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Essential micronutrient fortification of cereal grains for enhanced nutrition

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SUMMARY – Mineral nutrient deficiencies are responsible for a number of preventable diseases, including anemia (iron deficiency) and heart disease (selenium deficiency). Cereals have low bioavailability of essential minerals. People in developing countries, whose diet is primarily based on cereals, are largely affected. The aim of this project is to engineer cereal plants with appropriate genes to obtain grains with substantially enhanced contents of iron (Fe), selenium (Se) and zinc (Zn). We obtained the iron-storing protein ferritin (from *Glycine max)* and phytase (from *Aspergillus fumigatus*) that is responsible for degrading phytate which limits Fe absorption in the gut. We cloned three enzymes involved in the phytosiderophore biosynthetic pathway: nicotianamine aminotransferases A and B (from *Hordeum vulgare*) and nicotianamine synthase 1 (from *Oryza sativa*). Two iron transporters were also cloned: the iron regulated metal transporter 1 (from *Oryza sativa*) and the iron- phytosiderophore transporter (from *Hordeum vulgare*). We cloned the ATP sulfurylase for Se accumulation and a Zn transporter, both from *Arabidopsis thaliana*. All traits are being introduced simultaneously into the same cereal plant(s): rice and maize, using a combinatorial transformation approach. To the best of our knowledge, these experiments represent the most comprehensive experiments to date, for achieving substantial micronutrient fortification of important cereal crops.

Introduction

Micronutrients such as vitamins or trace minerals, are elements that the body must obtain from outside sources. Micronutrients like iron (Fe), zinc (Zn) and selenium (Se), are essential to the body in small amounts because they are either components of enzymes, or act as cofactors in controlling chemical reactions. Iron is a carrier of oxygen in red blood cell haemoglobin; it transports electrons within cells; and it is an integral part of important enzyme systems in various tissues. Populations with iron deficiency risks are infants, children, adolescents and pregnant women. Iron deficiency is probably the most common nutritional deficiency disorder in the world. Zinc is present in all body tissues and fluids and it is an essential component of a large number (>300) of enzymes. It participates in the genetic expression of polynucleotide transcription; it is important in immune system (humoral immunity) functioning, and it stabilizes molecular structures of cellular components. Populations most at risk of being zinc deficient are infants, children, adolescents and pregnant women. Zn deficiency may result in low psychomotor and mental development (children), poor pregnancy, poor immune function, tiredness and retarded growth among other symptoms. More than 30% of people are Zn deficient (White and Broadley, 2005). Selenium is implicated in the protection of body tissues against oxidative stress, in the maintenance of defences against infection and in the modulation of growth and development. Some enzymes contain Se and this micronutrient also optimizes the biological functioning of Zn and Fe in humans, and it has anticarcinogenic properties. Se deficiency may result in hypothyriodism, weakened immune system, Keshan disease (heart), Kaschin-Beck disease (epithelial degeneration) and leads to heart disease. Fifteen percent of the population are Se deficient (White and Broadley, 2005).

Material and methods

Gene cloning

The NCBI nucleotide sequence database (http://www.ncbi.nlm.nih.gov/) was used to search for sources

of these genes, and we were able to identify different sequences that could be used for the cloning of these genes. One microgram total RNA was used for RT-PCR. The names, sources, accession numbers and sequence lengths of the genes are given in Table 1. Primer combinations used to amplifying the sequences, are listed in Table 2. All primer combinations give the full length cDNA for the genes. All RT-PCR products were cloned into the pGEM vector (Promega) and sequenced. By using the BLAST program (http://www.ncbi.nlm.nih.gov/), we confirmed 100% homology of the cloned sequences, to the reported genes.

Table 1. Summary of the genes to be used in transformation experiments. A brief description of the genes, their Genbank accession numbers, the source plant tissue for cloning of the genes and the technique used, and the transformation vector employed to make constructs: pAL76 (constitutive) and pTO126 (seed-specific), are listed

Mineral	Genes	Gene Products	Accession number	Sequence length	Source	Tissue	DNA/RNA	Plasmid
Iron	IRT1	Iron regulated metal transporter	AB070226.1	1125 bp	*Oryza sativa*	Root	RNA-cDNA	pAL76
	NAS1	Nicotianamine Synthase 1	AB021746.2	999 bp	*Oryza sativa*	Root	RNA-cDNA	pAL76
	NAAT-A	Nicotianamine aminotransferase	D88273.2	1402 bp	*Hordeum vulgare*	Root	RNA-cDNA	pAL76
	NAAT-B	Nicotianamine aminotransferase	AB005788.1	1656 bp	*Hordeum vulgare*	Root	RNA-cDNA	pAL76
	HvYS1	Iron-phytosiderophore transporter	AB214183.1	2037 bp	*Hordeum vulgare*	Root	RNA-cDNA	pAL76
	Ferritin	Ferritin	M64337	780 bp	*Glycine max*	Leaf	RNA-cDNA	pTO126
	PHYA3	Phytase	AJ419776	1331 bp	*Aspergillus fumigatus*	Provided by Dr. E. Mullaney; USDA, Louisiana		pTO126
Zinc	ZAT	Zn transporter	NM_130246.2	1197 bp	*Arabidopsis thaliana*	Leaf	RNA-cDNA	pAL76
Selenium	APS1	ATP sulfurylase	NM_113189.2	1392 bp	*Arabidopsis thaliana*	Leaf	RNA-cDNA	pAL76

Table 2. Sequences of primers used to amplify full length sequences for all required genes

Mineral	GENES	Position	Primer sequence
Iron	IRT1	Forward	5'- A GGATCC ATG GCG ACG CCG CGG ACA CTG GTG CCC ATT CTG-3'
		Reverse	5'- A GAATTC TCA CGC CCA CTT GGC CAT GAC GGA C-3'
	NAS1	Forward	5'- A GGATCC ATG GAG GCT CAG AAC CAA GAG GTC G-3'
		Reverse	5'- A GAATTC CAT AAT ATA GTG CGC CTT TCG ATC GTC CGG CTG T-3'
	NAAT-A	Forward	5'- A GGATCC ATG GTA CAC CAG AGC AAC GGC CAC G-3'
		Reverse	5'- A AAGCTT CTA ACA ACC ATT TAT AGA ATT CTT C-3'
	NAAT-B	Forward	5'- A GGATCC ATG GCC ACC GTA CGC CAG AGC GAC G-3'
		Reverse	5'- A GAATTC CTA GCA ATC ATC GCT CGA ATT TCT C-3'
	HvYS1	Forward	5'- A GGATCC ATG GAC ATC GTC GCC CCG GAC CGC A-3'
		Reverse	5'- A AAGCTT TTA GGC AGC AGG TAG AAA CTT CAT G-3'
	HvYS1	Forward	5'- A GGATCC ATG GAC ATC GTC GCC CCG GAC CGC A-3'
		Reverse	5'- A AAGCTT TTA GGC AGC AGG TAG AAA CTT CAT G-3'
	Ferritin	Forward	5'- ATG GCT CTT GCT CCA TCC AAA GTT-3'
		Reverse	5'- GGC TAT TCA AGA TTA AGC AGC ATC-3'
	PHYA3	Forward	5'- TGT AGA GTC ACC TCC GGA CTG GCA GTC -3'
		Reverse	5'-CCG CGG CTA AGC AAA ACA CTC CG-3'
Zinc	ZAT	Forward	5'-A GGATCC ATG GAG TCT TCA AGT CCC CAC CAT A-3'
		Reverse	5'-A GAATTC TTA GCG CTC GAT TTG TAT CGT GAC A-3'
Selenium	APS1	Forward	5'- A GGATCC ATG GCT TCA ATG GCT GCC GTC TTA A-3'
		Reverse	5'- A GAATTC TTA CAC CGG AAC CAC TTC TGG TAG T-3'

Plasmid construction

The full length sequences of all genes (Tables 1 and 2) were excised from the pGEM vector and subcloned either into pAL76 (Christensen and Quail, 1996), which contains the *Ubi-1* promoter and its first intron, and an *Agrobacterium tumefaciens nos* transcriptional terminator, or into pTO126 which contains a rice glutelin endosperm specific promoter (Leisy *et al*., 1990; Drakakaki *et al*., 2005).

Rice and corn transformation and plant regeneration

Immature maize zygotic embryos (*Zea mays* L., cv M37W) will be cultured as described by Mehlo *et al*. (2000). After 10 days of culture on callus induction media, bombardment will be performed using 70 μg of coated gold particles (Christou *et al*., 1991). The embryos will be incubated on high-osmoticum containing medium (0.2 M mannitol, 0.2 M sorbitol) for 5-6 h prior to bombardment and 10-16 h after bombardment. The gold particles will be coated with DNA at a molar ratio of 3:1 (linear minimal transgene cassettes of gene of interest:selectable marker cassette, which is derived from the pAHC20 plasmid that contains the *bar* selectable marker gene; Christensen and Quail 1996), for cotransformation (Christou *et al*., 1991). Bombarded callus will be selected on phosphinothricin-supplemented medium as described previously (Mehlo *et al*., 2000). A minimum of 100 independent transgenic events will be generated in order to assure the recovery of an adequate number of transgenic plants containing, and expressing, all input genes.

Mature rice embryos (*Oryza sativa* L., cv EYI 105) will be excised and cultured as described (Sudhakar *et al*., 1998; Valdez *et al*., 1998). After 5 days of culture, bombardment will be carried out using 70 μg of coated gold particles (Christou *et al*., 1991). The rice embryos will be incubated on high-osmoticum containing medium (0.2 M mannitol, 0.2 M sorbitol) for 5-6 h prior to bombardment. The gold particles will be coated with a molar ratio of 3:1 (linear minimal transgene cassettes of the gene of interest:selectable marker cassette (hygromycin phosphotransferase, *hpt*) for cotransformation (Sudhakar *et al*., 1998; Valdez *et al*., 1998). Bombarded embryos will be selected on hygromycin-supplemented medium, and a minimum of 25 independent transgenic events will be generated.

Results

Transformation vectors expressing all the genes described in Tables 1 and 2 were successfully constructed and will be transformed into the respective cereal crops in due course.

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