprived of potential DNA contamination via DNase treatment, including Ribolock RNase inhibitor to minimize RNA degradation during the clean-up procedure (extended ThermoFischer Scientific RNA purification protocol). Secondly, a solution containing 1µg of the extracted mRNA was converted to cDNA through the iScript cDNA synthesis kit (Bio-Rad), which contains random primers in addition to the polydT oligonucleotides.

3.1-2. Restriction-ligation assembly reactions

Vector construction followed an iterative process dependent on restriction-ligation reactions, named Golden Braid 3.0 cloning (Vazquez-Vilar et al., 2017). This is a cloning technology that allows the exchange and assembly of DNA fragments in a modular framework. As such, the creation of single-gene or multi-gene expression vectors is simplified. It is supported by an extensive software, which assists in the *in-silico* prediction of all necessary reactions, and respective outcomes. The Golden Braid destination vectors (pDGBα, pDGBΩ) are binary vectors, that function as recipients for the assemblies, and they contain different selection markers to allow for counter-selection. The insertion of a given assembly into a vector relies on specific restriction enzymes, namely *BsaI*, for alpha vectors (pDGBα), or *BsmBI*, for omega vectors (pDGBΩ), (Thermo Fischer Scientific), as well as T4 ligase (Thermo Fischer Scientific). Each reaction was prepared as a 10 µL aqueous mixture containing 75 ng of the destination vector, 60 fM of the desired DNA fragment, 5U of the appropriate restriction enzyme (*BsaI* or *BsmBI,* depending on the destination vector), 3U of T4 ligase and 2 µL of T4 ligase buffer (5x). The reaction mixture underwent 25 digestion/ligation cycles of 2 minutes at 37℃ and 5 minutes at 16℃, followed by three temperature steps, first, 37℃ for 5 minutes, second, 50℃ for 30 minutes, third, 80℃ for 10 minutes. The whole reaction was transformed in *Escherichia coli* TOP10 competent cells through heat-shock. Briefly, a bacterial aliquot of 200 µL was thawed for 10 minutes, after which the reaction mixture was added and incubated for 30 minutes in ice. The bacterial suspension was heat-shocked at 42℃ for 45 seconds, followed by 1 hour growth (37℃, 200 rpm) after adding 800 µL of liquid Super Optimal Broth (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 10 mM $MgCl_2$, 10mM $MgSO_4$, 20 mM glucose, pH 7). The resulting bacterial suspension was plated in solid Luria-Bertani (LB) agar plates supplemented with 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside acid (40 µg/mL) and isopropylthio-β-galactosidase (0.5 mM) allowing the white/blue selection of clones, as well as specific antibiotics, namely, chloramphenicol (50 µg/mL) for the UPD2 vectors, kanamycin (50 µg/mL) for alpha vectors and spectinomycin (100 µg/mL) for omega vectors, and allowed to grow, at 37℃ overnight.

White colonies, thereby indicating a successful restriction-ligation reaction through the loss of the *lacZ* gene, were selected for colony PCR and the positive clones used to start liquid cultures of LB medium (37℃, 200 rpm, overnight) supplemented with the proper antibiotic. Liquid cultures were later used for plasmid extraction using the GeneJET MiniPrep Plasmid extraction kit (Thermo Fischer Scientific). To assess if the assemblies were correct, the vectors were subjected to sequencing, restriction analysis or specific PCR reactions.

3.1-3. Domestications

The first step in the construction of the DNA modules to be utilized in the Golden Braid system is to 'domesticate' them. This involves obtaining specific DNA fragments, named GBParts, from appropriate templates through PCR amplification with Phusion High-Fidelity DNA Polymerase (Thermo Fischer Scientific) and assembling them in UPD2 vectors. Briefly, GBParts are amplified with specific GB primers that have a terminal region composed by a *BsmBI* recognition sequence, a cleavage site for cloning into UPD2 and a four-nucleotide barcode. These four-letter codes determine the position of each DNA fragment within the vector in the next step of restriction-ligation. In figure 9, a schematic representation of the domestication reactions is depicted.

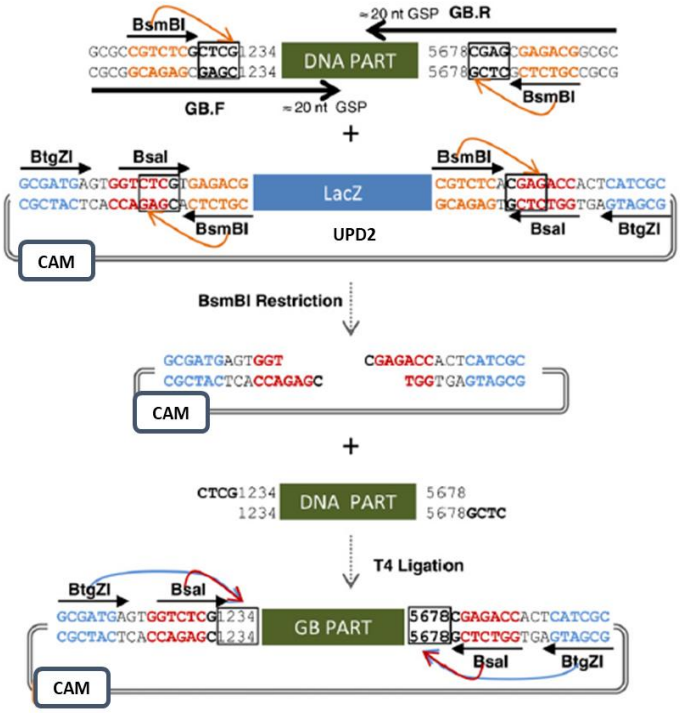


Figure 9 - Schematic representation of the standard domestication reactions for the formation of GBParts (Adapted from Sarrion-Perdigones et al., 2015: figure 3, pp 1622). The domestication occurs through PCR amplification of the target sequence with standard GBprimers (GB.F and GB.R), which contain a gene specific primer (GSP) and a tail region that includes a *BsmBI* recognition site, the cleavage site for cloning into pUPD2, and the four-nucleotide barcode (1234 and 5678). The resulting GBPart can be cleaved by *BsaI* to produce 1234 and 5678 flanking overhangs. *BsmBI* recognition sequences are in orange in the DNA sequence; *BsaI* and *BtgZI* are in red and blue, respectively. Enzyme cleavage sites are highlighted in a box. Abbreviations: CAM – chloramphenicol resistance marker gene.

Out of all the different DNA fragments present in table 5, four of them were synthetically produced (GenScript), to facilitate the cloning procedure, namely, coding sequence 1(as single a B3-B5 fragment); coding sequence 8 (as a B4 fragment); coding sequence 10 (as a B5 fragment); and terminator 4 (as a

B6-C1 fragment). Additionally, to ensure higher expression in tuber tissue and facilitate synthetic manufacturing, both coding sequences 1 and 10 were codon optimized for *S. tuberosum* expression using IDT codon optimization tool (https://eu.idtdna.com/CodonOpt). The other fragments were obtained through standard PCR amplification.

Table 5 - DNA fragments that compose the different GBParts utilised in this work and their significance in the Golden Braid system. Abbreviations: Ts – tissue-specific promoter; IP – inducible promoter; T – terminator; CDS – coding sequence.

ID	Function	Fragment Size (bp)	Obtained from
TsP1	**GBPart** Promoters (A1-B2)	642	pP-YBI (Diretto et al., 2007)
TsP2		1525	pL0 (Rocha-Sosa et al., 1989)
TsP3		1122	gDNA
IP		719	gDNA
CP		1026	Addgene kit # 1000000076
N	Nested PCR for RsP	3000	gDNA
T1	**GBPart** Terminators (B6-C1)	966	gDNA
T2		642	gDNA
T3		441	gDNA
T4		249	Synthetic Construct (IDT DNA)
T5		495	Addgene kit # 1000000076
NESTED	Nested PCR for T3	1585	gDNA
CDS1	**GBPart** Coding Sequence (B3-B5)	3795	Synthetic Construct (GenScript)
CDS2		1234	gDNA
CDS3		768	cDNA
CDS4		1077	pCAMBIA-1300 (Singh et al., 2017)
CDS5		1032	pYLMF-B (Zhu et al., 2017)
CDS6	**GBPart** Coding Sequence (B3)	2757	pMOD35hGA (Storozhenko et al., 2007)
CDS7		1875	PAT_HF (De Lepeleire et al., 2018)
CDS8	**GBPart** Coding Sequence (B4)	72	Synthetic construct (GenScript)
CDS9	**GBPart** Coding Sequence (B5)	1398	pMOD35hGA (Storozhenko et al., 2007)
CDS10		1545	Synthetic construct (GenScript)

The correct size bands were selected in an agarose gel and purified using the GeneJET PCR product purification kit (Thermo Fischer Scientific) and quantified in a Nano Drop Spectrophotometer. The purified fragments were subject the first restriction-ligation reaction, namely the domestication reaction, in which 60 fM of each fragment were added to 75 ng of UPD2 vector, together with *BsmBI* and T4 ligase. Then, the resulting solution was used to transform *E. coli* TOP10 competent cells, which were plated in LB plates supplemented with 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside acid (40 µg/mL) and isopropylthio-β-galactosidase (0.5 mM) and chloramphenicol (50 µg/mL). White colonies were tested through colony PCR using vector specific primers (T_a = 54 ℃; Extension time = 3 minutes)

and the positive clones were targeted for plasmid extraction. Afterwards, the plasmids sequence was confirmed through sequencing (Macrogen) and their nucleotide sequences deposited in the Golden Braid database.

3.1-4. Domestication of C-terminal tagged coding sequences

To construct the C-terminal tagged versions of the expression cassettes, the purified UPD2 fragments were used as a template for new amplification, with the same forward primer as before and a new reverse primer containing in its tail, the coding sequence for different tags, before the STOP codon (Figure 10). For coding sequence 1, a single patch of 467 bp covering the initial 5' region was synthesized and used in a domestication reaction with a second patch of 3452 bp, which was obtained through PCR amplification of the remaining sequence for the synthetic CDS1 structure (B3-B5) (Figure 10). This was required, because there were identical regions duplicated within its sequence, hampering amplification of the full coding sequence. Furthermore, to secure tag expression *in planta*, their sequences were codon optimized for expression in *S. tuberosum*.

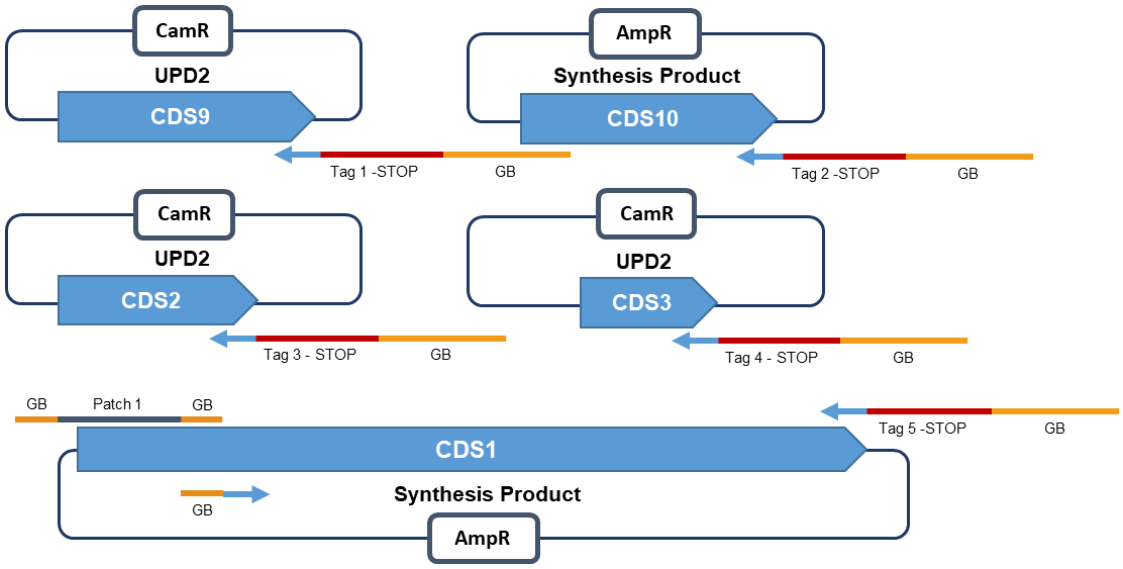


Figure 10- Schematic representation of the amplification-based method for constructing the C-terminally tagged versions of certain coding sequences, namely, CDS9, CDS10, CDS2, CDS3 and CDS1. For all, with the exception of CDS1, a new reverse primer (Tag-STOP-GB) was synthesized, in which after the gene specific region (coloured blue), the specific tag coding sequence was present (with codon optimization for S. tuberosum expression) followed by the STOP codon (Tag–STOP) and the Golden braid specific tail (GB). As for CDS1, the first 467 bp were synthetically produced with Golden braid specific 3' and 5' ends and the remaining sequence was amplified, as all the others, with a GB forward primer and a Tag-STOP-GB reverse primer. Abbreviations: CamR – chloramphenicol resistance marker gene; KanR – kanamycin resistance marker gene.

3.1-5. Domestication of the recombination site

The recombination site (RS) was obtained through the annealing of two complementary oligonucleotides, which contained Golden Braid specific tails (figure 11), so that the annealed double-stranded fragment could be directly cloned into an UPD2 vector and used in subsequent reactions.

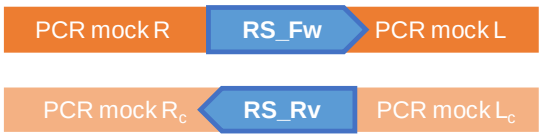


Figure 11 - Schematic representation of the construction of the recombination sites. These were designed as if they were complete transcription units (A1-C1). To achieve this, the oligonucleotides were synthesized (Integrated DNA Technologies) with the golden braid tails (PCR_mock_right and PCR_mock_left) that would be added if they underwent a PCR reaction with the GB primers. Abbreviations: R – Right, L – Left; R_c – Right complementary; L_c – Left complementary.

In brief, the oligonucleotides were subject to an annealing protocol, in which equimolar amounts of each oligo were mixed in annealing buffer (10mM Tris-HCL pH 8.0, 1mM EDTA pH 8.0, 50mM NaCl), and annealed using a program as follows: 5 minutes at 95 ºC; 1 minute at a range of temperatures, starting at 95 ºC and decreasing 1ºC until the melting temperature (77 ºC); 30 minutes at 77 ºC; 1 minute at a range of temperatures, starting at 77 ºC and decreasing 1 ºC until 25ºC. The obtained double-strand DNA fragment was directly used in a domestication reaction, as described previously.

3.1-6. Multipartite assembly

The second set of restriction-ligation reactions aims to assemble multiple previously domesticated GBParts into expression cassettes, named transcription units (TU). This occurs through a *BsaI*-mediated restriction, allowing the orderly assembly of the different GBParts into a single TU, the order of which relies on the four-letter codes present before and after each fragment. In figure 12, schematic representation of how the transcription units are assembled and the different codes for each position is depicted. In figure 13, the assembly of the previously amplified GBParts is represented.

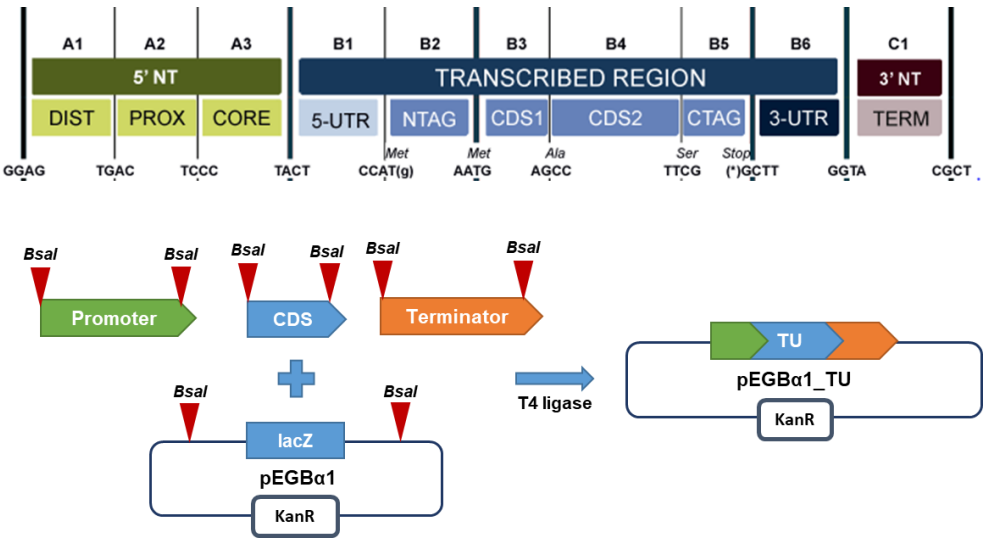


Figure 12 - Schematic representation of Multipartite assembly (Adapted from Golden Braid 2.0 User's Manual: figure 7.1.1, pp). Top: Components of a transcriptional unit in the Golden Braid language and respective four-letter codes for each fragment. Bottom: Multipartite assembly relies on a BsaI restriction, both in the different fragments that compose the transcriptional unit, here represented as promoter, CDS (coding sequence) and terminator, and the destination vector, an α vector. Afterwards, the orderly assembly of the various elements depends on the four-letter codes present at both the 3' and 5' end of each fragment. Abbreviations: KanR – kanamycin resistance marker gene.

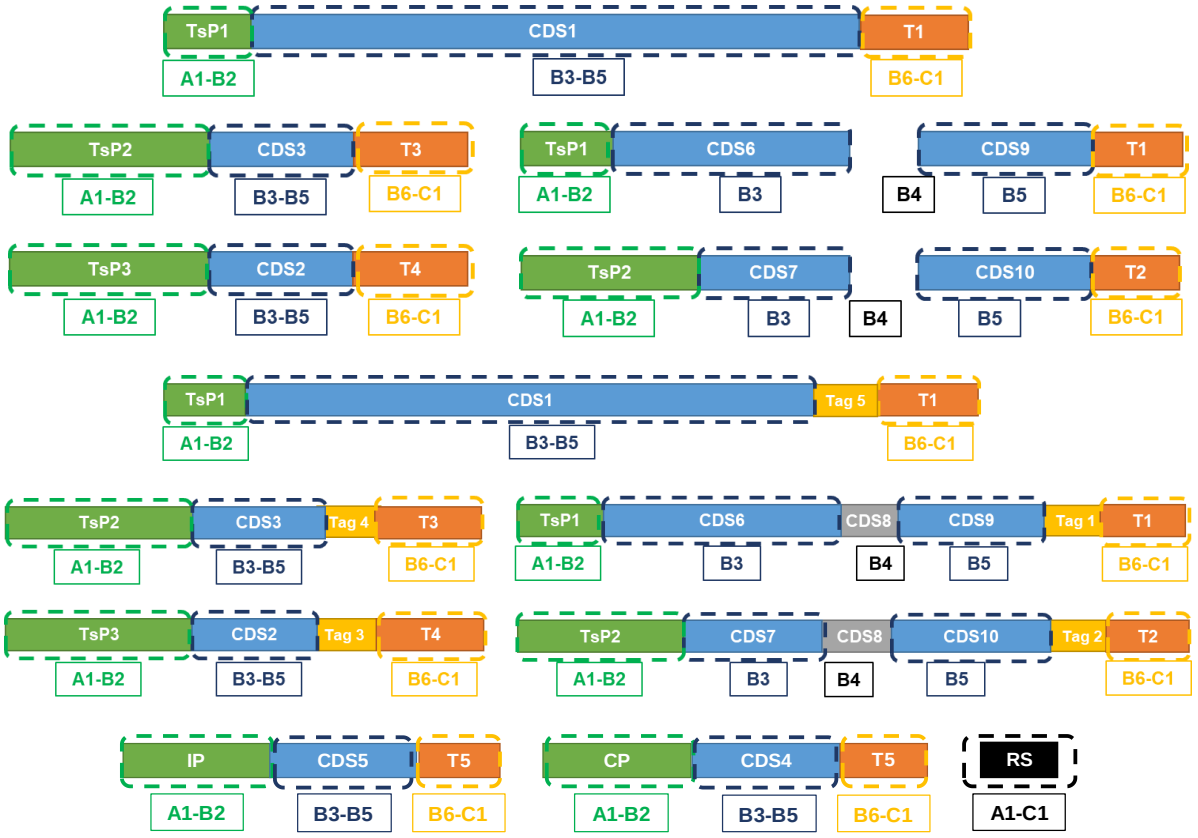


Figure 13 - Representative scheme for assembly of the different DNA fragments that were domesticated and their role in the Golden Braid language. Abbreviations: TsP – tissue-specific promoter; IP – inducible promoter; T – terminator; CDS – coding sequence; RS – recombination site. The promoters are in green, terminators in orange and coding sequences in blue and grey. The peptide tags utilized are represented in yellow.

The purified UPD2 vectors containing the different GBParts were subject the second restriction-ligation reaction, in which 60 fM of each vector was added to 75 ng of alpha vector, together with *BsaI* and T4 ligase. Then, the reaction result was used to transform *E. coli* TOP10 competent cells, which were plated in LB plates supplemented with 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside acid (40 µg/mL) and isopropylthio-β-galactosidase (0.5 mM) and kanamycin (50 µg/mL). White colonies were tested through colony PCR using GBPart specific primers and the positive clones were targeted for plasmid extraction. Afterwards, the plasmids sequence was confirmed through restriction analysis (1h, at 37 ºC), and their nucleotide sequences deposited in the Golden Braid database.

3.1-7. Bipartite assembly

The third set of restriction-ligation reactions aimed at combining different transcriptional units in a single expression vector (Binary assembly), which requires that the vectors in which each TU is present are complementary. Essentially, these reactions can only occur from two complementary alpha vectors (alpha 1 and alpha 2) to a third omega vector (omega 1 or omega 2), and they are mediated by the creation of *BsmBI* sticky-ends. Moreover, the inverse is also possible, using two complementary omega vectors (omega 1 and omega 2), in a *BsaI*-mediated reaction, to insert the TU into a third vector (alpha 1 or alpha 2). In figure 14, a schematic representation of these reactions is present.

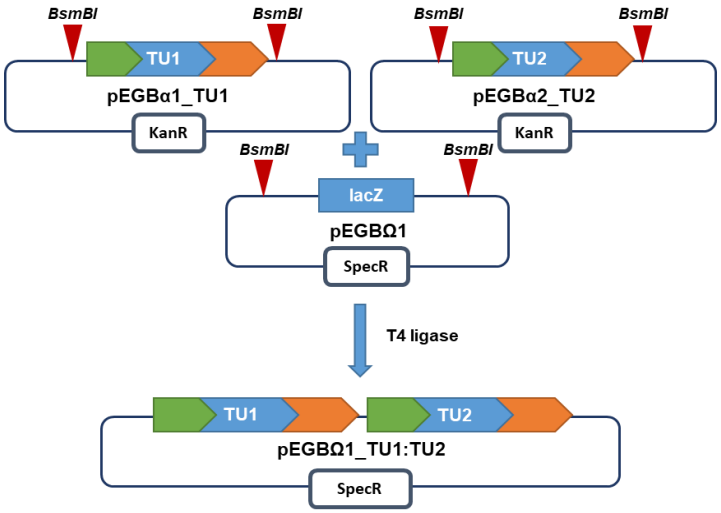


Figure 14 - Schematic representation of a Binary assembly reaction (Adapted from Golden Braid 2.0 User's Manual: figure 7.2.1, pp 13). The creation of multigene vectors (binary assembly) in Golden Braid depends on the combination of vectors containing assembled TUs from different levels, namely, α1/ α1R with α2/ α2R through BsmBI restriction, or Ω1/ Ω1R with Ω2/ Ω2R through BsaI restriction. The TUs will then orderly assemble in a third destination vector, which in the case of a BsmBI restriction will be an omega vector, and in the case of BsaI restriction, an alpha vector. Abbreviations: KanR – kanamycin resistance marker gene; SpecR – spectinomycin resistance marker gene.

The purified TU-containing alpha vectors were subject the third set of restriction-ligation reactions, in which 60 fM of each vector was added to 75 ng of omega vector, together with *BsmBI* and T4 ligase. The schematic representation of the different reactions is in figure 15. Then, the reaction result was used to transform *E. coli* TOP10 competent cells, which were plated in LB plates supplemented with 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside acid (40 µg/mL) and isopropylthio-β-galactosidase (0.5 mM) and spectinomycin (100 µg/mL). White colonies were tested through colony PCR using GBPart specific primers and the positive clones were targeted for plasmid extraction. Afterwards, the plasmids sequence was confirmed through PCR analysis and restriction, and their nucleotide sequences deposited in the Golden Braid database. All subsequent reactions were of this type and followed the same logic of action.

A)

(1) α1::TsP1_CDS6_CDS8_CDS9-Tag1_T1 **(TU_4T) +** α2R::TsP2_CDS7_CDS8_CDS10-Tag2_T2 **(TU_5T)** → ***BsmBI*** → **Ω2::TU_4T::TU_5T**

(2) α1::TsP3_CDS2-Tag3_T4 **(TU_3T) +** α2R::TsP2_CDS3-Tag4_T3 **(TU_2T)** → ***BsmBI*** → **Ω2::TU_3T::TU_2T**

(3) α1::TsP1_CDS1-Tag5_T1 **(TU_1T) +** α2::Stuffer **(SF)** → ***BsmBI*** → **Ω2::TU_1T_SF**

(4) α1::TsP1_CDS1-Tag5_T1 **(TU_1T) +** α2::Stuffer **(SF)** → ***BsmBI*** → **Ω1R::TU_1T_SF**

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(5) Ω1R::KanR (KanR) + Ω2::TU_4T::TU_5T → ***Bsa I*** → **α2::KanR::TU_4T::TU_5T**

(6) Ω1R::KanR (KanR) + Ω2::TU_3T::TU_2T → ***Bsa I*** → **α2::KanR::TU_3T::TU_2T**

(7) Ω1::Stuffer (SF) + Ω2::TU_3T::TU_2T → ***Bsa I*** → **α1::SF::TU_3T::TU_2T**

(8) Ω1R::KanR (KanR) + Ω2::TU_1T_SF → ***Bsa I*** → **α2::KanR::TU_1T::SF**

(9) Ω1::Stuffer (SF) + Ω2::TU_1T_SF → ***Bsa I*** → **α1R::SF::TU_1T::_SF**

---

(10) α1::SF::TU_3T::TU_2T **+** α2::KanR::TU_4T::TU_5T → ***BsmBI*** → **Ω2R::SF::TU_3T::TU_2T::KanR::TU_4T::TU_5T**

(11) α1R::SF::TU_1T::_SF **+** α2::KanR::TU_4T::TU_5T → ***BsmBI*** → **Ω2::SF::TU_1T::SF::KanR::TU_4T::TU_5T**

(12) Ω1R::TU_1T_SF **+** Ω2R::SF::TU_3T::TU_2T::KanR::TU_4T::TU_5T → ***BsmBI*** → **α2::SF::TU_1T::TU_4T::TU_5T::KanR::TU_3T::TU_2T**

B)

(I) α1::TsP1_CDS6_CDS8_CDS9_T1 **(TU_4) +** α2R::TsP2_CDS7_CDS8_CDS10_T2 **(TU_5)** → ***BsmBI*** → **Ω2::TU_4::TU_5**

(II) α1::TsP3_CDS2_T4 **(TU_3) +** α2R::TsP2_CDS3_T3 **(TU_2)** → ***BsmBI*** → **Ω1::TU_3::TU_2**

(III) α1::TsP1_CDS1_T1 **(TU_1) +** α2::Stuffer **(SF)** → ***BsmBI*** → **Ω1R::TU_1_SF**

(IV.A) α1::RS **(TU_8) +** α2::CP_CDS4_T5 **(TU_6)** → ***BsmBI*** → **Ω1::TU_8::TU_6**

(IV.B) α1::IP_CDS5_T5 **(TU_7) +** α2::RS **(TU_9)** → ***BsmBI*** → **Ω2::TU_7::TU_9**

(IV) Ω1::TU_8::TU_6 **+** Ω2::TU_7::TU_9 → ***Bsa I*** → **α1::TU_8::TU_6::TU_7::TU_9**

(V) Ω1::TU_3::TU_2 **+** Ω2::TU_4::TU_5 → ***Bsa I*** → **α2:: TU_3::TU_2:: TU_4::TU_5**

(VI) Ω1R::TU_1_SF **+** Ω2::TU_4::TU_5 → ***Bsa I*** → **α2::TU_1::SF::TU_4::TU_5**

(VII) α1::TU_8::TU_6::TU_7::TU_9 **+** α2:: TU_3::TU_2:: TU_4::TU_5 → ***BsmBI*** → **Ω2::TU_8::TU_6::TU_7::TU_9::TU_3::TU_2:: TU_4::TU_5**

(VIII) Ω1R::TU_1_SF **+** Ω2::TU_8::TU_6::TU_7::TU_9::TU_3::TU_2:: TU_4::TU_5 → ***Bsa I*** → **α2::SF::TU_1::TU_8::TU_6::TU_7::TU_9::TU_3::TU_2::TU_4::TU_5**

Figure 15 - Schematic representation of the binary assembly reactions for the creation of the vectors with cassettes expressing epitope tags (A) and vectors without epitope tags (B). Abbreviations: TsP – tissue-specific promoter; IP – inducible promoter; T – terminator; CDS – coding sequence; RS – recombination site; Stuffer - S. lycopersicum intergenic region (stuffer fragment). KanR – kanamycin resistance marker gene; SpecR – spectinomycin resistance marker gene.

3.2- Quantitative assessment of the expression level for a tissue specific promoter in potato

3.2.1- Plants and Growth conditions

Two *Solanum tuberosum* cv. Desiree representatives of the transgenic line TPPA (De Lepeleire et al., 2018) and two wild-type plants were obtained through vegetative propagation, by cutting uniform sections at the internodes, and underwent a 2 week growth period in vitro in half-strength MS media (Murashige and Skoog) , under 16/8 h light/dark daily cycles. Afterwards, the plantlets were transferred to soil and grown in day-long light cycles, at 24℃. Leaf and root tissue was collected after 6 weeks of growth.

3.2.2- RNA extraction and cDNA synthesis

The synthesis of cDNA from *S. tuberosum* leaf and root tissue was initiated with mRNA extraction using the GeneJET plant RNA purification kit (Thermo Fischer Scientific), then the solution was cleaned up with a DNase treatment, including Ribolock RNase inhibitor (Thermo Fischer Scientific) to minimize RNA degradation during the clean-up procedure. Secondly, a solution containing 1µg of the extracted mRNA was converted to cDNA through the iScript cDNA synthesis kit (Bio-Rad), which contains random primers in addition the polydT oligonucleotides.

3.2.3- qPCR analysis

For the RT-qPCR, the KAPA Sybr fast qPCR kit (KAPA Biosystems) was utilized as a master reaction mix, following manufacturers recommendations. Each run consisted of a denaturation step at 95°C for 3 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing for 20 seconds, at the appropriate temperature for each primer pair, and extension at 72°C for 30 seconds. The collected data was analysed using the qBASE software based on the $2^{-\Delta\Delta}$Ct method (Hellemans et al., 2007; Livak and Schmittgen, 2001).

4- Results

4.1- Vector construction

4.1.1.- Domestication reactions

The first step in the Golden Braid (GB) cloning strategy consists on the domestication of the all the DNA sequences, that are aimed to be used in the vectors. This process consists on a PCR amplification using specific primers, containing a GB tail, that will allow the subsequent cloning of these fragments in an UPD2 vector (domestication). The Golden Braid online software aids in the design of these primers and produces an *in-silico* representation of the final domesticated fragment. For some sequences, it is necessary to remove internal *BsmBI* and *BsaI* restriction sites, as these would interfere with subsequent cloning reactions. This is achieved through the PCR amplification of the target sequence in patches (small fragments), using specific primers that will introduce single mismatches in the restriction site, disrupting it. This was the case for coding sequences 9 (2 patches), 7 (2 patches) and 3 (3 patches).

Additionally, for two DNA fragments, namely, terminator 3 and the root-specific promoter, a nested PCR was performed before the domestication reaction, as these were difficult regions to amplify using only GB-primers. The nested PCR consisted on the amplification of a larger genomic region, containing the sequence of interest, using standard primers. This DNA fragment would be then used as a template for the domestication reaction of the fragment of interest. All the amplifications were performed with the Phusion-High Fidelity DNA polymerase, in 50 µL reactions, out of which 10 µL were used to see the result in an 1% agarose gel electrophoresis (figure 16).

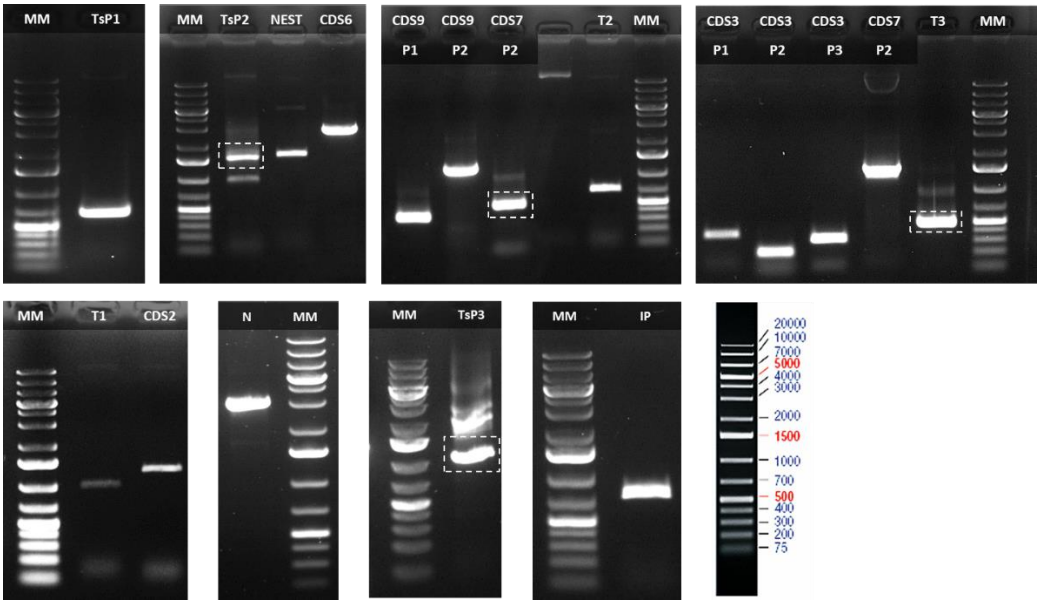

Figure 16 - Electrophoresis in an 1% agarose gel of the PCR amplification with Phusion High-Fidelity DNA polymerase for the domestication reactions. Abbreviations: MM – Gene Ruler 1kb Plus DNA ladder (Thermo Fischer); TsP1 – tissue-specific promoter (624 bp); TsP2 – tissue-specific promoter 2 (1525); NEST – Genomic fragment for the amplification of T3 (1585 bp); CDS6 – Coding sequence 6 (2757 bp); CDS9 - P1 – Coding sequence patch 1 (356 bp); CDS9 - P2 – Coding sequence 9 patch 2 (1106 bp); CDS7 - P1 – Coding sequence 7 patch 1 (1448 bp); CDS7 - P2 – Coding sequence 7 patch 2 (490 bp); T2 – Terminator 2 (642 bp); CDS3 – P1 – Coding sequence 3 patch 1 (352 bp); CDS3 – P2 – Coding sequence 3 patch 2 (195 bp); CDS3 – P3 – Coding sequence 3 patch 3 (308 bp); T3 – Terminator 3 (441 bp); T1 – Terminator 1 (966 bp); CDS2 – Coding sequence 2 (1234

bp); N – Genomic fragment for the amplification of the Root-specific promoter (3000 bp); TsP3 – tissue-specific promoter 3 (1122 bp); IP -Inducible promoter (719 bp).

After confirmation that the correct size band was amplified for all the DNA fragments of interest, these were purified. Then, they were used in the *BsmBI*-mediated domestication reactions, with a normalized concentration of 60 fM, to ensure equimolar ratios of the different patches (or fragments) and UPD2 vector. The resulting solution was transformed into competent *E. coli* TOP10 cells, which were grown in LB media supplemented with X-Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside acid), IPTG (isopropylthio-β-galactosidase) and chloramphenicol. The X-Gal and IPTG allow the visual identification of blue and white colonies, being that the white colonies are representative of successful cloning of the desired DNA fragment in place of the *lacZ* gene, while the chloramphenicol allows the selection of only transformed bacteria. White colonies for each transformation event were targeted for colony PCR, using UPD2 specific primers. As such, only colonies in which the *lacZ* gene was replaced by the fragment of interest will have the correct band size (figure 17).

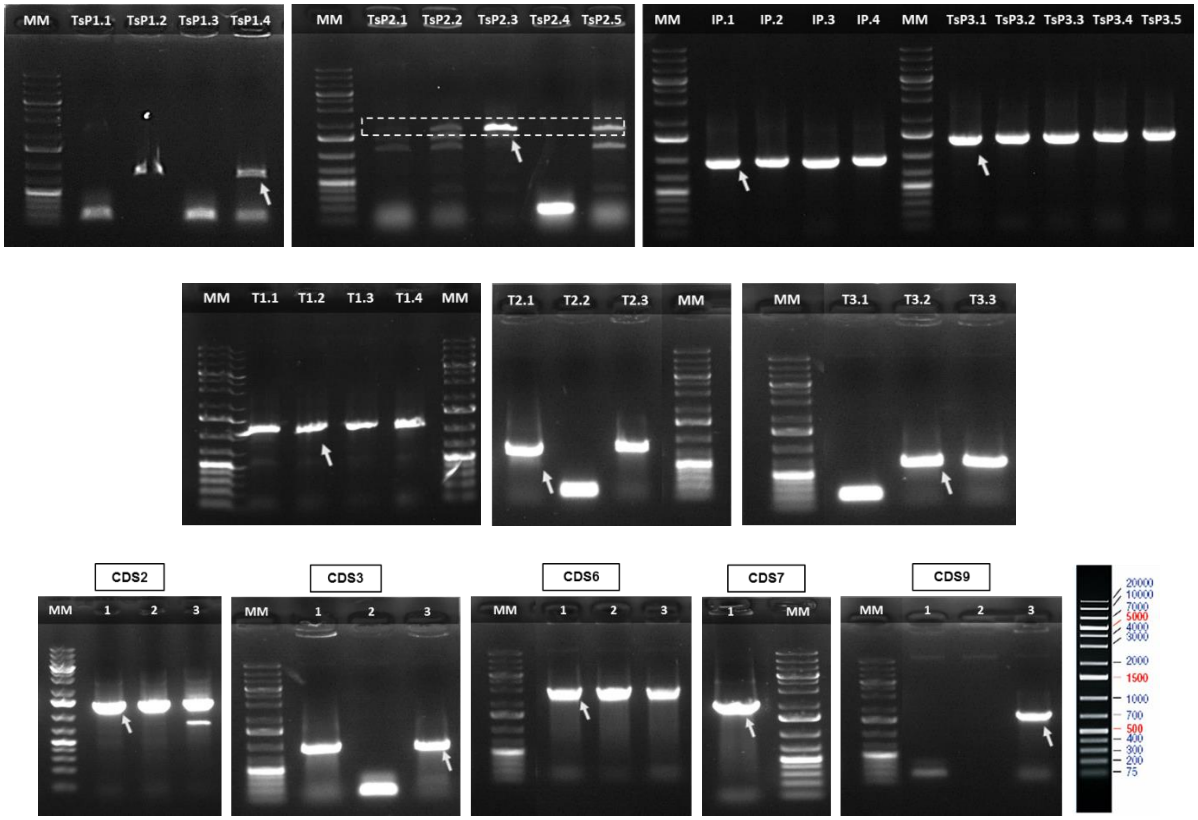

Figure 17 - Electrophoresis in an 1% agarose gel of the colony PCR for screening the domestication reactions results. Arrows point to the colony that was targeted for vector extraction. Abbreviations: MM – Gene Ruler 1kb Plus DNA ladder (Thermo Fischer); colonies TsP1.1 to TsP1.4 – Tissue-specific promoter 1 (624 bp); colonies TsP2.1 to TsP2.5 – Tissue-specific promoter 2 (1525); colonies TsP3.1 to TsP3.5 – Tissue-specific promoter (1122 bp); colonies IP.1 to IP.4 - Inducible promoter (719 bp); colonies T1.1 to T1.4 – Terminator 1 (966 bp); colonies T2.1 to T2.3 – Terminator 2(642 bp); colonies T3.1 to T3.3 – Terminator 3 (441 bp); CDS2_colonies 1 to 3 – Coding sequence 2 (1234 bp); CDS3_colonies 1 to 3 – Coding sequence 3 (768 bp); CDS6_colonies 1 to 3 – Coding sequence 6 (2757 bp); CDS7_colony 1 – Coding sequence 7 (1895 bp); CDS9_colonies 1 to 3 – Coding sequence 9 (1398 bp);

Considering the colony PCR results, one colony was picked from each plate to start a liquid culture, which is indicated in figure 17 by an arrow. These cultures were used to extract the vectors containing the domesticated fragments. Finally, they were submitted for sequencing, to ascertain that the domestication reaction didn't insert any mutations in the original sequence.

4.1.1-1 Domestication of C-terminal tagged coding sequences

Four coding sequences were targeted for the addition of peptide tags in their 3´terminus, namely, CDS1, CDS2, CDS3, CDS9 and CDS10. However, this was only performed after having all the desired fragments domesticated. As such, the “re-domestication” only required the PCR amplification of the sequence of interest from a purified UPD2 vector, with a modified reverse primer, in which the tag of interest would be present before the STOP codon (figure 12). Additionally, for coding sequence 1, due to difficulties in amplification of the whole sequence, an artificial patch was produced (Patch 1 = 467 bp) and used in a domestication reaction with a second patch (Patch 2 = 3452 bp), obtained through normal PCR amplification, with the modified reverse primer. Once more, the PCR reactions were performed with the Phusion-High Fidelity DNA polymerase, in 50 µL reactions, out of which 10 µL were used to see the result in an 1% agarose gel electrophoresis. The result of these reactions is depicted in figure 18.

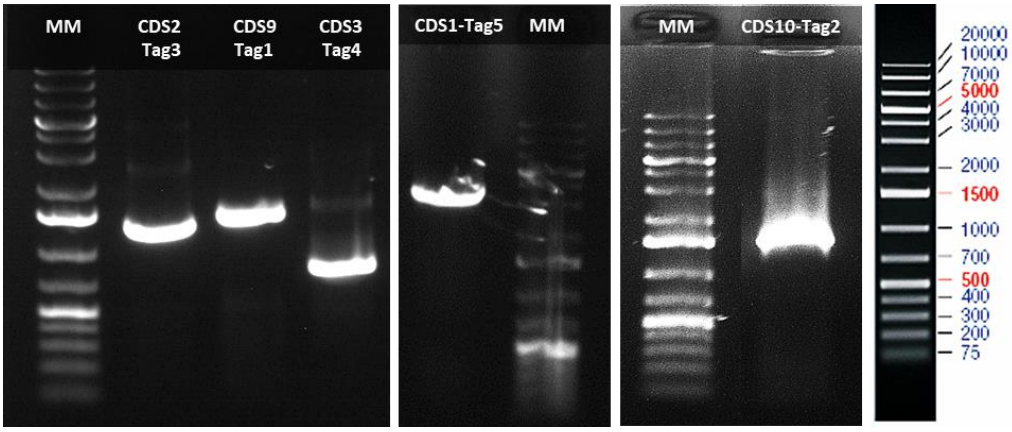

Figure 18 - Electrophoresis in an 1% agarose gel of the PCR amplification for the introduction of a peptide tag in the 3’ end of CDS1, CDS2, CDS3, CDS9 and CDS10. Abbreviations: MM – Gene Ruler 1kb Plus DNA ladder (Thermo Fischer); CDS9_Tag1 – Coding sequence 9 with a tag(1419 bp); CDS3_Tag4 – Coding sequence 3 with a tag (795 bp); CDS2_Tag3 – Coding sequence 2 with a tag (1264 bp); CDS1-Tag5 – Patch 2 of coding sequence 1 with a tag (3476 bp); CDS10_Tag2 – Coding sequence 10 with a tag (1572 bp).

After obtaining the new coding sequence with the tag, the PCR fragments were purified and used in a new domestication reaction, again with all fragments normalized to 60 fM, mediated by a *BsmBI* restriction. The resulting solution was used to transform competent *E. coli* TOP10 cells through heat-shock. Then, white colonies were selected for screening through colony PCR, the result of which is present in figure 19. Coding sequence 1 was screened using specific primers for the 3’ terminal region where the tag would be inserted, but for all the other coding sequences screening was performed with UPD2 specific primers.

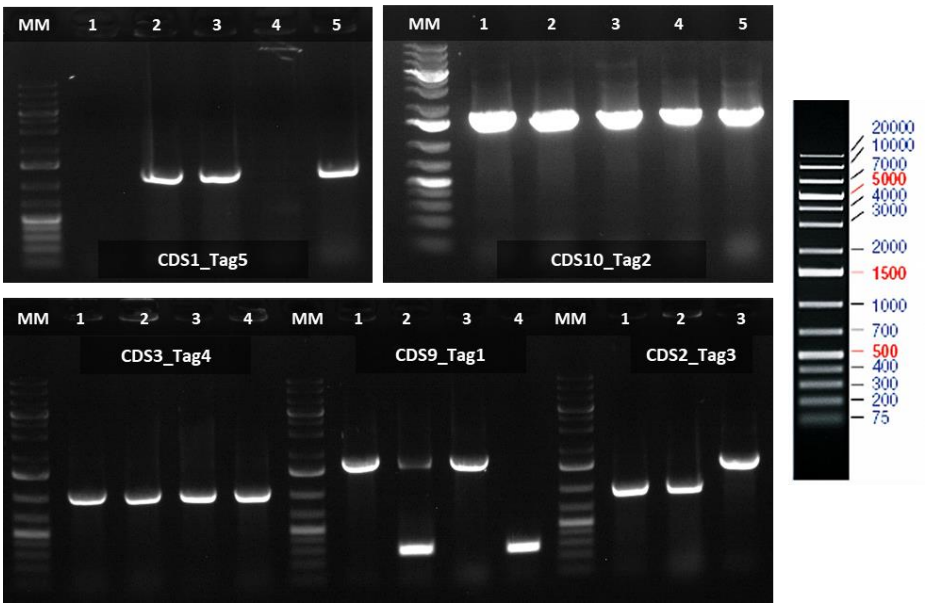

Figure 19 - Electrophoresis in an 1% agarose gel of the colony PCR for confirming the presence of the tagged coding sequences in the UPD2 vector. Abbreviations: MM – Gene Ruler 1kb Plus DNA ladder (Thermo Fischer); CDS9_Tag1 (colony 1 to 4) – Coding sequence 9 with a tag(1419 bp); CDS3_Tag4 (colony 1 to 4) – Coding sequence 3 with a tag (795 bp); CDS2_Tag3 (colony 1 to 3) – Coding sequence 2 with a tag (1264 bp); CDS1_Tag5 (colony 1 to 5) – 3' terminal section of coding sequence 1 with a tag (1058 bp); CDS10_Tag2 (colony 1 to 5) – Coding sequence 10 with a tag (1572 bp).

For each transformation event, a positive colony was selected for starting a liquid culture and subsequent plasmid extraction. The purified plasmids were then sequenced to guarantee that the tag was indeed introduced and that the new amplification didn't insert mutations in the coding sequences. After this, it was possible to proceed to next phase of Golden Braid cloning.

4.1.2.- Multipartite assembly (transcription unit formation)

A transcription unit (TU) is a modular gene expression structure, that is easily customized to ensure that the gene (or genes) aimed for expression are under the user preferred regulatory sequences. The domestication reactions allow the adaptation of the DNA fragments to the Golden Braid 'language', which guarantees that during the formation of the transcription units, each fragment will be orderly assembled. As such, after finalizing the domestication reactions, one can proceed to constructing the transcription units, as described in the material and methods section (table 6).

Table 6 - List of all the assembled transcription units. Abbreviations: TU- Transcription Unit; TsP - tissue-specific promoter; IP – inducible promoter; T – terminator; CDS – coding sequence; RS – recombination site.

ID	Transcription Unit	ID	Transcription Unit
TU_1	α1::TsP1_CDS1_T1	TU_4T	α1::TsP1_CDS6_CDS8_CDS9-Tag1_T1
TU_1T	α1::TsP1_CDS1-Tag5_T1	TU_5	α2R::TsP2_CDS7_CDS8_CDS10_T2
TU_2	α2R::TsP2_CDS3_T3	TU_5T	α2R::TsP2_CDS7_CDS8_CDS10-Tag2_T2
TU_2T	α2R::TsP2_CDS3-Tag4_T3	TU_6	α2::CP_CDS4_T5
TU_3	α1::TsP3_CDS2_T4	TU_7	α1::IP_CDS5_T5
TU_3T	α1::TsP3_CDS2-Tag3_T4	TU_8	α1::RS
TU_4	α1::TsP1_CDS6_CDS8_CDS9_T1	TU_9	α2::RS

All the vectors containing the domesticated DNA parts were normalized to a 60 fM concentration and used for the transcription unit formation reaction, which is an identical program to the domestication, only difference being that it is dependent on a *BsaI* restriction-ligation reaction. After, this aforementioned reaction mixture was used to transform *E. coli* TOP10 competent cells through heat-shock. The bacteria were plated in LB media supplemented with X-Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside acid), IPTG (isopropylthio-β-galactosidase) and kanamycin. This allows blue/white selection, determining correct assembly of the TU in the destination alpha vector, as well as selection for transformed bacteria.

White colonies were targeted for initiating overnight liquid cultures, that allowed for plasmid extraction. Next, the purified plasmids were subject to restriction analysis, to ascertain that the extracted vector had a correctly assembled transcription unit. For this, *in-silico* predictions of the restriction patterns obtained from each reaction were produced in the Vector NTI software. The summary of this analysis is present in table 7.

Table 7 - Summary of the in-silico predictions (Vector NTI) of the restriction pattern obtained for each TU-containing alpha vector. Abbreviations: RE – restriction enzyme; TU ID – Identification of the assembled transcription unit.

Reaction	TU ID	RE	Expected fragments (bp)
1	TU_1	BamHI	8577, 3119, 87
2	TU_4	AvaI	6556, 1887, 1541, 1131, 630, 480
3	TU_5	AvaI	5090, 2597, 1131, 776, 773, 630, 480
4	TU_6	AvaI	4200, 2636, 1131, 630, 480
5	TU_5T	HindIII	8961, 2138, 980
6	TU_3	AvaI	6303, 1131, 630, 480, 470
7	TU_2	AvaI	6873, 1131, 630, 480
8	TU_4T	AvaI	6556, 1905, 1544, 1131, 630, 480
9	TU_1T	AvaI	8163, 1403, 1131, 630, 480
10	TU_2T	AvaI	6903, 1131, 630, 480
11	TU_3T	AvaI	6303, 1131, 630, 480, 470
12	TU_8	AvaI	4168, 1131, 630, 480
13	TU_9	AvaI	4170, 1131, 630, 480
14	TU_7	AvaI	6383, 1131, 630, 480

For all TU-containing vectors, with the exception of TU_5T, (Table 8 - reaction 5) and TU_1 (Table 8 - reaction 1), the restriction was performed with *Ava I*, guaranteeing that there would be a minimum of four cut sites. As for the other two vectors, the restriction was done with *Hind III* and *Bam HI*, respectively. Additionally, in all the restriction reactions performed, at least one of the cut sites would be specific to the TU present in the vector. After this, the restriction outcome was analysed through a 1% agarose gel electrophoresis (figure 20).

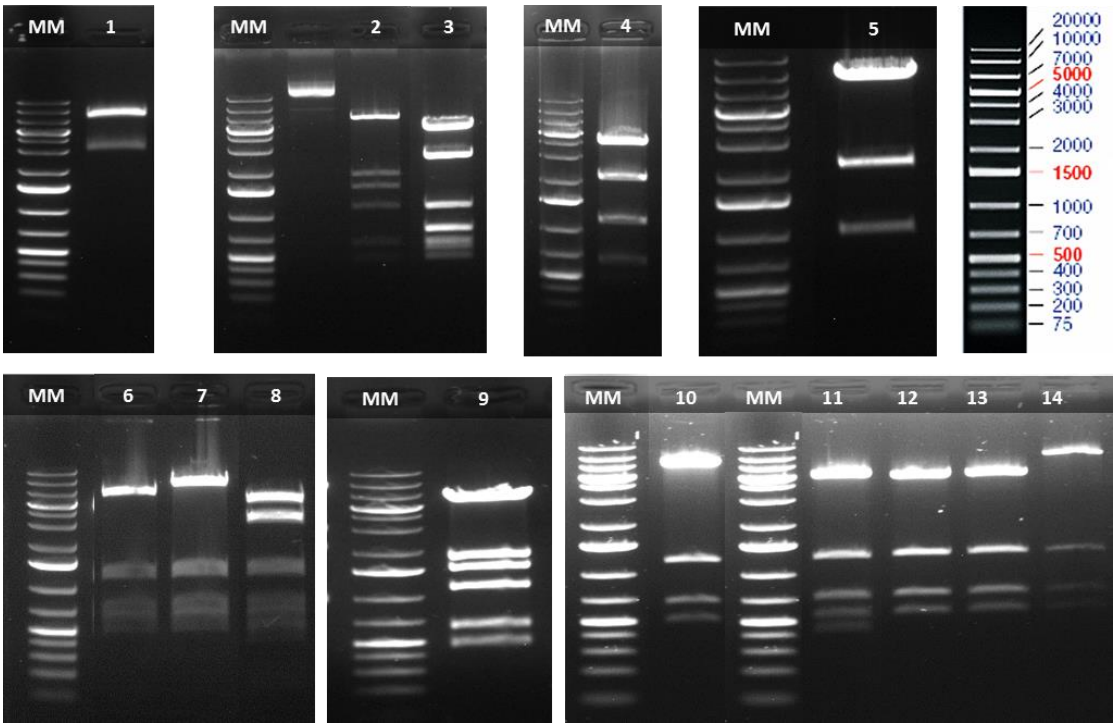

Figure 20 - Electrophoresis in an 1% agarose gel of the restriction reactions with the TU-containing alpha vectors. Each reaction was performed with 500 ng of pure plasmid, 1U/uL of restriction enzyme and 1X restriction buffer. The restrictions had a duration of 1 hour and were set at 37 ºC. Abbreviations: MM – Gene Ruler 1kb Plus DNA ladder (Thermo Fischer); 1 – TU_1; 2 – TU_4; 3 – TU_5; 4 – TU_6; 5 – TU_5T; 6 – TU_3; 7 – TU_2; 8 – TU_4T; 9 – TU_1T; 10 – TU_2T; 11 – TU_3T; 12 – TU_8; 13 – TU_9; 14 – TU_7.

The restriction patterns for all the TU-containing alpha vectors matched the *in-silico* predictions, however, in reaction 9 (figure 20), there is a clear extra band above the expected 1.4 kb restriction fragment. This is possibly genomic DNA contamination that wasn't properly removed during the vector purification step, since all the other bands correspond specifically to the predicted restriction pattern. Specifically, the 1131, 630 and 480 bp bands obtained from backbone specific restriction, the 1403 bp band obtained from restriction between the 3' end of coding sequence 1 and 141 bp downstream of the terminator 1, and the 8163 bp band obtained from backbone specific restriction and TU-specific restriction. After this confirmation step, it was time to proceed to next step in vector assembly (Bipartite assembly).

4.1.2.- Bipartite assembly

The Golden braid cloning strategy makes the creation of multigene expression vectors an easier process, as it follows an iterative "construction" protocol, in which each step increases slightly the level of complexity. Bipartite assembly constitutes the higher level of assembly, in which two complementary vectors will have their DNA inserts combined in a third vector. This process was repeated several times to allow different TU combinations, in light of enhancing one, two or three micronutrients (figure 15). These reactions had as a goal to create a set of expression cassettes with peptide tags (herein referred as tagged vectors), and a separate set without tags (herein referred as non-tagged vectors). The latter containing a self-excision recombination system module, that allowed controlled removal of the selection marker gene.

First, TU-containing alpha vectors were combined in a single omega vector, with a *BsmBI*-mediated restriction-ligation reaction. Because coding sequence 1 (both with and without the strep tag), wasn't composed by two individual transcription units, it was combined with a stuffer vector, in a reaction equal to the former. The Golden Braid kit provides a collection of different level complementary stuffer vectors, which assist for bipartite assemblies. In this case, the stuffer vector contained a small non-coding intergenic sequence of *S. lycopersicum* DNA in place of the TU.

The resulting solution was used to transform competent *E. coli* TOP10 cells through heat-shock and plated in LB media supplemented with X-Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside acid), IPTG (isopropylthio-β-galactosidase) and spectinomycin. White colonies for both sets of reactions (tagged and non-tagged vectors) were selected for screening through colony PCR. The results were then visualized through electrophoresis in a 1% agarose gel (figure 21).

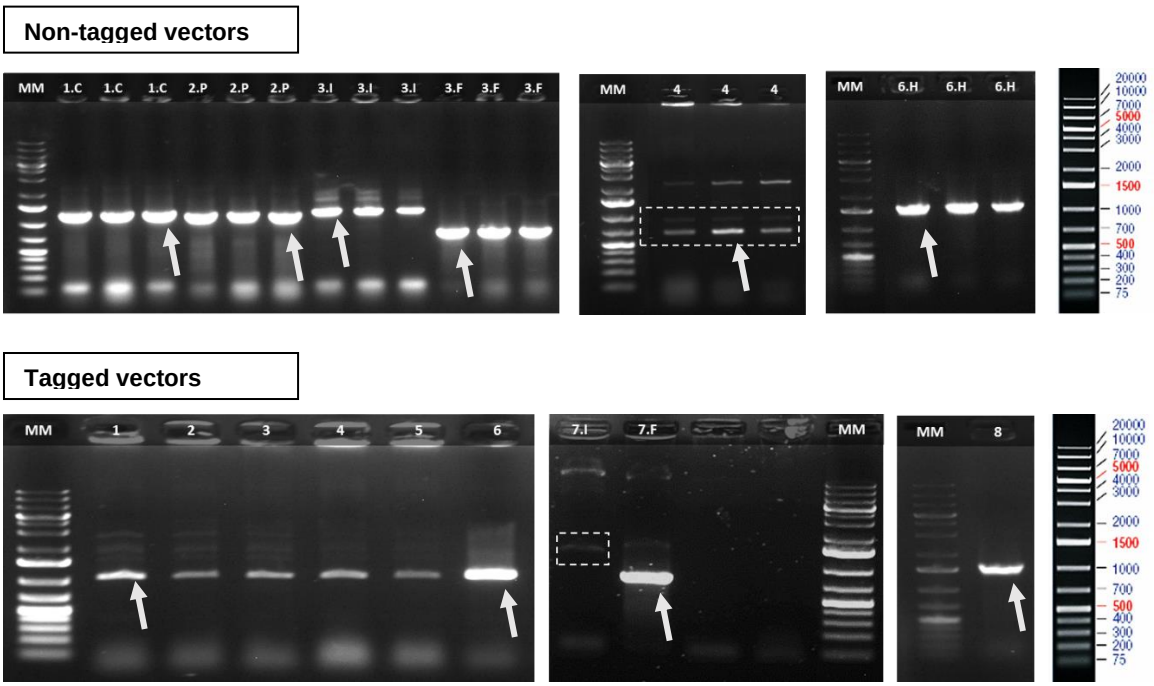

Figure 21 - Electrophoresis in a 1% agarose gel for the visualization of the colony PCR reactions result for the first set of bipartite assemblies. The colonies selected for plasmid extraction are indicated by an arrow. Abbreviations: MM – Gene Ruler 1kb Plus DNA ladder (Thermo Fischer); SF – stuffer fragment (S. lycopersicum intergenic region). **Non-tagged vectors:** 1 – Assembly IV.B (Amplification of a 1 kb region); 2 – Assembly IV.A (Amplification of a 1 kb region); 3.I – Assembly II (Amplification of a 1.2 kb region); 3.F – Assembly II (Amplification of a 768 bp region); 4 – Assembly III (Amplification of a 650 bp region); 6.H – Assembly I (Amplification of a 1.5 kb region). **Tagged vectors:** 1/2/3 – Assembly 3 (Amplification of a 1 kb region); 4/5/6 – Assembly 4 (Amplification of a 1 kb region); 7 – Assembly 2 (7.I – Amplification of a 1.2 kb region , 7.F – Amplification of a 768 bp region); 8 - Assembly 1 (Amplification of a 1.5 kb region).

For each transformation event, a single colony was selected (as indicated by an arrow in figure 21), taking in to account the colony PCR screening, to start liquid cultures. These were used for plasmid extraction. As such, vectors aiming for the enhancement of one micronutrient without tags were finalized. However, for the corresponding set of tagged vectors, a second restriction-ligation reaction, mediated by *BsaI*, had to be done. This time, each vector was combined with a second omega vector, bearing a kanamycin expression cassette (Ω1R::SF::Pnos_NptII_Tnos). This was a necessary step because whilst the non-tagged vectors would be combined with a self-excision plant selection marker gene

module, the tagged vectors needed a marker gene, so that the identification of transformed plants is facilitated.

Once more, the resulting solution was used for the transformation of *E. coli* TOP10 competent cells through heat-shock. After plating in LB plates supplemented with X-Gal (5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside acid), IPTG (isopropylthio-β-galactosidase) and kanamycin, and a growth period of 24 hours, white colonies were selected for screening with colony PCR. The result of those reactions was visualized through electrophoresis in a 1% agarose gel (figure 22).

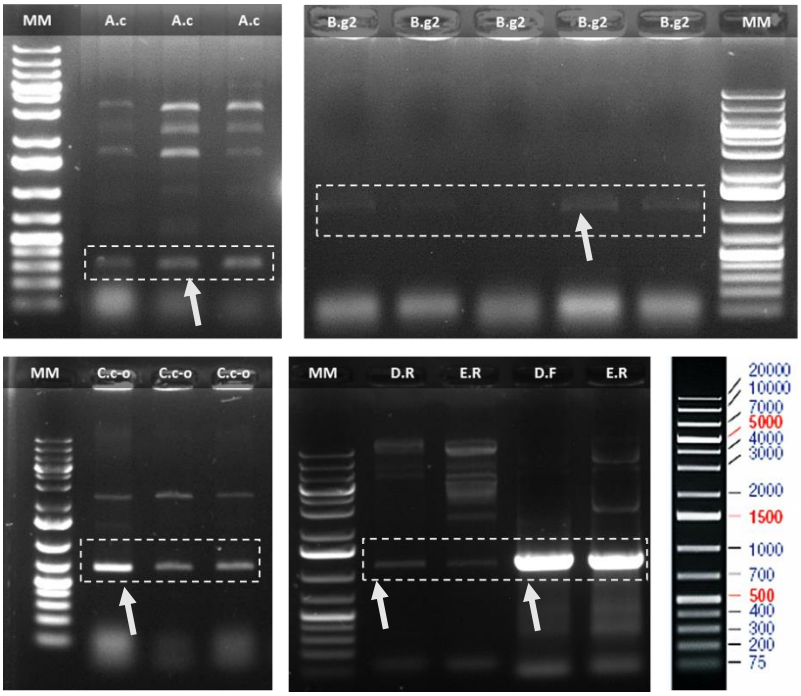

Figure 22 - Electrophoresis in a 1% agarose gel for the visualization of the colony PCR reactions result for the second set of bipartite assemblies. The colonies selected for plasmid extraction are indicated by an arrow. Abbreviations: MM – Gene Ruler 1kb Plus DNA ladder (Thermo Fischer); A.c – Assembly 8 (Amplification of a 289 bp region); B.g2 – Assembly 5 (Amplification of a 1.1 kb region); C.c-o – Assembly 9 (Amplification of a 650 bp region) D – Assembly 7 (D.R - Amplification of a 1.1 kb region, D.F – Amplification of a 1.2 kb region); E – Assembly 6 (D.R - Amplification of a 1.1 kb region, D.F – Amplification of a 1.2 kb region).

Again, the colonies indicated by an arrow in figure 22 were selected for starting new liquid cultures, that allowed for plasmid extraction and purification. As such, all the single micronutrient vectors, both with and without tags, were completed.

Next, two combinations of micronutrients were aimed for the following set of bipartite assemblies, namely, folate with iron and folate with β-carotene. This was performed for both the vectors with the tags, and without tags. In addition, the self-excision recombination module was constructed. All these were dependent on restriction-ligation reactions, either *BsaI* or *BsmBI* mediated depending on whether the destination vector was an alpha or omega vectors, respectively. These were used for the transformation of competent *E. coli* TOP10 cells through heat-shock. Following overnight incubation, white colonies were picked for screening through colony PCR. The result of those reactions was visualized through electrophoresis in a 1% agarose gel (figure 23).

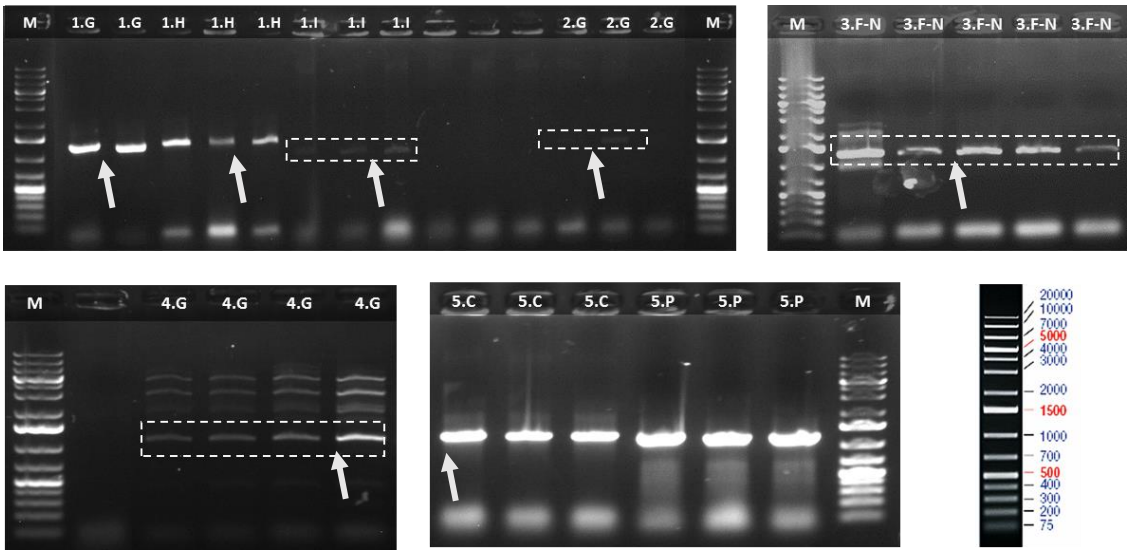

Figure 23 - Electrophoresis in a 1% agarose gel for the visualization of the colony PCR reactions result for the third set of bipartite assemblies. The colonies selected for plasmid extraction are indicated by an arrow. Abbreviations: M – Gene Ruler 1kb Plus DNA ladder (Thermo Fischer). 1- Assembly V (1.G – Amplification of a 1.4 kb region, 1.H – Amplification of a 1.5 kb region, 1.I – Amplification of a 1.2 kb region) ; 2- Assembly VI (2.G - Amplification of a 1.4 kb region); 3 – Assembly 10 (3.F-N - Amplification a 1.2 kb region); 4- Assembly 11 (4.G – Amplification of a 1 kb region); 5 – Assembly IV (5.C - Amplification of a 1 kb region; 5.P - Amplification of a 1 kb region).

Each transformation event yielded at least one colony that provided a positive result in the colony PCR (indicated with arrows in figure 23), so I could proceed with a liquid culture and plasmid extraction.

The final set of reactions allowed for the presence of all micronutrient-enhancing cassettes in a single expression vector. This was achieved by two subsequent restriction-ligation reactions for the non-tagged vector, and one restriction-ligation reaction for the tagged vector (figure 15). First, the self-excision recombination module was combined with α2:: TU_3::TU_2:: TU_4::TU_5, in an omega vector. The resulting solution was used to transform *E. coli* TOP10 competent cells, and these were plated in LB supplemented with spectinomycin. White colonies were used for screening the plates with colony PCR (figure 24).

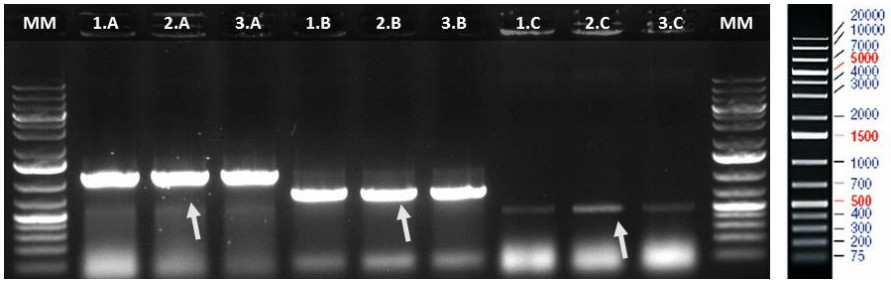

Figure 24 - Electrophoresis in a 1% agarose gel for the visualization of the colony PCR reactions result for the combination of the self-excision recombination module with Assembly 5. The colony selected for plasmid extraction is indicated by an arrow. Abbreviations: MM – Gene Ruler 1kb Plus DNA ladder (Thermo Fischer); 1 to 3 – white colonies 1 to 3; A - Amplification of a 1 kb region; B - Amplification of a 768 bp region; C – Amplification of a 490 bp region.

Colony number 2 from this transformation event was used to start a new liquid culture, that allowed for plasmid extraction. Finally, the two last bipartite assembly reactions were performed, both for the tagged and non-tagged vectors. Because, the final vector in both sets is an alpha vector, they were mediated by a *BsaI* restriction-ligation. Following transformation of *E. coli* TOP10 competent cells, white colonies were chosen for screening through colony PCR (figure 25).

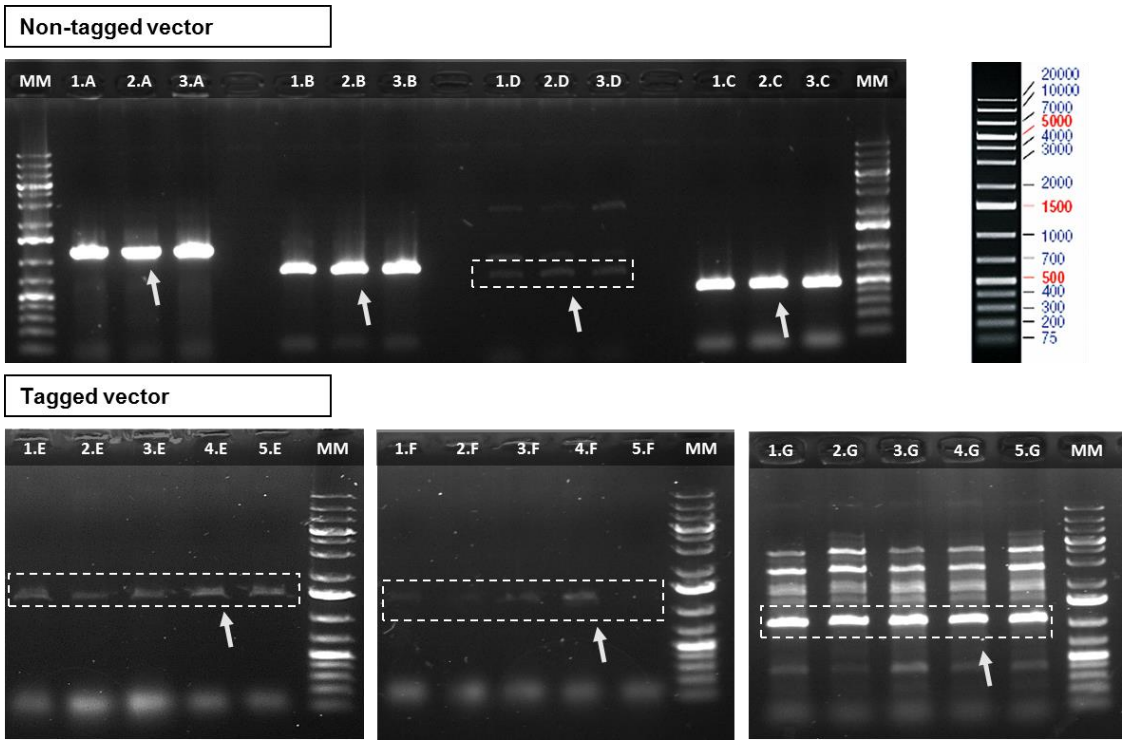

Figure 25 - Electrophoresis in a 1% agarose gel for the visualization of the colony PCR reactions result for the last two bipartite assembly reactions. The colonies selected for plasmid extraction is indicated by an arrow. Abbreviations: MM – Gene Ruler 1kb Plus DNA ladder (Thermo Fischer). **Non-tagged vector:** 1 to 3 – white colonies 1 to 3; A - Amplification of a 1 kb region; B - Amplification of a 768 bp region; D - Amplification of a 650 bp region; C – Amplification of a 490 bp region. **Tagged vector:** 1 to 5 – white colonies 1 to 5; E – Amplification of a 1.4 kb region; F – Amplification of a 1.2 kb region; G - Amplification of a 1 kb region.

Taking into account the colony PCR results, one positive colony was selected from each transformation event, to start new liquid cultures. These were used for plasmid extraction and purification.

Once all vectors had been assembled and purified, it was necessary to further confirm that they matched the *in-silico* sequence predictions. To do so, a series of PCR analysis were performed with the pure vectors. These consisted on the amplification of specific sequences that should exist in the purified vector, if the fragments were correctly inserted. The results of this analysis were visualized through electrophoresis in a 1% agarose gel (figure 26 and figure 27).

ID	Binary Assembly	ID	Binary Assembly
1	Ω2::TU_4T::TU_5T	7	α1::SF::TU_3T::TU_2T
2	Ω2::TU_3T::TU_2T	8	α2::KanR::TU_1T::SF
3	Ω2::TU_1T_SF	9	α1R::SF::TU_1T::_SF
4	Ω1R::TU_1T_SF	10	Ω2R::SF::TU_3T::TU_2T::KanR::TU_4T::TU_5T
5	α2::KanR::TU_4T::TU_5T	11	Ω2::SF::TU_1T::SF::KanR:: TU_4T::TU_5T
6	α2::KanR::TU_3T::TU_2T	12	α2::SF::TU_1T::TU_4T::TU_5T::KanR::TU_3T::TU_2T

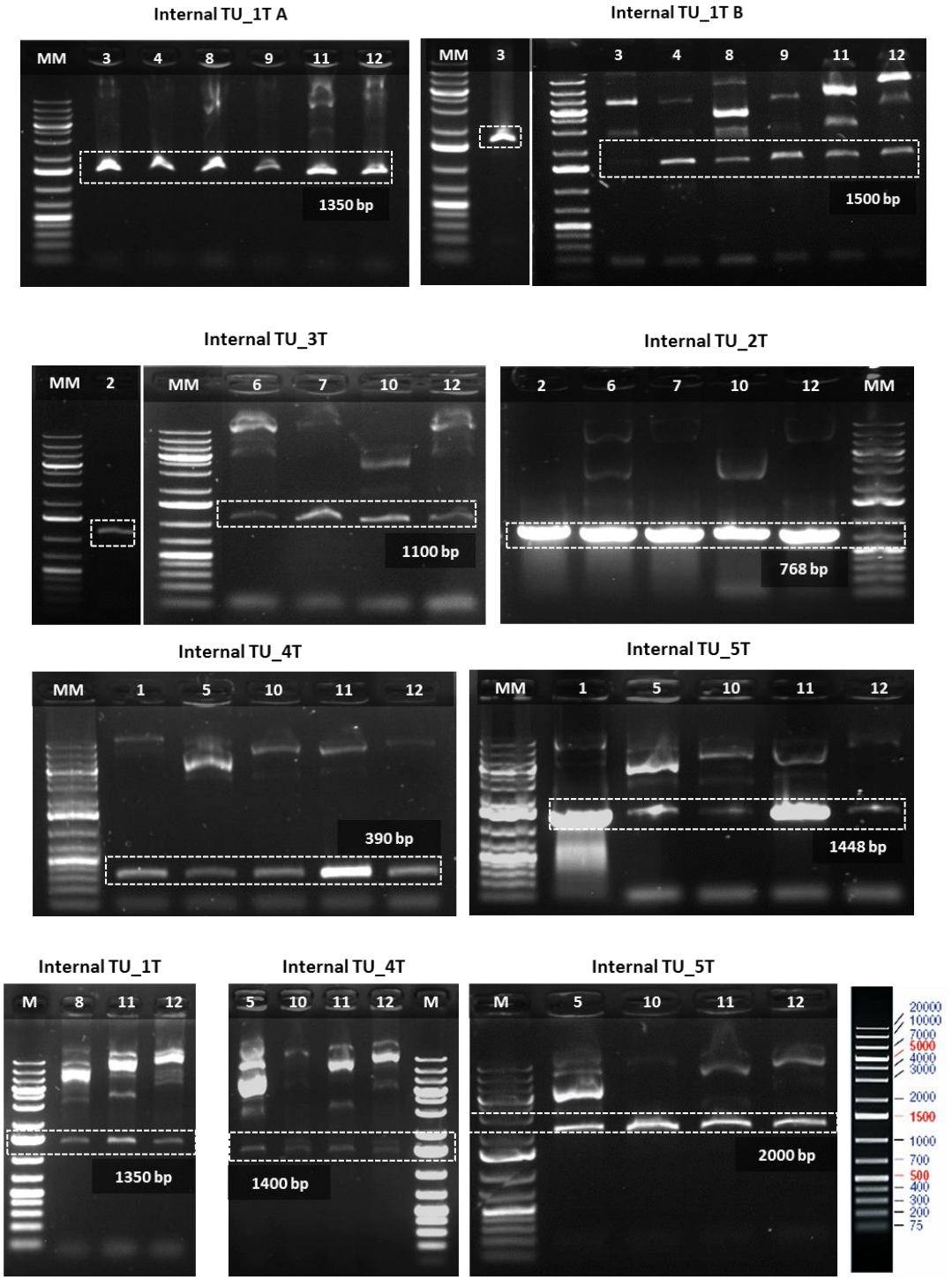

Figure 26 - Electrophoresis in a 1% agarose gel for the visualization of the PCR analysis for tagged vectors. The different binary assemblies are represented by numbers 1 to 12, as shown in the table over the gels. Above each gel, the TU whose internal fragment was targeted is indicated, as well, as the expected band size. **Abbreviations:** MM – Gene Ruler 1kb Plus DNA ladder (Thermo Fischer); TU – Transcription unit.

ID	Binary Assembly
I	Ω2::TU_4::TU_5
II	Ω1::TU_3::TU_2
III	Ω1R::TU_1::SF
IV	α1::TU_8::TU_6::TU_7::TU_9
V	α2:: TU_3::TU_2:: TU_4::TU_5
VI	α2::TU_1::SF::TU_4::TU_5
VII	Ω2::TU_8::TU_6::TU_7::TU_9::TU_3::TU_2:: TU_4::TU_5
VIII	α2::SF::TU_1::TU_8::TU_6::TU_7::TU_9::TU_3::TU_2::TU_4::TU_5

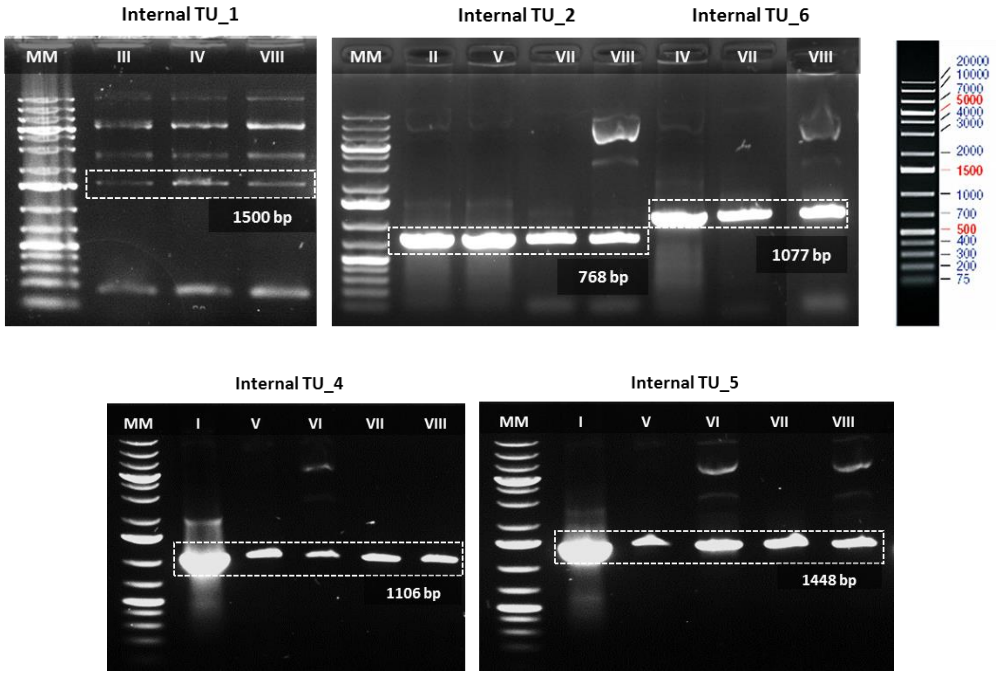

Figure 27 - Electrophoresis in a 1% agarose gel for the visualization of the PCR analysis for the non-tagged vectors. The different binary assemblies are represented by numbers I to VIII, as shown in the table over the gels. Above each gel, the TU whose internal fragment was targeted is indicated, as well, as the expected band size. **Abbreviations:** MM – Gene Ruler 1kb Plus DNA ladder (Thermo Fischer); TU – Transcription unit.

As it can be verified in both figures 26 and 27, the sets of assembled vectors allowed the amplification of correct size fragments, correspondent to sections of some of the transcription units constructed. Next, to confirm that the primer pairs utilized weren't amplifying unspecific regions of the purified vectors, or possible residual DNA fragments, a set of negative and positive control reactions were performed.

The positive controls templates consisted of purified and sequence verified UPD2 vectors (figure 28 - TOP), containing different domesticated fragments, or TU-containing alpha vectors (figure 28 - BOTTOM), namely, α1::TsP1_CDS1-Tag5_T1, α1::TsP1_CDS6_CDS8_CDS9_T1 and α2R::TsP2_CDS7_CDS8_CDS10_T2. The negative control template for all reactions was the α1::TsP3_CDS2_T4 vector, except the one concerning the Internal TU_3T amplicon, which had as template the α2R::TsP2_CDS3_T3 vector. All the reactions were performed equally to the PCR analysis, with the same number of cycles and identical annealing temperatures.

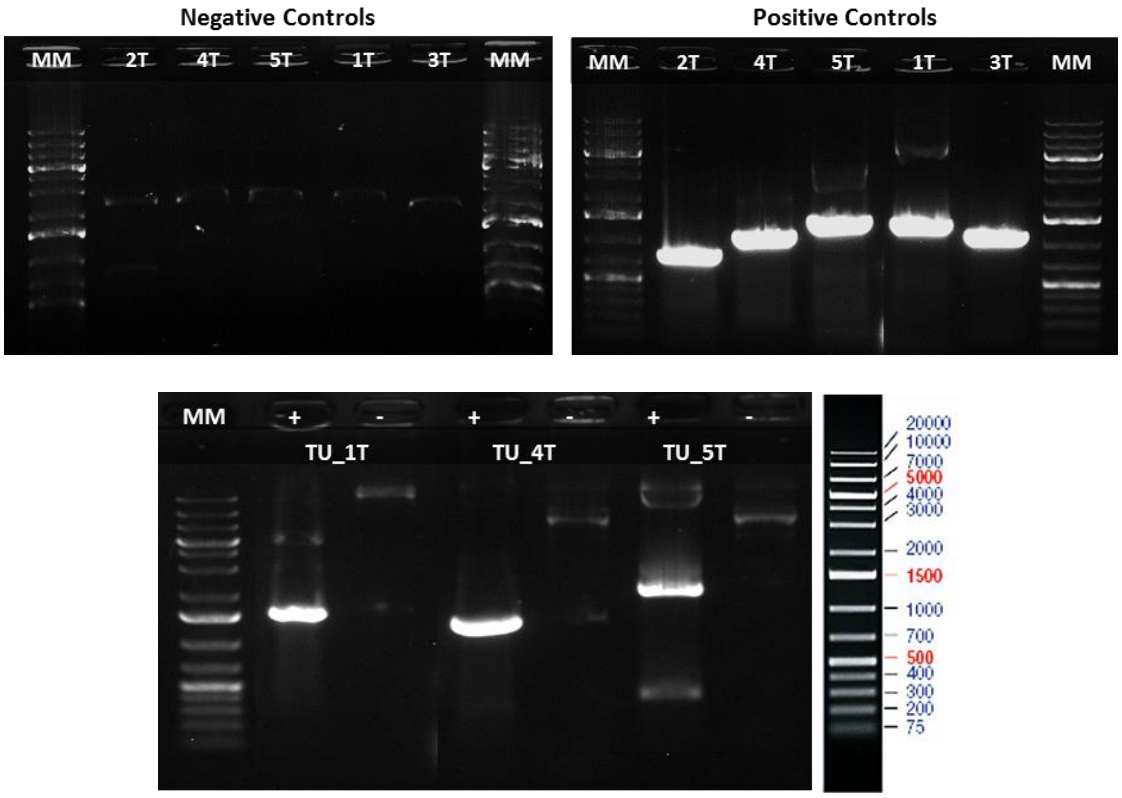

Figure 28 - Electrophoresis in a 1% agarose gel for the visualization of the positive and negative controls of the PCR analysis for the both the non-tagged and tagged vectors. Each reaction aimed for the amplification of an internal fragment within the different created TUs. **TU internal fragments :** 2T –768 bp amplicon ; 4T – 1.1 kb amplicon ; 5T – 1.448 kb amplicon ; 1T - 1.35 kb amplicon; 3T - 1.1 kb amplicon; TU_1T – 1.5 kb amplicon; TU_4T - 1.4 kb amplicon; 5T - 2 kb amplicon. **Abbreviations:** MM – Gene Ruler 1kb Plus DNA ladder (Thermo Fischer); '+' – positive control; '-'– negative control.

As depicted in figure 28, all the primer pairs utilized for analysing the pure vectors were specific for their sequences. Also, they did not lead to the amplification of a similar size fragment in a vector that didn't contain the sequence of interest.

The final step in ascertaining vector sequence was a restriction analysis, followed by sequencing, which was performed in the tagged final vector. It underwent a restriction reaction with *Not I* FD and *EcoRI,* as well as a linearization with *SmaI*. The result was visualized through an electrophoresis in a 1% agarose gel (figure 29).

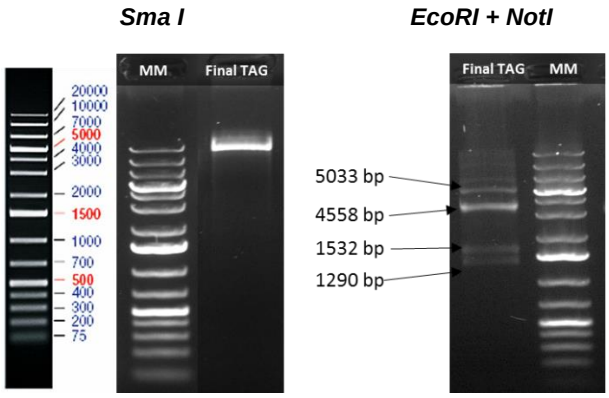

Figure 29 - Electrophoresis in a 1% agarose gel for the visualization of restriction analysis performed in the final tagged vector. In the left, vector linearization (30 kb) achieved through *SmaI* restriction, and in the right, restriction pattern after digestion with

*No*t I FD and *EcoRI* (18309, 5033, 4558, 1532, 1290 bp). Abbreviations: MM - Gene Ruler 1kb Plus DNA ladder (Thermo Fischer); Final TAG - α2::SF::TU_1T::TU_4T::TU_5T::KanR::TU_3T::TU_2T (SF - *S. lycopersicum* intergenic region (stuffer fragment), KanR – kanamycin resistance marker gene, TU – Transcription unit).

As it can be seen in figure 29, some degradation occurred in the digestion reaction with *EcoRI* and *NotI* FD, since the biggest band (approximately 18 kb) couldn't be visualized. However, the linearization reaction allowed to ascertain that the vector is indeed the correct size, since the single 30 kb fragment is clearly seen. Due to the vector's size, only a small region was targeted for sequencing, namely, one of the iron associated transcription units.

Two different primers were used for the sequencing, the first would bind approximately 900 bp upstream of the coding sequencing, allowing the analysis of the correct assembly of the promoter and coding sequence inserts in the TU. The second primer would bind in the 3' terminal region of the coding sequence, which would allow to see the correct insertion of the terminator downstream of the coding sequence. Both primers showed the correct assembly of this TU, which guaranteed that the Golden Braid cloning system was indeed effective in the construction of the vectors.

4.2 - Quantitative assessment of the expression level for a tissue specific promoter in potato

The majority of plant genetic engineering projects use constitutive promoter sequences, that help in gene overexpression, but sometimes at the cost of yield or physiological impairments (Lim *et al.,* 2012). In this sense, one of the main goals of this project was to utilize tissue-specific promoter sequences, that would drive a high transgene expression.

In search of good promoters, the capacity of a promoter from *S. tuberosum* in driving high expression, within the root tissue, was assessed. As such, the first step was to identify the promoter specific sequence and try to domesticate it, from a genomic DNA sample of *S. tuberosum*. However, two different sequences were targeted (tissue-specific promoter 4.1 (TsP4.1) and tissue-specific promoter 4.2 (TsP4.2)), and no amplification occurred. As such, a more careful analysis of the publicly available sequences was performed (The Potato Genome Sequencing Consortium, 2011).

First, the presence of a characteristic motif present in all promoters from this gene family was verified. This motif is a 22 bp insert, and it constitutes the main structural difference between the two different classes that constitute this gene family in *S. tuberosum* (Twell and Ooms, 1988). Typically, it is present near the TATA box in the promoter region, upstream of the primary transcript. Notably, both promoter sequences lacked the characteristic 22 bp insert.

Next, mapping these sequences against the most recent genome assembly for *S. tuberosum*, to try and assess already available annotation of these regions was performed. Both tested sequences here were mapped to chromosome 8, however these alignments contained a high number of gaps. Also, this section of the genome has scarce annotation and is highly repetitive, so it remained unclear whether these sequences are indeed corresponding to regulatory sequences of this gene family.

In a last attempt to verify if this promoter could be used in this work, a promoter from the same family, present at the laboratory was tested through qPCR. This promoter had already been used in a previous project (De Lepeleire et al, 2018), yielding high expression of folate enhancement genes in potato tubers. For this analysis, 2 sets of plants were grown in soil until an adult stage (approximately 2 months). The analysis included one wild-type and one transgenic potato plant, transformed with 2 sets of vectors aiming for folate genes overexpression, under the control of the tissue-specific promoter 4.3 (TsP4.3). The experiment consisted in the comparison of its endogenous gene expression, with one of the transgenes that was being regulated by this promoter. Three tissue samples were collected from leaf, root and tuber in each set of plants. After, RNA extraction from each sample, the cDNA was synthesized. This served as the template for the quantitative expression assay (figure 30).

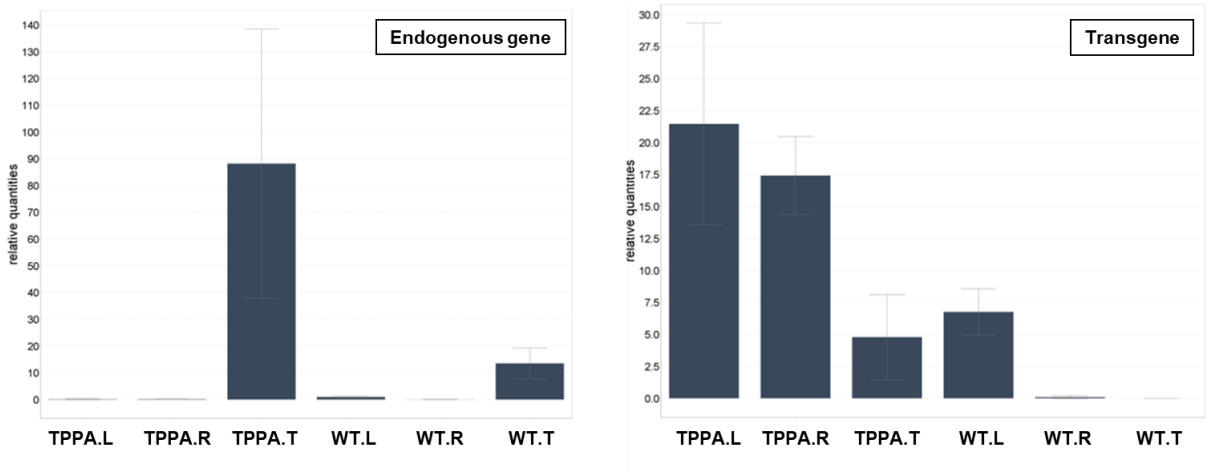

Figure 30 - Relative expression analysis of a transgene and endogenous gene in leaf, root and tuber tissue of *S. tuberosum* transgenic potato plant A (TPPA) and wild-type plants (value 1 represents WT mean relative expression for that tissue). Expression analysis was conducted using quantitative real-time PCR adult potato plants. Data analysis was performed using the qBASE software based on the 2–ΔΔCt calculation method. Normalization was achieved using *S. tuberosum* Elongation factor 1α and ribosomal protein L8 as reference genes (Nicot et al., 2005). The error bars represent standard deviation. Abbreviations: WT – S. tuberosum wild-type; TPPA – transgenic potato plant A; L – leaf tissue; R – root tissue; T – tuber tissue.

In transgenic plants (TPPA), endogenous gene expression was mainly identified in tuber tissue, with a very low, almost inexistent expression in root and leaf tissue. As for transgene expression, the higher expression in root and leaf tissue when comparing to the tuber, is clear. The wild-type plants recorded a much lower expression of the endogenous gene in tuber tissue, when comparing to the transgenic plants. Finally, as expected, the wild-type expression of the transgene was lower than the expression in transgenic plants.

This promoter belongs to a multicopy gene family that shares more than 95% sequence homology, which difficult the identification and annotation of the different genes, and respective regulatory sequences. After analyzing, these three promoter sequences, it remained still unclear whether they would allow replication of previous results, amounting to good root expression. As such, more experimental work is needed to review, and perhaps rectify previous work.

5- Discussion

Optimizing micronutrient status in the general population is a major concern for global public health, since MNDs pose various detrimental effects on human health. They rarely occur in isolation, as the populations that are more afflicted by it, typically rely on monotonous diets, with low intake of micronutrient-rich foods. The creation of transgenic multi-biofortified crops could help alleviate this problem, particularly in rural areas with little access to other alternatives. With this in mind, the main goal of this work was to develop the molecular tools that could allow the creation of a multi-biofortified potato. This improved variety would contain a higher concentration of folate, iron and beta-carotene (provitamin A) in, which constitute three of the most problematic micronutrient deficiencies worldwide.

5.1- Designing a metabolic engineering strategy

The creation of nutritionally enhanced crops depends on genetically engineering various metabolic pathways, to achieve higher micronutrient concentration, accumulation or/and stability. This is dependent on the transformation of plants with multi-gene expression vectors, that can target different aspects of micronutrient biosynthesis and metabolism. As such, this project aimed for the creation of two separate sets of expression vectors. One of the sets would promote micronutrient enhancement and accumulation, whilst containing a selection-marker gene self-excision module. This is to meet current public acceptance issues, facilitating the delivery of the modified tubers, to the populations in need. The other set of vectors would serve a more fundamental research purpose, as it would contain tagged coding sequences, that will hopefully allow a better understanding of protein interaction and micronutrient metabolism. This is of high relevance, as to our knowledge, there is no information regarding possible physiological modifications or adaptations in plants with higher amounts of multiple micronutrients. Nor is the interaction of these different metabolic pathways completely clarified yet. Additionally, this line of vectors could help to identify possible bottlenecks, or even allow insights into post-transcriptional regulation of the different proteins.

To allow for an enhancement of the three micronutrients, a literature mining was performed, to select genes that have clear evidence of enhancing micronutrient levels, either through a higher biosynthesis, accumulation or stability. This search had three main guidelines, first, the metabolic engineering strategy to enhance the micronutrient was successful in fortifying the plant, second, the strategy can be translated to potato tubers, and third, there were no described detrimental effects associated with the strategy. Through the careful combination of different elements from robust metabolic engineering strategies, this project aimed for integrating the current state-of-the-art. In addition to the selection of the appropriate genes, associated with a given desired trait, it is of outmost importance to pay proper attention to the regulatory regions controlling their expression.

There are significant differences in development and anatomy between monocot and dicot plants, so it is important to not assume that knowledge obtained from one group can be directly transferred to the other (Manavella and Chan, 2006). This implies that the regulation modules driving gene expression must be adapted to the plant of interest. Despite this, most transgenic crops have constitutively expressed genes, under the regulation of the 35S cauliflower mosaic virus promoter (Brisson et al.,

1984) or maize ubiquitin-1 promoter (Cornejo et al., 1993). These promoter sequences provide the whole plant with transgene expression. However, their usage is sometimes inadequate, leading to detrimental effects in plant physiology or crop yield (Hsieh et al., 2002; Kasuga et al., 1999). Furthermore, both these promoters have been proved to be susceptible to epigenetic transgene silencing (Meng et al., 2003; Rajeevkum et al., 2015). This has led to a general insecurity in overexpressing transgenes in a systemic fashion (Potenza et al., 2004).

To tailor the plant metabolism, without overloading it or compromising critical physiological processes, it is best to have temporal and tissue-specific expression of the introduced transgenes. This is not only dependent on the utilization of tissue-specific promoters, but also optimal terminator sequences, that can increase transcript stability and facilitate in the messenger RNA processing (Xing et al., 2010). In fact, when multiple gene cassettes coexist in the same expression vector, different terminators should be used to minimize gene silencing events, since they are promoted by the repetitive use of the same sequence (Xing et al., 2010). Again, despite the well-established role of terminators in gene expression, most genetic engineering projects still rely only on the usage of the nopaline synthase (Nos) and octopine synthase (OCS) terminators from *Agrobacterium tumefaciens*, or the 35S terminator from the cauliflower mosaic virus (Diamos and Maison, 2018).

In Diamos and Mason, 2018, the ability of different terminators in influencing GFP synthesis was assessed, to compare the three previous terminators efficiency with other less used sequences. In light of their work, as well as other authors, four distinct terminator sequences were selected.

Lastly, one of the main goals of this project was the utilization of the potato tuber as a sink tissue for the stable accumulation of folate, iron and beta-carotene. This is dependent on the utilization of tissue specific promoters, as such, two of the selected promoter sequences lead to tuber specific gene expression. The third utilized promoter controlled the expression of coding sequence 2, which encodes a protein located in the root tissue. This meant that expression needed to be high in the root tissue, and not the tuber, as such, a tomato root-specific promoter was chosen. The choice of this promoter was possible because these two species, *S. tuberosum* and *S. lycopersicum*, are very closely related (Rodriguez et al., 2009; The Tomato Consortium, 2012).

5.2- Assessing the potential of a candidate root-specific promoter from *S. tuberosum*

A promoter sequence was investigated for its potential in driving root specific expression in potato. Two different sequences were identified and targeted for amplification from the *S. tuberosum* genomic DNA, however no amplificon was obtained after multiple attempts. Also, both the sequences lacked a characteristic 22 bp insert that should be present in this type of regulatory sequence. This typical motif is present upstream of the transcription initiation position, and it was absent in both targeted sequences. A third promoter, similar to the previous two, present in the laboratory was assessed, as it had been utilized in a previous project, although for tuber expression, not root. Once more, the characteristic motif was absent, however transgenic plants with genes under its control had already been produced, so a quantitative expression analysis was performed.

This preliminary test aimed for the comparison of expression, in different potato tissues, of one transgene under the control of this promoter and the endogenous gene expression, in both transgenic and wild-type plants. Our results fail to replicate previous reports, as endogenous gene expression in *wt* subjects is only detected in tuber tissue, with leaf and roots having a very low expression value (figure 30). In transgenic plants, endogenous gene expression was equal to what had been observed for *wt* plants, however transgene expression under the regulation of this promoter was indeed higher in root tissue, than in tubers (figure 30). The standard deviation in this assay was high (figure 30), so little conclusions can be drawn without a more thorough analysis and more technical repeats.

Despite of its negative result, this primary assay was relevant in the sense that it enhanced the need for updating expression data regarding regulatory sequences and genes, that were retrieved prior to the Genomics Revolution, in the early 2000s (Peterson et al., 2018). This is because more robust sequencing techniques were since developed, that allowed the sequencing of a multitude of different genomes, which helps in the verification process of the retrieved sequences. Most of the times, if not all, the newly curated sequences weren't assessed again for their role in gene expression. This creates problems when researches try to use the new sequence, basing off the older expression study. Additionally, most plant genomes are not yet properly annotated, which creates many difficulties when utilizing repositories with curated information, since they are often incomplete and complicated to navigate. Finally, most genetic engineering projects utilize promoters that are known to be successful in attaining the desired expression pattern but haven't been thoroughly characterized. This means that a promoter region has been identified, however a formal expression analysis was not yet performed. This makes difficult their translation into other projects, as well as comparison with other well characterized promoters.

All things considered, it appears clear that more efforts need to be put in annotating genomes, and consequently, updating DNA sequences databases, whilst (re)assessing expression patterns of the curated sequences.

5.3- Implementation of novel cloning methodologies: benefits and shortcomings

The rapid development of synthetic biology frameworks is aiding in the construction of different types of vectors, and in this case particularly, it is making the construction of multi-gene expression vectors an easier, high throughput method (Nora et al., 2018). So far, different assembly strategies have been proposed, but systems based in type IIS restriction enzymes show greatest promise, as they aim for the creation of standard and interchangeable DNA parts (Vazquez-Vilar et al., 2017). These enzymes produce four-nucleotide sticky ends in each DNA fragment, which can be efficiently joined together by a T4 ligase. Also, these reactions occur in a single tube, which enhances yield and facilitates technical preparations. The Golden Braid technology consists in one of these modular DNA assemblies, based off Golden Gate cloning. This strategy enables an almost infinite increase of complexity, through the binary assembly of separate transcription units (TU) in a single destination vector. In addition, it facilitates the exchange of domesticated DNA fragments and TUs, between distinct research units, due to its modular and inter-changeable nature.

Golden braid cloning can be divided into three separate phases, namely, domestications, multipartite assembly and bipartite assembly. The first phase consists in the adaptation of the DNA fragments to the Golden Braid language, through PCR amplification using primers containing specific GB tails. This is a key step to ensure exchangeability of all utilized parts, facilitating their usage in future projects. In addition, all the newly created DNA parts are deposited in a comprehensive online repository, that allows the prediction of their assembly in different vectors, according to the user's preferences.

The application of the UPD2 vector as a universal entry vector, for all domestications, allowed the utilization of the same DNA fragments in more than one cassette, without requiring any adjustments to the vector. Second, because the domestication reaction leads to the introduction of the four-letter codes in the DNA fragments, the vector backbone remains unchanged. This allows greater flexibility when choosing the vectors for assembly, because we don't need to concern about compatibility of the different DNA fragments with the vector. Only in subsequent reactions is this a concern, to allow for higher complexity assembly. Moreover, once all the domestication products are verified through sequencing, and following reactions have a very residual chance of introducing mutations, in theory, no more sequencing should be required.

Some difficulties have arisen in the domestication phase, namely, when utilizing the online software for analysing possible internal *BsaI*/*BsmBI* restriction sites. It occurred that one of these sequence modifications suggested by the program led to an aminoacid substitution in the final protein. This was easily fixed by switching the problematic codon, by a synonymous counterpart, however it points to the fact that manual verification is necessary to ensure that no problematic modifications occur. Furthermore, a major flaw of the online domestication software is the impossibility of utilizing sequences smaller than 100 bp, preventing the simple analysis of some interesting DNA motifs, regulation sequences or recombination sites. As was the case for the cloning of the recombination sites. Nevertheless, the technical team behind the cloning framework was able to aid in this problem, by manually introducing this sequence, but it remains an issue for future projects, with similar sequences.

The second phase of this cloning strategy allows the composition of transcription units, which can be as simple, or as complex as desired, allowing the customization of the typical promoter::CDS::terminator structure accordingly. In fact, each TU can be composed of up to ten different components, that will all be assembled in a single step. At this phase, the only requirement is that the DNA fragments have been previously domesticated and introduced in a UPD2 vector. Then, the four-letter codes present in each fragment will determine its position within the transcription unit. This modular nature allowed an easy adaptation of the different transcription units (figure 13), whilst simplifying the actual assembly protocol.

Finally, the third phase consists in the pairwise combination of TUs in a third destination vector. This final step can be repeated various times to create high complexity multi-gene expression vectors. Here, the user needs to keep in mind that each TU has to be in a complementary level vector. This means that combinations can only be performed between alpha 1 (or 1R) and alpha 2 (or 2R) vectors, or omega 1 (or 1R) and omega 2 (or 2R) vectors. Then, these TUs are combined in a third opposite level vector, in a *BsaI* (for alpha-level reactions) or *BsmBI* (for omega-level reactions) restriction-ligation reaction, depending on the vectors in which the TUs were assembled.

A major plus of utilizing this cloning technology consists in the fact that the online software is user-friendly and provides standard, simple protocols for each assembly step. In addition, it has experimental data obtained from previously assembled vectors, demonstrating different expression patterns from various regulatory regions. This is particularly helpful when the user is wondering about the utilization of a specific DNA fragment, provided in the Golden Braid kit, for a given vector construction. Another advantage of this system is that the vectors utilized in multipartite and bipartite assembly, namely alpha and omega, are binary vectors. This means that at any given point they can be used for the transformation of the desired plant, which shortens the timeline of vector construction for transformation, allowing rapid testing of different designs *in vivo*.

Despite the simplicity of the provided protocols, some technical difficulties emerged during the construction of these high complexity vectors. Whilst multipartite assemblies were easily obtained, bipartite assemblies were a lengthy process, with numerous attempts at each level of increased complexity. Perhaps due to the ever-increasing size of the produced vectors, which difficult the bacterial transformation step. Evidence of this was the presence of fewer colonies in each transformation step. Sequentially, there were some complications in the purification of vectors, which made the process of confirming if they were correctly assembled more difficult. Genomic DNA contaminations were very frequent, so the plasmid isolation protocol had to be optimized, to obtain very pure solutions.

Altogether, the GB framework is a simple to use cloning strategy, that had enough flexibility to allow the creation of all the envisioned vectors and permitted this to happen relatively quickly. In the end, 24 DNA fragments were domesticated, from genomic samples or pure plasmid preparations; 14 transcription units were assembled, and 20 binary assemblies were performed. This amounts to a total of 59 constructed vectors, going up to 30 kilobases, that will serve for the enhancement of one, two or three micronutrients in tuber tissue.

6- Conclusions

Developing and distributing transgenic multi-biofortified plant crops could help alleviate micronutrient malnutrition, particularly in rural areas with little access to other alternatives. This relies on the selection of appropriate food vehicles, as well as micronutrients for the target population. In this sense, the tailoring of effective molecular tools could allow obtaining these modified plant foods. Once the goal is the enhancement of multiple micronutrients, multi-gene expression vectors are necessary, for the plant transformation. The utilization of the Golden Braid cloning framework for the creation of these expression vectors facilitates the genetic engineering process. Since, it also allows complete customization of the different vectors, whilst providing simple to follow technical protocols. As such, obtaining two complete sets of binary expression vectors followed an iterative and fast methodology, which aided in achieving the main goal of this thesis. In addition, the preliminary expression study for the *S. tuberosum* promoter reinforced the need for updating DNA sequence repositories, with newly curated information. Also, it shed a light on the need for good and thorough expression studies that a allow a more in-depth characterization of regulatory sequences in gene expression. In the future, the resulting transgenic potato tubers will be used to assess the value of this strategy in the enhancement of folate, provitamin A and iron concentrations, whilst assessing the potential micronutrient interactions and impact in plant physiology.

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