

Development of Rice and Maize with Multiple Essential Nutrients Through Simultaneous Multi-Pathway Engineering

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Abstract

Food insecurity is one of the most important social issues we face today, with nearly one billion people enduring chronic hunger and an additional two billion suffering from nutrient deficiencies, most in the developing world. The nutrients in the human diet ultimately come from plants. Consequently biofortification of staple food crops at source offers unique opportunities to contribute towards addressing this challenge in a sustainable way. Here we discuss the biofortification of crops, focusing on transgenic approaches which offer the most rapid way to develop high-nutrient commercial cultivars, at least technically. We will use specific examples from our own work to introduce the advantages of multigene transfer and demonstrate how extraordinary levels of multiple essential vitamins can be achieved in the edible part of the plant.

Keywords: Rice, maize, nutrients, biofortification, metabolic engineering, multigene transfer

Strategies

Many transgenic strategies are available to enhance the nutritional value of crops; these offer a rapid way to introduce desirable traits into elite varieties. Transgenic strategies differ from other approaches in that novel genetic information is introduced directly into the plant's genome. The chosen approach depends predominantly on whether the nutritional compound is synthesized *de novo* by the plant or obtained from the environment.

Organic molecules, such as amino acids, fatty acids and vitamins, are synthesized by the plant. Increasing the nutritional value requires some form of metabolic engineering with the aim of increasing the amount of these desirable compounds, decreasing the amount of a competitive compound or even extending an existing metabolic pathway to generate a novel product (Capell and Christou 2004). The subsequent sections set the stage and discuss examples that illustrate how transgenic approaches can be used to enhance the nutrient content of crops for specific essential vitamins specifically for maize and rice, using multigene strategies.

A focus on multigene transfer (MGT), the key limiting step in the engineering of complex metabolic pathways in plants

MGT allows researchers to achieve goals that were once impossible – the import of entire metabolic pathways, the expression of entire protein complexes, the development of transgenic crops simultaneously engineered to produce a spectrum of added-value compounds (Naqvi et al 2010). The simultaneous transfer of multiple genes into plants enables researchers to study and manipulate entire metabolic pathways, express multimeric proteins or protein complexes, and study complex genetic control circuits and regulatory hierarchies. Early transformation methods for plants were developed with the implicit intention to introduce one or two genes (usually a primary transgene and a selectable or screenable marker) and have been optimized on that basis (Twyman et al 2002a). Although MGT can be achieved using such methods, they operate under the law of diminishing returns and thus large populations of plants must be screened to identify rare individual lines with the sought-after genotype. More recently, researchers have attempted to address these limitations by developing new transformation methods that recognize the desire to introduce multiple transgenes into plants

and express them in a coordinated manner. Essentially all these methods aim to achieve the recombination of a SMART locus (Naqvi et al 2010), i.e. one containing stable multiple arrays of transgenes.

MGT through cotransformation of unlinked genes

Cotransformation is the simultaneous introduction of two or more transgenes. This allows plants carrying multiple transgenes to be produced in one generation, often with all the transgenes integrated at the same locus thus preventing segregation. Cotransformation can be achieved using two broad approaches, one involving linked genes (multiple genes on the same plasmid) and one involving unlinked genes (different genes on different plasmids). Both approaches can be used with both major strategies for gene transfer to plants. In the case of *Agrobacterium*-mediated transformation, several transgenes can be included within the same T-DNA or supplied as separate T-DNAs. In the case of direct DNA transfer, transgenes can be linked on a single plasmid or provided on separate plasmids (Twyman et al 2002b).

When two transgenes are used, the linked and unlinked cotransformation strategies are equally efficient. In both cases, the transgenes tend to integrate at a single locus, although the precise arrangement of unlinked T-DNAs depends on the bacterial strain (Twyman et al 2002b). As the number of transgenes increases, any linked cotransformation strategy with conventional vectors becomes much less efficient owing to vector instability, the lack of unique restriction sites during iterative cloning and the fact that larger input DNA sequences are more likely to fragment. The unlinked transformation strategy holds up much better, although only in the case of direct DNA transfer, as multiple T-DNAs tend to integrate inefficiently. Methods such as particle bombardment with multiple input plasmids can generate transgenic plants carrying all the input genes with high cotransformation efficiency (Agrawal et al 2005; Zhu et al 2008; Naqvi et al 2009).

Applications of multigene transfer (MGT) in the metabolic engineering of maize and rice

We developed a combinatorial nuclear transformation method to dissect and modify the carotenoid biosynthetic pathway in maize (and subsequently rice), resulting in the rapid production of a diverse population of multiplex transgenic plants (Zhu et al 2008). In this study, five transgenes [maize *psy1*, *Pantonea ananatis crtI*, *Gentiana lutea* lycopene β -cyclase (*Glycb*), *G. lutea* β -carotene hydroxylase (*Glbch*) and *Paracoccus* spp. β -carotene ketolase (*ParacrW*)] controlled by different endosperm-specific promoters were transferred into a white maize variety deficient for endosperm carotenoid synthesis using an unlinked direct transfer

strategy (Zhu et al 2008). Transgenic plants expressing different enzyme combinations and showing distinct metabolic phenotypes were generated, allowing the identification and complementation of rate-limiting steps in the pathway.

Using the same strategy we generated transgenic maize plants expressing four transgenes affecting three metabolic pathways [maize *psy1* and *P. ananatis crtI* for carotenoid synthesis, rice dehydroascorbate reductase (*Dhar*) for ascorbate recycling and *E. coli* GTP (guanosine triphosphate) cyclohydrolase (*folE*) for folate synthesis] (Naqvi et al 2009). Elevated levels of β -carotene, folate and ascorbate were measured in the endosperm, showing that MGT can be used for the simultaneous modification of different metabolic pathways with the same overall aim, increased nutrition, to provide the first step towards 'super-nutritious' crops engineered to provide adequate amounts of all essential nutrients.

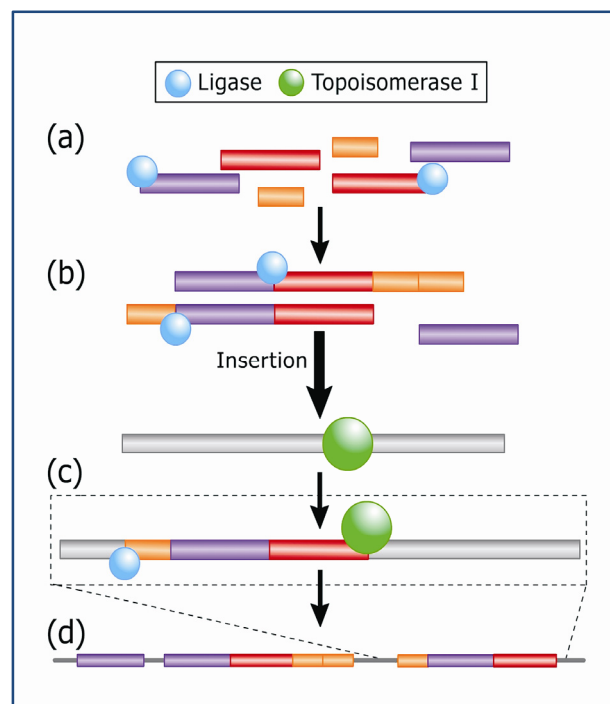


Figure 1. Transgene integration in plants (a-b). Established transgene integration models indicate that linear DNA fragments associate with DNA ligase (a), which converts them into concatemers (b). c = When these interact with a genome break, often at the site of topoisomerase I activity, the DNA ligase stitches the concatemer into the site as part of the natural DNA repair process, perhaps promoting further repair complexes to form. D = This generates a cluster of transgenes and concatemers interspersed with host DNA (Kohli et al 2006)



Figure 2. Multivitamin corn generated through combinatorial nuclear transformation using maize *psy1* and *P. ananatis crtI* for carotenoid synthesis, rice dehydroascorbate reductase (DHAR) for ascorbate recycling, and *E. coli* GTP (guanosine triphosphate) cyclohydrolase (*folE*) for folate synthesis (Naqvi et al 2009); 100-200 g of grain provides full recommended daily intake (RDI) of β -carotene (as a sole source of vitamin A), more than enough folate, and about 20% of the RDI of ascorbate; β -carotene 60 $\mu\text{g/g}$ dry wt (DW), (*PSY1+CrtI*); 200 $\mu\text{g/g}$ DW folate (*folE*); 110 $\mu\text{g/g}$ fresh wt of ascorbate (DHAR)

Transgene Integration - the key to successful MGT

Transgene integration following both direct DNA transfer and *Agrobacterium*-mediated transformation involves illegitimate recombination, often at the sites of naturally occurring DNA breaks and at sites with microhomology to the incoming DNA (Somers and Makarevitch 2004). Both T-DNA and the plasmids used for direct DNA transfer tend to concatenate before integration and the presence of filler genomic DNA between individual transgenes or T-DNAs indicates that integration events might cluster at sites with damaged DNA and involve similar processes (Kohli et al 2003; Kohli et al 2006; Svitashv et al 2002; Mehlo et al 2000). Two-phase transgene integration mechanisms have been proposed to explain transgene concatenation before integration and the transgene clusters, with single copy transgenes and concatemers interspersed with filler DNA (Svitashv et al 2002; Kohli et al 1998; Pawlowski and Somers, 1998). Direct DNA transfer

favors the integration of discrete DNA fragments at a single transgenic locus, which is why cotransformation with different plasmids or linear fragments leads to SMART loci often containing all the transgenes in the input mix. By contrast, because large multiplex loci are generated less frequently with *Agrobacterium*, T-DNA methods for multigene transfer rely on the presence of multiple transgenes linked on a large input DNA fragment, otherwise transgenes tend to integrate at separate locations and segregate in later generations.

What does the future hold?

Although MGT can be viewed as a goal in itself, the practical limitations might reflect what happens after integration, e.g. interactions between transgenes, rearrangements and silencing. The limitations of MGT need to be tested, with the impact of promoter use being particularly important. Repetitious use of the same promoter was once considered inadvisable owing to the likelihood of transcriptional silencing, but we have created many multiplex transgenic plants carrying five or more genes under the same promoter with no untoward effects (Naqvi et al 2009). Such nutritionally enhanced plants will need to be fortified with agronomic traits such as resistance to pests and diseases, parasitic weeds and ability to endure abiotic stresses when deployed in the field. Toxicity and allergenicity testing in the context of risk assessment is also a mandated requirement.

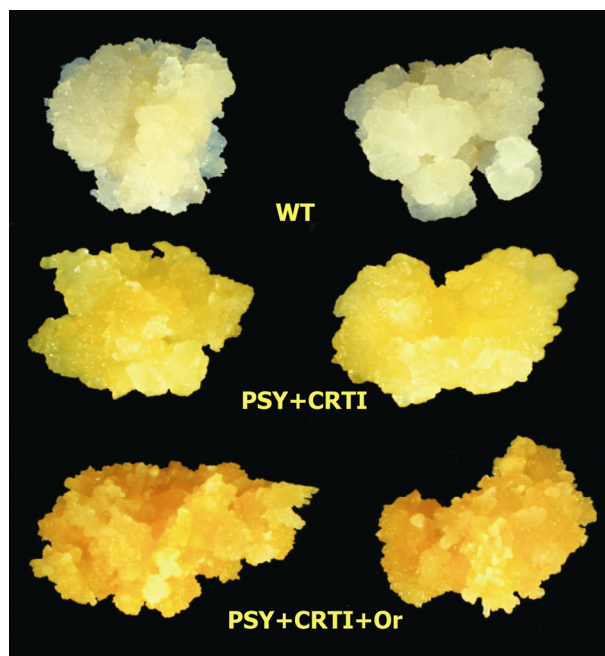


Figure 3. Rice callus generated through combinatorial nuclear transformation using maize *psy1*; *Pantonea ananatis crtI* for carotenoid synthesis and *Arabidopsis Orange (Or)* gene

A major barrier to adoption is the expensive, time consuming and onerous regulatory approval process for the commercial release of such crops and we need to see dramatic improvement in this area if there is any hope of seeing the fruit of such research on biofortified GE crops, particularly in publicly funded laboratories from reaching the people who need it the most, poor and malnourished people in the developing world.

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