Increasing Nutrients in Genetically Modified Organisms

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Applications of Genetic Engineering and Biotechnology

Martin CRETTOL
Eliot PORTEVIN

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1 Preface

About 800 million people suffer from hunger in the world, and some other 2 billion are affected by some kind of nutritious deficiency. Finding a durable solution to global malnutrition is essential, especially as the population is projected to increase from 7.3 to 9.6 billion by 2050 (fao.org, see References). This would imply an increase of 70% of global food supply. This also means devastating deforestation, decimation of rare animal species, and exploitation of captive-bred animals in terrible conditions: many are not ready to sacrifice their planet for the sake of having children. An innovative, sustainable solution must be found.

This article presents our research on genetically modified organisms (GMOs) as a possible way out of this situation. GMOs have been heavily criticised, and have acquired a bad reputation in the general public. For example, lay people consider GMOs as toxic and harmful for nature. However, this hasn't been scientifically proven; the real concern about GMOs is rather that they might affect biodiversity and the ecosystem around them in general. For instance: some genetically fortified species might take over other crops, due to a lack of control over them. The scientific community does agree that a reasonable use of biotechnology and the application of some strict laws/rules could overcome such problems however; one can therefore be confident that GMOs can be used safely. The potential that they offer is the increase in the nutritional value of some food, therefore reducing the amount of food needed for a human to stay healthy. In this article, we will focus on the scientific procedure to increase the concentration in nutrients in food. Not all nutrients will be covered (vitamins, minerals, etc.), instead we will focus on a single - essential - compound: the Vitamin C.

Vitamin C deficiency can lead to anaemia, debility, exhaustion, spontaneous bleeding, and more importantly scurvy, a disease found in sailors at the 15- and 16- centuries, who couldn't eat any fresh plant products for months. This was and is to this day, considered to be one of the most important illnesses derived from nutrient deficiency in the history of humanity. These symptoms and diseases remain relatively rare, but if the amounts of Vitamin C in nutrition were to decrease (which some argue is already happening), or food containing vitamin C to become scarce, they would become quite abundant. In order to counter such an eventuality, some research has started to be done regarding firstly, regarding how Vitamin C is produced, and secondly how we could reproduce this process to increase the nutritional value of our food. We think this research is particularly interesting, since it suggests that one doesn't necessarily need to intervene in the DNA of the studied species. In fact, some natural features alone (see end of Introduction) are able to do a portion of the expected work. Some scientists even exclusively base their research on these factors.

We wonder, firstly, how the amount of Vitamin C can be increased, by which process, if this process is natural or not, how much time it can take, and whether it could be applied efficiently at a global scale. We are expecting, however, that many other questions will come to us while working on the topic.

2 Introduction

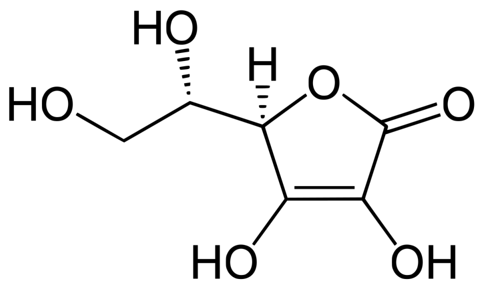

Figure 1: Skeletal formula of L-Ascorbate

According to Wikipedia, a vitamin is an organic substance, necessary in small amounts (less than 100 mg/day) for the metabolism of a living organism, which cannot be synthesised in sufficient quantities by this organism.
The vitamin C molecule is called L-Ascorbate (ASC). Its name is derived from the Greek prefix *a* (without) and the French word for the scurvy disease (scorbut). It is synthesised by fungi, protozoa, plants, and many animals. In some animals, including humans and guinea pigs, the gene coding for the enzyme responsible for the production of ASC has mutated over the course of evolution and is now unable to synthesise it, making it a vitamin for these animals. Plant derived foods are the main dietary source for ASC, but it can also be found in meats, such as cow liver (it is an ASC synthesising organ in animals). Such meat is rather irrelevant however, since one cannot have a diet mainly consisting of cow liver.

The recommended dietary allowance (RDA) in Vitamin C is a controversial subject, and many countries don't have the same recommendations, the given values ranging from 40 in the UK to 110 mg/day in France. These recommendations change when it comes to people who are more exposed to negative effects of oxidants, such as smokers, pregnant and breast-feeding women (the recommended amount increases by around 20 mg). The scientific community, however, is thinking of m odifying the RDA to 200 mg / day, as ASC intake over the current RDA has a noticeable influence in reducing the risk of cardio-vascular diseases, respiratory tract infections, and cancer (Locato et al. 2013).

Fruits and Vegetables	mg ASC/100g
Kakadu Plums	≈ 3000
Guava	243
Currant	200
Pepper	146
Rocket	110
Kiwi	85
Brussels Sprout	81
Broccoli	77
Orange	57
Strawberry	54
Lemon	50
Endive	35
Tomato	23
Potatoes	15
Apple	8
Carrot	4

Figure 2: Amount of ASC in different fruits and vegetables in mg/100g

As mentioned above, in this climate change context, ASC in food may become a challenge for human health. This is why, over the past 40 years, research in Biology has started to investigate how ascorbic acid is metabolised, and how we could increase this production. This research was mainly based on potatoes but also on fleshy fruits such as tomatoes and strawberries. The possibility to increase the ASC content of potatoes by breeding them was proved to be meaningful very long ago. Indeed, potatoes are an omnipresent species in developing countries, thus being relevant in supplying vitamin C: they do readily have a moderate amount of ASC, and people are eager to consume them because of their low cost and high caloric value.

Nowadays, the industrial production of vitamin C is not only inefficient, but the industrially produced molecule also isn't absorbed as well as natural ASC in fruits by humans. This leads to a, if not desperate, important need in functional and eco-friendly techniques to increase Vitamin C content in fruits and vegetables. Some of these techniques, that do not require using biotechnology, have already been proven useful, such as increasing light exposure, or a little salt stress for potatoes (Mittova et al., 2004).

3 Bioengineering techniques to increase Ascorbic Acid in plants

A biosynthetic pathway is a sequence of biochemical processes occurring within a living organism; most of the time, enzymes are needed to catalyse the reaction(s). In order to synthesize ASC, four different biosynthetic pathways can be used, according to the species involved. These pathways are named pectin degradation, the Smirnoff-Wheeler pathway, the animal pathway, and another animal-like pathway (see Figure 3). The Smirnoff-Wheeler pathway was discovered in 1998 and is named after its discoverer (Wheeler et al., 1998).

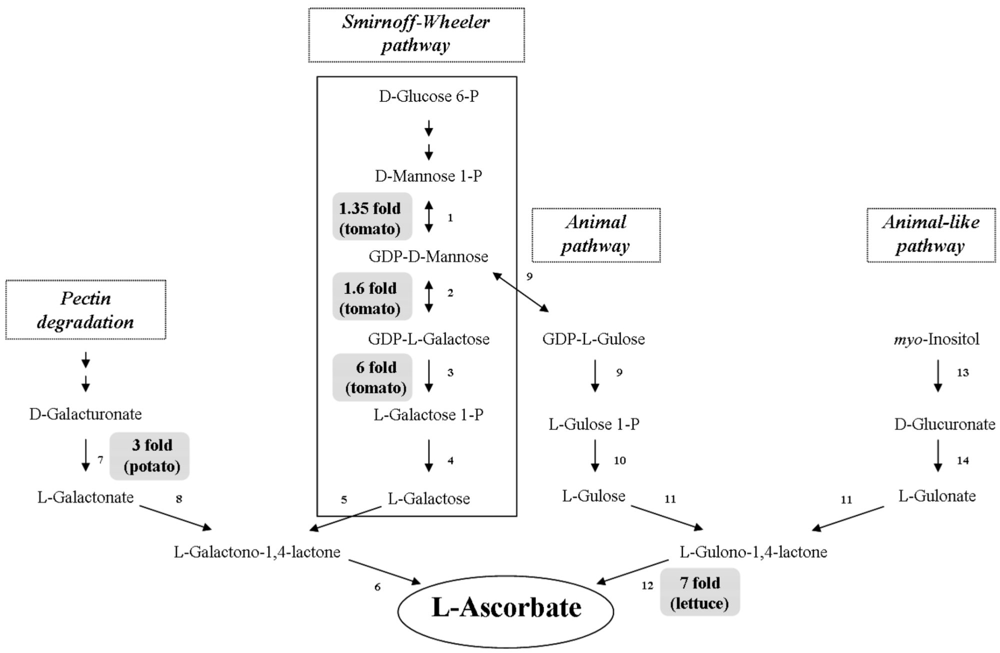

Figure 3: ASC biosynthetic routes and ASC increases in bio-engineered crops (Locato et al., 2013)

In order to increase the ASC production in plant-based food, two main methods have been used :
The first one is to overexpress a gene coding for the enzymes synthesizing chemical products in the pathway leading to ASC production. The overexpression of genes coding for enzymes involved in the Smirnoff-Wheeler pathway was experimented many times with different genes. The most successful experiment linked with the S-W pathway was made by overexpressing the gene GDP-L-galactose phosphorylase (GGP) which is linked to the production of L-Galactose, the penultimate step of ASC production (see Figure 3) (Bulley et al., 2012). This experiment was conducted with potatoes, strawberries and tomatoes. The genes inserted in the genome of potatoes were taken both from Arabidopsis (a flower which had already proven to increase the ASC content) and from another potato species, whereas the gene inserted in tomatoes and strawberries was taken from tomatoes exclusively. The genes were then promoted with a modified cauliflower mosaic virus 35S promoter and a polyubiquitin promoter (only for potatoes). These promoters were a sure value, because they are often used in the context of transgenic plant

creation. Despite these favourable conditions, there was a downside to the results: whilst the ASC content in tomatoes was increased from three to six times, the fruits themselves had lost their seeds, and the tissue surrounding the seeds had jellied (see picture). With the strawberries, quite the opposite happened: the fruits were still healthy, but only some of them had an increased amount of ASC, and the best that was obtained was a doubling of the initial value.

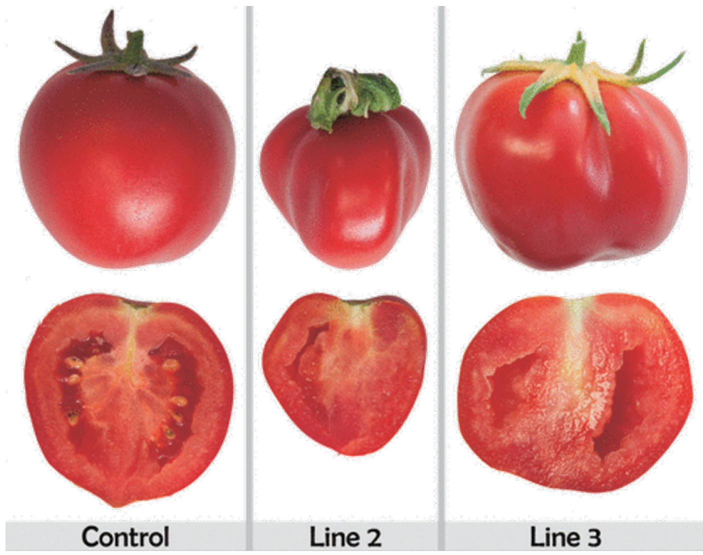

Figure 4: Normal tomato and two transgenic tomato lines
(Bulley et al., 2012)

Another experiment was conducted with the overexpression of a rat gene coding for L-Gulono-1,4-Lactone (GLOase) (see Figure 3) (Jain and Nessler, 2000). The gene was under the same promoter as before (the 35S), before being introduced in tobacco and lettuce. The results were similar to those with the potato: a 7-fold ASC content increase in tobacco, and a 4 to 7-fold ASC content in lettuce.

The second method to increase ASC content is linked to genes encoding the recycling process of ASC, respectively dehydroascorbate reductase (DHAR), and monodehydroascorbate reductase (MDHAR). Both of these genes catalyze the reaction leading to the creation of an ascorbate from an oxidised ascorbate radical (Stevens et al. , 2007). Several experiments were made using the overexpression of the DHAR gene, which was willing to recycle more ASC (Chen et al., 2003; Naqvi et al., 2009). Both experiments proved that a DHAR overexpression leads to an ASC content increase (2 to 4-fold for the former and 6-fold for the latter). In the first experiment, a DHAR cDNA was isolated from wheat and introduced into tobacco and maize. In the second one, the researchers tried to increase the content not only of ASC but also of two other vitamins in south-african corn: Beta-carotene and folate (A and B9). The second one was much more successful. With MDHAR, an experiment was conducted, whereby the gene was overexpressed in some plants, and silenced in others (Gest et al., 2013). Surprisingly, the transgenic plants with the silenced MDHAR gene produced much more ASC than the ones with the overexpressed genes. Moreover the plant with the silenced gene reacted positively to light (the ASC level increased) whereas, for the other one, the ASC content decreased with a big light exposure. The reason of this mechanism is for yet unknown but it could be hypothesized that a MDHAR activity decrease/increase would switch on/off an unidentified pathway leading to ASC control, by which the intensity would be defined by the light exposure.

4 Documentation in research institutions

In order to get a better idea of how these two methods were discovered, how they are put into application and by whom, we decided to go visit a research institution, and to interview a person working on the same topic. Pierre Baldet, a researcher working at the INRAE in Bordeaux with over 60 publications to his name, was nice enough to accept this request. However, the epidemical situation we are living stopped us from seeing him in the flesh, so the questions that follow were asked an answered online.

What does your work consist of?

I am a researcher at INRAE (National Research Institute for Agriculture, Food and the Environment), in the Joint Research Unit "Fruit Biology and Pathology" of the Villenave d'Ornon research center in Gironde within the Metabolism team (https://www6.bordeaux-aquitaine.inrae.fr/bfp). I study the metabolism of ascorbic acid (VitC) in fruit, and more particularly in tomatoes, a model species for the study of fleshy fruits. Concretely, I try to understand what mechanisms control the production of ascorbic acid in a plant and more precisely in the fruit.

What do you do on a daily basis?

I plan experiments with members of my team, I carry them out, from the cultivation of the plants to their analysis, their interpretation etc..... We discuss a lot before setting up an experiment in order to define which biological question we want to answer. I am also involved in the supervision of students from the University of Bordeaux at the Bachelor 3, Master 1 & 2 levels and also PhD students. I take part in weekly meetings of the Metabolism team. Once or twice a year, I may be asked to give a 2-hour course to Master 2 students. I write articles in order to give feedback of my work to the scientific community, these articles are published in international journals in the Plant Biology part. I apply for funding calls (fellowships, grants), in order to continue my research, to finance scholarships, to purchase some equipment...

In your opinion, is vitamin C research a fairly common research subject?
I don't know about all the teams interested in ASC research in the world, I only know those who work in the plant field, or rather those who publish regularly. Research in Biology has a vast amount of research possibilities, there is room for everyone. Of course there is competition, so everyone tries to be original in their research.

Do you think we need more researchers / students who care about it? How many people make up your team?
Yes and no: our team is small, it is made up of an INRAE researcher (myself), two lecturers from the University of Bordeaux, a technician (INRAE) and a PhD student and M2. We are surrounded by other researchers who all have different skills like us and this is the richness of our job; when we don't know something or need help for an experiment, we can go see our colleague who knows and he will help us! Even if very little people work on this topic, science is a big unity and people from another department will come and help us, because you don't always need to be an expert in a specific domain to make things work out.

Do you work in partnership with other institutes?
Yes of course, it is strongly encouraged to collaborate with laboratory colleagues but also elsewhere, it is a philosophy that is very widespread among researchers and therefore we have collaborations within the INRAE in France, but also with foreign groups: I for one have colleagues in Germany, in the UK, in Spain, in Argentina.... And the list goes on!

What have been the recent findings on ASC metabolism?
As far as the metabolism and biosynthesis of ascorbic acid is concerned, everything has been rather well known and uncontested for the last 15 years, but everything remains to be studied regarding the mechanisms that control the production of ascorbic acid in plants and also in fruits. These are the main source of Vitamin C for humans, but we still don't know how everything is regulated, for example we know that light has a positive effect on the production of ascorbic acid but the underlying mechanisms remain unknown, etc... What we do know is that whatever the food (fresh, canned, processed, etc), there is always ascorbic acid added because it is a powerful antioxidant.

What are the different techniques on which you base your research? Do you think human intervention in the ASC production can have negative effects on the given food (calorie loss, etc.)?
Research in plant biology is a world in perpetual evolution which benefits from discov-

eries in many fields of physics and chemistry. New technologies are therefore introduced each year, allowing us to make huge leaps in our research. For example, techniques in genome sequencing of living organisms allow us to make new discoveries which would have been unimaginable 10 years ago. The most recent discovery in that domain is the CRISPR technology. In short, the list of techniques I use is very varied, ranging from basic biochemistry to PCR, gene sequencing, bioinformatics, enzymology, genetics to plant physiology. One must understand that an organism that does not produce ascorbic acid cannot survive in the presence of oxygen -this includes all living things on Earth except for microorganisms that have other antioxidant strategies-. If their ASC production is reduced, plants have severe health problems, an important lack of development, reduced resistance to stress... If this production is increased however, I believe the effects can only be beneficial. Nevertheless, one must understand how it all works before playing the sorcerer's apprentice, which is my job.

What devices do you use?
The list is too long to be defined, but you can be assured that we have a wide variety of equipment in the laboratory, ranging from small devices that can be placed on a bench, to the largest that can occupy half a room (which are often very expensive, some of them being worth up to hundreds of thousands of euros!).

Do you think you can, in the long term, meet the need for vitamin C with the help of developing techniques? More specifically, do you think you can apply them on a large scale (taking into account the cost and possible side effects of your manipulations)?
This is the ultimate goal of our research at the INRAE: to understand plants and in particular the plants that feed us in order to improve their functioning while preserving the environment in which they live, and us humans at the same time. However, industrialising ASC production is something we are still far from achieving, so it is hard to imagine whether it will be possible or not.

Is there a process/technique that you had to abandon? If so, what were the reasons for discontinuing it?
Yes, as a matter of fact, it is rather usual to abandon a technique in favor of a new one that is better in terms of saving time, money, but more importantly gives more precise results. For example, when I started my PHD 30 years ago, one needed a week to purify a few micrograms of an enzyme obtained from several kilos of spinach; one could then spend a whole thesis working on this same enzyme. Nowadays however, one can do the same work in only a month, because it is now possible to synthesise milligrams of this protein with plasmids and bacteria, or any other eukaryotic or prokaryotic organism. A second example in the progress of biotechnology is the production of insulin. 30 years ago, people with diabetes would receive insulin extracted and purified from pig pancreas, whereas we can now produce human insulin in bacteria...

5 Discussion

Research in Biology is, as said Pierre Baldet, in constant evolution. As soon as a technique, rule or function proves to be useful and functional, another comes into play, either by contradicting the previous, or by enabling better results. In our case, two biotechnological methods were imagined and applied, whereby it was made possible to increase the ASC content of fruits and vegetables by overexpressing or silencing their genes. A few years ago, this wouldn't have been imaginable, or at least not realistic. However, at the speed science goes, one cannot expect that the podium these processes are standing on will stay undisputed for long. The progress that has been made can therefore not be considered as a long term solution, but rather as a door that was opened, leading to the world of ASC bioengineering. Indeed, it was seen that these techniques weren't flawless: the tomatoes that were studied ended up being sterile, and their shape altered. They certainly weren't ready for commercial use, yet they achieved the initial goal of the research: increasing ASC content.

Albert Szent-Györgyi once said: "A discovery is said to be an accident meeting a prepared mind". Although we can expect the pathway that will be followed in the next years by scientists, it is hard to predict what is to come: one can quite confidently assure that research will go on regarding over-expressing, and maybe silencing genes. However, the studies we have considered go back to the early 2000s. Since then, a revolution has taken place in genetic engineering: the discovery of the CRISPR technology. Without going too much into the details, CRISPR is a family of DNA sequences found in the genomes of prokaryotic organisms such as bacteria and archaea. These sequences are derived from DNA fragments of bacteriophages that had previously infected the prokaryote. They are used to detect and destroy DNA from similar bacteriophages during subsequent infections. In short, DNA sequencing and gene modifications can now be done at a much greater scale than twenty years ago, enabling multiple genes to be over-expressed and other silenced in our case. To our knowledge, this technology has not been investigated very much up to now, or at least, there are few publications that deal with it. Nevertheless, the technique that will produce fruits with great ASC concentration will most certainly be using the CRISPR-Cas9 protein, which has already shown to be so useful. One must bear in mind however, that no matter how well scientists do in this domain, everyone is not ready to eat some genetically modified food. Studies proving their sanity would have to be numerous to convince most people that they can consume them without taking any risks. Even so, some might still refuse to consume the products because of their ethical opinion, or their relation to natural products. There is already an important controversy regarding what we are eating these days: should we only eat fruits and vegetables with "eco-friendly" or "bio" labels? Should we buy food that comes from another land? Many think so, and some even decide to stop buying their food in supermarkets, to exclusively consume their own garden products, which generally are healthier and more nutritional. The mind behind these decisions is totally understandable, and even recommendable, but it does not align with the goal of this research. Some people will therefore have to make a choice: to be self-sustainable, or to count on science. Interesting to note is that if they decide not to consume GMOs, it will also enable more fruits to be available for others. After all, if everyone was able to make their own vegetables and to only eat local food, there would be no need for industrialised fruits. The arguments favourable to the use of these GMOs on the market are not numerous, but they speak for themselves: if the mutations that they have undergone only modify their ASC content, and don't have an effect on their shape, nutritional value and sanity overall, there is no reason for them not to be used. On the contrary: there are only benefits: people will need to eat less, yet they will be healthier. Such important modifications would also mean that the fruits are tested regularly, guaranteeing their quality to the customer. To top things off, the ability to increase ASC content would translate an incredible understanding of the science behind the formation of fruits, which could lead to some further experimentation on them, and ultimately to the creation of the perfect fruit: that wouldn't rot, that would be juicy and sweet, and greatly nutritional.

6 Summary

It has been seen that L-Ascorbate is vital for humans, since we are not able to synthesise it and that vitamin C deficiency can cause scurvy, which is considered to be the most important illness caused by vitamin deficiency in human history. It has also been seen that the recommended dietary allowance can greatly vary depending on the country, ranging from 40 mg to 110 mg per day. We then investigated two different biotechnological methods to increase ASC content in plant-based food, namely over-expressing and silencing genes that are directly or indirectly linked to ASC production. It came to our attention however, that some tomatoes that had undergone a big ASC increase didn't have seeds anymore, and that their flesh was jellified. To complete our investigation, we also reached out to Pierre Baldet, who gave us a concrete idea of what the work of researcher consists of. The gathered information enabled us to understand the progress made in agro-biotechnology regarding vitamin C, which led to a - even if imprecise - conclusion regarding the research to upcome, and its goals.

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