

Review

Increased Production of Nutriment by Genetically Engineered Crops

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Plants are the basis of human nutrition and have been selected and improved to assure this purpose. Nowadays, new technologies such as genetic engineering and genomics approaches allow further improvement of plants. We describe here three examples for which these techniques have been employed. We introduced the first enzyme involved in fructan synthesis, the sucrose sucrose fructosyltransferase (isolated from Jerusalem artichoke), into sugar beet. The transgenic sugar beet showed a dramatic change in the nature of the accumulated sugar, 90% of the sucrose being converted into fructan. The use of transgenic sugar beet for the production and isolation of fructans will result in a more efficient plant production system of fructans and should promote their use in human food. The second example shows how the over-expression of the key enzyme of flavonoid biosynthesis could increase anti-oxidant levels in tomato. Introduction of a highly expressed chalcone isomerase led to a seventyfold increase of the amount of quercetin glucoside, which is a strong anti-oxidant in tomato. We were also able to modify the essential amino acid content of potato in order to increase its nutritional value. The introduction of a feedback insensitive bacterial gene involved in biosynthesis of aspartate family amino acids led to a sixfold increase of the lysine content. Because the use of a bacterial gene could appear to be controversial, we also introduced a mutated form of the plant key enzyme of lysine biosynthesis (dihydrodipicolinate synthase) in potato. This modification led to a 15 times increase of the lysine content of potato. This increase of the essential amino acid lysine influences the nutritional value of potato, which normally has low levels of several essential amino acids. These three examples show how the metabolism of primary constituents of the plant cell such as sugar or amino acids, but also of secondary metabolites such as flavonoids, can be modified by genetic engineering. Producing fructan, a soluble fiber, increasing the level of flavonoids, an antioxidant, in tomato or increasing the level of essential amino acids in potato are all clear examples of plant genetic modifications with possible positive effects on human nutrition.

Key teaching points:

- Research indicates that dietary fructans, such as inulin, and flavonoids may have important health-promoting properties. Current dietary consumption of these nutriment may be suboptimal for realizing their benefits.
- Sugar beets have been genetically engineered to contain enzymes that efficiently convert sucrose to fructans. These sugar beets could provide an additional source of fructans for use as a food ingredient in a greater variety of foods.
- Initial studies have shown it is possible to engineer tomatoes genetically to overexpress the enzyme responsible for synthesis of flavonol in tomatoes.
- A single amino acid residue change in the potato enzyme responsible for lysine synthesis makes it much less susceptible to feedback inhibition such that the concentration of lysine in potato can be greatly increased.
- Modern biotechnology is proving to be a powerful tool to improve the nutritional quality of crop foods when combined with traditional plant breeding and genetic resource management.

INTRODUCTION

Plants not only form the basis of the human food chain, but they are also an important means to improve human health and

well-being. Several plant-derived compounds are known to prevent or delay diseases such as arteriosclerosis or cancer. Moreover, the plant kingdom contains a plethora of compounds that can serve as pharmaceutical drugs.

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To better exploit the potential of plants, humans developed techniques to domesticate and improve plant species. Biotechnology is among the more powerful of these techniques. Crop biotechnology is not a single technique, but encompasses a number of methods ranging from clonal micropropagation of elite cultivars and dihaploids plant production to genetic engineering. Genetic engineering of plants became possible at the beginning of the 80's for some model plants, but soon the range of plants amenable to this technique increased. It is now possible to modify crop plants such as maize, wheat, canola, potato or sugar beet genetically, although differences still remain in the efficiency of the techniques when applied to these different species.

In the first phase, genetic engineering of plants mainly focused on the creation of plants expressing resistance toward herbicides and pests. A second phase began when genetic engineering began to be used to improve the quality of the crops in terms of final users. At Plant Research International we use both plant genomics and modern breeding approaches to better exploit the potential of plants. We propose in this article to highlight three examples where genetic engineering led to modification/improvement of crop quality with potential impacts on human nutrition.

TOWARD THE DEVELOPMENT OF AN ALTERNATIVE SOURCE OF FRUCTAN; FRUCTAN PRODUCTION IN ENGINEERED SUGAR BEET

Definition/Applications/Production

Fructans are polymers of fructose synthesized by about 15% of flowering plants. The nature of the link between the fructose moieties (β 2-1 or β 2-6) determines the type of fructans. Currently, industrial applications (food and non-food) are based on fructan molecules presenting the β 2-1 linkage. These fructans, also called inulin, are extracted from chicory (*Cichorium intybus*) roots. The chemical properties of inulin molecules vary with the length of the polymer. Short molecules, with a degree of polymerization (DP) of 3 to 6, are used as low calorie sweeteners, the sweetening power decreasing with the extension of the molecule. These short molecules are mainly prepared by fermentation of sucrose with an *Aspergillus*-derived fructosyl transferase enzyme. On the other hand, long molecules (DP 6 to 60) mimic fat and can be used as fat replacer in food preparation (e.g. ice cream).

Place of Fructans in the Diet and Health Promoting Properties

It has been estimated that the American diet provides an average of 2.6 g of inulin and 2.5 g of oligofructose per day [1]. The main components of the diet contributing to this intake are wheat and onions. In Europe the daily intake has been estimated

to be relatively higher, between 4 to 12 g per day [2]. But this intake can peak to 20 g per day after eating a bowl of onion soup.

The effects of inulin on human health are more and more documented and have been reported before (see reviews by Roberfroid and Delzenne, 1998 [3] and Boeckner et al., 2001 [4] and special issues of *The Journal of Nutrition* 1999:129S and *The American Journal of Clinical Nutrition*, 2000: 71S).

Inulin is not digested in the upper part of the digestive tract, and more than 85% of the ingested inulin reaches the colon. There the endogenous flora ferments it. This fermentation results in the production of short chain fatty acids that are presumed to be responsible for the health promoting effects of inulin. One well-documented activity of inulin is its beneficial effect on the micro-flora encompassing the bifidobacteria. Both *in vitro* assays and animal and human studies confirmed that the consumption of inulin results in an increase of the number of bifidobacteria. This bifidogenic effect is observed with a consumption of as little as 5 g of inulin per day. A number of health promoting properties are associated with bifidobacteria, such as inhibition of pathogenic bacteria growth, production of vitamins (B group), reduction of blood ammonia concentration and decrease of cholesterol level. Their antagonistic effect against pathogenic bacteria could prove very useful in improving the survival of probiotics after ingestion and in helping to restore normal intestinal flora after antibiotic therapy.

Beside its effect on bifidobacteria population, consumption of inulin has been reported to improve blood lipid composition and mineral uptake while reducing the risk of colon cancer.

Production of Fructan in Genetically Engineered Sugar Beet

Sugar beet is one of the most efficient crops of the Western world and can yield up to 10 tons sucrose per hectare. If such a crop could be engineered to produce fructan, it would create an abundant source of fructan, allowing further development of food and non-food applications. Sugar beet is particularly well suited to produce fructan because it accumulates high levels of sucrose in the vacuole of its tap root cells (up to 500 mM). It was shown that the plant enzymes catalyzing the synthesis of fructan in chicory or Jerusalem artichoke are located in the vacuole and use sucrose as the primary substrate. Moreover, sugar beet is an industrial crop plant with a known agronomy and an established processing industry. All these characteristics make sugar beet one of the best candidates for the concept of "Plant as Factory" [5].

In order to transform sugar beet with the genes involved in fructan biosynthesis, two prerequisites had to be fulfilled. The genes responsible for fructan synthesis had to be cloned, and an efficient transformation protocol for sugar beet had to be developed.

The Genes

At the time this work was initiated (beginning of the 90's) no fructan genes coming from plants had been cloned. The

enzymes catalyzing the biosynthesis of fructan were identified, purified and characterized [6,7]. Two enzymes are necessary to catalyze the synthesis of long chain inulin molecules. The first enzymes catalyze the transfer of the fructose moiety from a sucrose molecule onto another sucrose molecule resulting in the synthesis of 1-kestose (DP3) and in the release of free glucose; this enzyme is called sucrose sucrose:fructosyltransferase (SST). The synthesis of longer molecules requires the action of fructan:fructan fructosyl transferase (FFT), which catalyzes the transfer of fructosyl moieties from one fructan molecule onto another fructan molecule. Micro sequencing of trypsin digest fragments allowed the design of degenerated primers that were used to amplify DNA fragments, which in turn were used to screen a cDNA library of Jerusalem artichoke tubers. Two cDNAs encoding *1-sst* and *1-fft* were cloned, and their function was assessed in transgenic petunia [8] and potato (data not published).

Transformation of Sugar Beet

Sugar beet was considered a recalcitrant crop when it came to genetic engineering, meaning that only a few successes had been reported and that protocols for genetic engineering of this crop allow only recovery of a few transformants. In our institute, sugar beet *in vitro* tissue culture and genetic transformation has been investigated for several years. This research led to the identification of a particularly responsive cell type, i.e., the stomatal guard cell [9]. Using various techniques it was shown that for sugar beet these specific cells have a high capacity of regeneration into plants. A transformation protocol was developed, based on the genetic transformation of guard cell protoplasts via polyethylene glycol-mediated DNA uptake [10].

Results

As a first step, to test the feasibility of fructan production in sugar beet, a vector harboring the *1-sst* gene from *Helianthus* and the *pat* selection marker was constructed. This construct was introduced into sugar beet guard cell protoplasts, and several transgenic lines were obtained and characterized [11]. Out of the transgenic lines analyzed, six showed expression of the transgene. Among these lines four showed the accumulation of short DP fructan (DP3, DP4 and DP5). One line was investigated further, and soluble carbohydrates were quantified by HPAEC-PAD. While the amount of sucrose in the control sugar beet tap root was 84.2 mg/g FW, the sucrose concentration in the transgenic tap root was only 7.9 mg/g FW. However, the transgenic tap root also accumulated 37.2, 22.5 and 4.8 mg/g FW of DP3, 4 and 5, respectively. The synthesis of these amounts of fructan would have required a total input of 68.8 mg/g FW of sucrose. The amount of sucrose left in the transgenic tap root was less than 10% of the amount in the control, indicating that over 90% of stored sucrose was channelled into fructan synthesis. Fructan synthesis is accompanied by a release of glucose. While the actual amount of glucose in the

transgenic tap root was 4.5 mg/g FW, the synthesis of fructan as observed for this tap root would have accounted for the release of 29.6 mg/g FW, showing that about 85% of the released glucose was re-metabolized.

Transgenic sugar beet proved to be a good candidate for the production of short chain fructan. The product as such could be used as a low calorie sweetener and compete with the fermentation-based production of oligofructose. In order to assess the possibility of production of long-chain fructans in sugar beet, we prepared a construct harboring the *Helianthus 1-sst* gene in combination with the *1-fft* gene. This construct was introduced into sugar beet, and fructan profiles were analyzed. We observed the accumulation of fructan with a high DP in some of the transgenic lines (data not published) showing that long chain fructan could be produced in sugar beet as well.

How Can Fructan Production in Sugar Beet Benefit Human Nutrition?

Inulin is nowadays extracted from chicory; although chicory agronomy is developing, it remains poor compared to the well-established agronomy and long history of selection of sugar beet. Production of inulin in sugar beet could promote a widespread culture of inulin producing plants allowing the production of large quantities of inulin that would favor a larger use of this ingredient in food. The actual diet of American or European citizens comprises already about 5 g per day of fructan, but human studies showed that inulin consumption could be increased to 20 g per day to reach an optimum health promoting effect without any secondary effects on the digestive tract.

The introduction of chosen genes in sugar beet will result in the production of tailor-made fructans. While the production of short chain fructans requires only one *1-sst* gene, long chain fructans can be produced when both a *1-sst* and a *1-fft* gene are introduced into sugar beet. Using genes isolated from different plant species should allow the fine tuning of the fructan profile accumulated by the transgenic crop. It will then be possible to produce *in planta* fructan types that require minimal technological processing. We predict that nutritionists will be able to define more precisely which types (chain length, profile, type of linkage) of fructan are the most useful ones in human nutrition. Once defined it will be possible to produce this specific fructan in transgenic sugar beet.

IMPROVING HEALTH CHARACTERISTICS: FLAVONOID BIOSYNTHESIS IN TOMATO

A biotechnological approach was used for increasing health-promoting compounds in tomato. Flavonoids comprise a large and diverse group of polyphenolic compounds that are ubiquitous in plants. Flavonoids are involved in many aspects of plant growth and development, such as pathogen resistance,

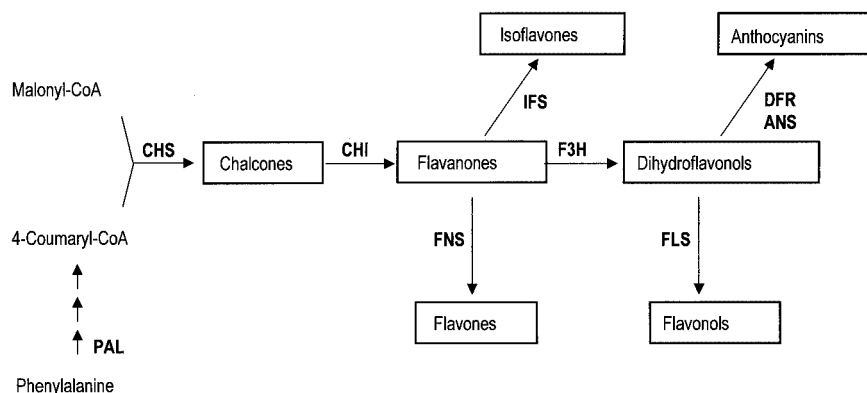


Fig. 1. Schematic overview of the flavonoid biosynthesis pathway. Boxed are the different flavonoid classes. PAL: phenylalanine-ammonia lyase, CHS: chalcone synthase, CHI: chalcone isomerase, F3H: flavanone-3-hydroxylase, IFS: isoflavone synthase, FNS: flavone synthase, FLS: flavonol synthase, DFR: dihydroflavonol reductase, ANS: anthocyanidin synthase.

pigment production and UV light protection. In addition, due to their antioxidant properties they are thought to be beneficial to human health [12]. Several epidemiological studies showed that increased consumption of flavonoids could help to protect against chronic diseases, such as cardiovascular disease. Many major food crops however, contain only small amounts of flavonoids in their edible parts or produce flavonoids that do not have optimal antioxidant characteristics.

Most of the enzymes involved in the biosynthesis of the

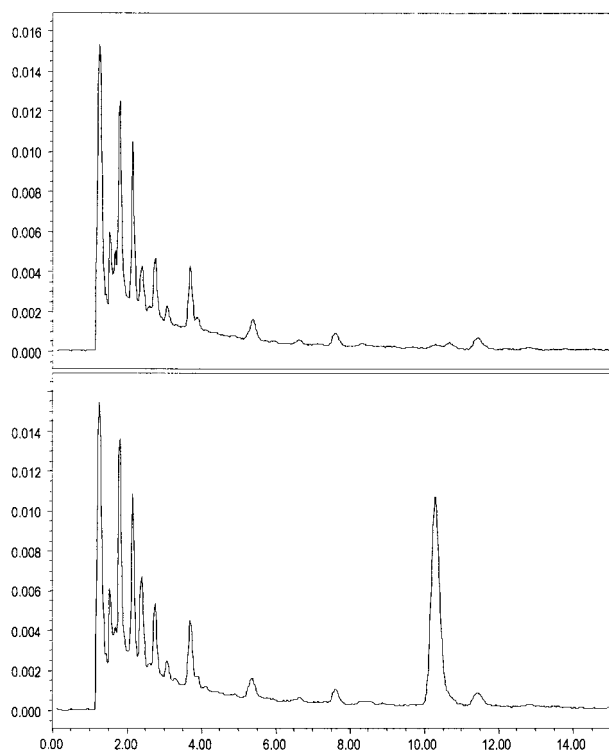


Fig. 2. Metabolite analysis of tomato peel (by high performance liquid chromatography). The upper panel shows the chromatogram of untransformed tomato, whereas the lower panel reveals the presence of a high level of flavonols in transgenic tomato.

different flavonoids have been well characterized, and their encoding and regulatory genes have already been isolated. The knowledge and availability of these genes gives us the tools to genetically up-regulate the overall flavonoid biosynthesis or to engineer the pathway towards new flavonoid species in crop plants.

Tomato, which is an important food crop worldwide, contains small amounts of flavonols in the peel of its fruit. Flavonols are a group of flavonoids that are very good antioxidants and are thought to protect against cardiovascular diseases. Both biochemical and gene expression data of tomato have suggested that one of the rate-limiting steps in flavonol biosynthesis in the peel could lie at the level of chalcone isomerase (*CHI*), a key enzyme in flavonoid biosynthesis (Fig. 1). Flavonol biosynthesis was up-regulated in tomato fruit peel by over-expressing the *CHI* gene from *Petunia* in transgenic tomatoes,

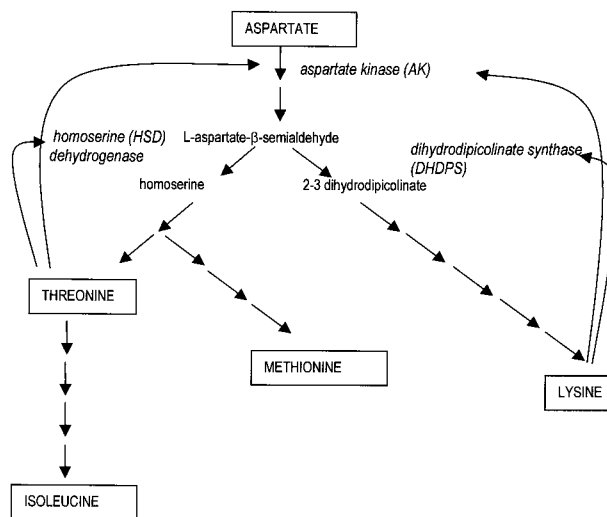


Fig. 3. Schematic overview of the aspartate family amino acid biosynthesis pathway. Only the major key enzymes are indicated in *italics*. Curved arrows represent feedback inhibition by the end-product amino acids.

origin that are one hundredfold less sensitive to feedback inhibition by lysine into potato. Expression of a feedback insensitive DHDPS enzyme resulted in a sixfold increase of lysine. Introduction of a feedback-insensitive AK enzyme in potato resulted in an eightfold increase of threonine and a twofold increase of methionine. The results with the bacterial feedback-insensitive enzymes have shown that we are indeed able to increase essential amino acid levels in potato in this way. However, it would be preferable if we did not have to introduce bacterial genes into the potato genome. Therefore, we isolated the potato gene encoding DHDPS and changed one amino acid residue to render the enzyme feedback-insensitive. Introduction of this desensitized potato gene back into potato resulted in a dramatic increase of the lysine content (Fig. 4). The lysine level reached up to 15% of the total amino acid level, whereas in untransformed plants this level is only 1%. Lysine is now becoming a bulk amino acid instead of a low-level amino acid.

We are currently using the genomics approach for further improvement of the lysine content in crops by exploiting gene expression analysis of the transgenic plants accumulating high lysine with DNA micro-array technology. Genes showing a different expression profile in the high lysine plants might form a new lead for further improvement of the lysine content in plants. DNA micro-array analysis will also be used together with high-throughput protein and metabolite analyses in a risk-assessment study to determine the extent to which the transgenic potato plants are altered by the technique.

CONCLUSION

In this paper we describe how it was possible to change the nature of the sugar accumulated in sugar beet, increase the level of flavonoids in tomato and modify the amino acid profile of potato. These different results were made possible by combining metabolic pathway elucidation, plant genomics and genetic engineering. This approach proved to be powerful for the manipulation of primary constituents of the plant (amino acids, sugar) and secondary compounds (flavonoids), both important in human nutrition. These examples illustrate how modern biotechnology can contribute to the improvement of human food. Once we have a better insight into biosynthetic routes leading to the formation of desired compounds, we will have the tools to improve both the quantity and quality of human food. Modern biotechnology will be a powerful tool in achieving this goal when combined with traditional plant breeding and genetic resource management.

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