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A Step Towards Developing Transgenic Plants with High Nutritional Quality

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With advances in recombinant DNA methods and transformation procedures, it is possible to transfer genes into crop plants from unrelated plants, microbes and animals. These transgenic plants have further provided wealth of information to understand the mechanism of gene expression such as tissue specific expression of seed storage proteins and others. Moreover, the availability of efficient transformation system for crop species is of intense interest to biotechnology, agrochemical and seed companies for the application of this technology to crop improvement. However, the application of this technology to rational plant-improvement is currently limited by a shortage of cloned genes for important traits. In our laboratory recently, as a first step towards developing transgenic plants, we have isolated two useful genes which are relevant to human health, namely essential aminoacids-rich storage protein gene from *Amaranthus* and Oxalate decarboxylase gene from *Collybia velutipes*.

Key Words: Transgenic plants, Gene expression, Seed storage proteins, Plant improvement, Cloned genes

Introduction

Plant breeders are routinely concerned with improving traits such as tolerance to disease or environmental stress, yield and quality of the commercial product and plant architecture. In some instances, the mechanistic basis for a particular trait is known, and therefore it is possible to envisage strategies for the rational improvement of plants by the introduction of one or few cloned genes. In the area of plant genetic engineering, however, rate limiting step in developing transgenic plants has been the isolation of the relevant genes. In this brief article, we have described the isolation of two novel genes that may be useful to plant improvement.

Molecular Cloning of Seed-specific Protein Gene from Amaranthus to Improve Nutritional Quality of Seed Proteins of Legumes and Cereals

Seeds are the major source of protein for most of the world's population. But most often they are deficient in one or the other essential amino acid. Cereals are most often deficient in lysine and in some cases in tryptophan and threonine. For years plant breeders have tried to improve the balance of essential amino acids of the important crop plants (Larkins 1982). Molecular approaches for improving the nutritional quality of seed proteins provide alternatives to the conventional approaches. One of the

ways by which the quality of their proteins can be improved is by the transfer of a heterologous gene that would encode for a protein with higher levels of the desired aminoacids.

Amaranth grain, because of its high nutritional value, has been identified as a crop with potential economic value. Its amino acid composition closely matches the optimum human requirement of amino acids. It is rich in lysine, tryptophan and methionine and so can be used as a source of a gene that encodes a protein of high nutritional value. In spite of good quality and quantity, these proteins have not yet been purified and characterized. Storage proteins accumulate in seeds in large amounts thereby determining the nutritional quality of the seed. As a first step in isolation of a gene encoding a protein of high nutritional values, two proteins of 54 kDa and 35 kDa were purified from the seeds of *Amaranthus hypochondriacus*. The 35 kDa protein was found to be soluble in low salt buffers (albumin type) while the 54 kDa polypeptide required high salt for its solubilization. Aminoacid composition of both the proteins was determined. Since 35 kDa protein had a well balanced aminoacids composition and high levels of the essential aminoacids, it was chosen for further work. Since the seed proteins are synthesized during a particular stage of development, their mRNA represent a large fraction of the total mRNA at that particular stage. This makes the cloning of cDNAs for the seed protein genes easier. The stage at which the 35kDa protein was being synthesized, was determined by SDS-PAGE and western blot analysis. The level of mRNA at that stage was then checked by immunoprecipitation of the *in vitro* translated product and was used for cDNA synthesis. Affinity purified AmA1 antibodies were used to isolate cDNA clones from a developing seed expression library constructed in a lambda gt11 expression vector. The cDNA clone (1.2 kilobases) has a single major open reading frame corresponding to a 304 amino acid polypeptide. The clone was confirmed by hybrid-selected translation and immunoprecipitation. AmA1 gene is expressed during early embryogenesis. Mature seeds even after one year of storage showed the presence of AmA1 mRNA, though the level was reduced. It suggests that it is very stable. Germinated seeds did not show any AmA1 mRNA. No RNA was detected in leaves and roots, suggesting that the expression is tissue specific. Such specificity of expression is due to sequences in the promoter and enhancer regions of the gene.

The amino acid composition as obtained from purified protein and the derived amino acid sequence shows a high proportion of essential aminoacids like lysine, leucine, threonine, phenylalanine, valine and sulfur amino acids (table 1, figure 1) that are otherwise deficient in the major seed proteins of legumes and cereals. It has relatively low level of glutamine compared to other seed storage proteins (Raina & Datta 1992). Interestingly, the AmA1 composition

Table 1 *Aminoacid composition of AmA1 as calculated by considering total residue number of each aminoacid from the sequence and their respective molecular weights**

Amino acids	AmA1	WHO
Trp	3.6	1.0
Met/Cys	3.9	3.5
Thr	5.1	4.0
Ile	6.1	4.0
Val	5.2	5.0
Lys	7.5	5.5
Phe/Tyr	13.7	6.0
Leu	9.2	7.0

* Anjana Raina and Asis Datta (1992). Molecular cloning of a gene encoding seed-specific protein with nutritionally balanced amino acid composition from *Amaranthus Proc. Natl. Acad. Sci. USA* **89** 11774-11778

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                                                        M  A  G  L  P
ATCAGATTAACATAATTTCACAATAAAAAAAAAAAAAAGAGCTTAAATGGCGGGATTACC          60

 V  I  M  C  L  K  S  N  N  N  Q  K  Y  L  R  Y  Q  S  D  N
AGTGATTATGTGCCTAAAATCAAATAACAACCAGAAGTACTTAAGATATCAAAGTGATAA          120

 I  Q  Q  Y  G  L  L  Q  F  S  A  D  K  I  L  D  P  L  A  Q
TATTCAACAATATGGTCTTCTTCAATTTTCAGCTGATAAGATTTTAGATCCATTAGCTCA          180

 F  E  V  E  P  S  K  T  Y  D  G  L  V  H  I  K  S  R  Y  T
ATTTGAAGTCGAACCTTCCAAGACTTATGATGGTCTTGTTCACATCAAATCTCGCTACAC          240

 N  K  Y  L  V  R  W  S  P  N  H  Y  W  I  T  A  S  A  N  E
TAACAAATATTTGGTTAGGTGGTCTCCCAATCATTATTGGATTACAGCATCAGCCAATGA          300

 P  D  E  N  K  S  N  W  A  C  T  L  F  K  P  L  Y  V  E  E
ACCAGATGAAAATAAAAGCAATTGGGCATGCACATTATTCAAACCACTTTACGTAGAAGA          360

 G  N  M  K  K  V  R  L  L  H  V  Q  L  G  H  Y  T  E  N  Y
AGGTAACATGAAAAAGGTTCGACTTTTGCACGTCCAATTAGGTCATTATACAGAAAATTA          420

 T  V  G  G  S  F  V  S  Y  L  F  A  E  S  S  Q  I  D  T  G
TACCGTTGGTGGGTCCTTCGTATCATACTTATTTGCCGAATCAAGTCAAATTGATACCGG          480

 S  K  D  V  F  H  V  I  D  W  K  S  I  F  Q  F  P  K  T  Y
CTCTAAAGACGTATTCCATGTCATAGATTGGAAATCAATCTTTCAATTTCCCAAAACATA          540

 V  T  F  K  G  N  N  G  K  Y  L  G  V  I  T  I  N  Q  L  P
TGTCACATTTAAAGGAAATAATGGAAAATATTTAGGGGTTATCACAATTAATCAACTTCC          600

 C  L  Q  F  G  Y  D  N  L  N  D  P  K  V  A  H  Q  M  F  V
ATGTCTACAATTTGGGTATGATAATCTTAATGATCCAAAGGTGGCTCATCAAATGTTTGT          660

 T  S  N  G  T  I  C  I  K  S  N  Y  M  N  K  F  W  R  L  S
CACTTCTAATGGTACTATTTGCATTAAATCCAATTATATGAACAAGTTTTGGAGACTCTC          720

 T  D  N  W  I  L  V  D  G  N  D  P  R  E  T  N  E  A  A  A
TACGGATAATTGGATATTAGTCGATGGGAATGATCCTCGCGAAACTAATGAAGCTGCTGC          780

 L  F  R  S  D  V  H  D  F  N  V  I  S  L  L  N  M  Q  K  T
GTTGTTTAGGTCGGATGTGCATGATTTTAATGTGATTTCGCTTTTGAACATGCAAAAAAC          840

 W  F  I  K  R  F  T  S  G  K  P  E  F  I  N  C  M  N  A  A
TTGGTTTATTAAGAGATTTACGAGTGGTAAGCCTGAGTTTATAAATTGTATGAATGCAGC          900

 T  Q  I  V  D  E  T  A  I  L  E  I  I  E  L  G  S  N  N
TACTCAAATTGTTGATGAAACTGCTATTTTAGAGATAATAGAATTGGGATCCAACAACTA          960

ATATATTGGATTGCTTTTAAGATTCAAATTAAAGTCTAGTTGTTAATGTAAGGAATAAAA         1020

CGTTGTAAGTCGTCTCTTTGGAAACAAGAGGGTTCTTCCTTGTATCATATCTCTATGGTC         1080

TCTTTCAGATTTTGACCATAAGATTACTATTAAATACTTGTAATGTGTTTGTCTGTGATG         1140

ATTACTCTTTGTTGGAATAAAATAATTGTTAGAATTATATTAC                          1183
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Figure 1. Nucleotide sequence of the 1183-bp AmA1 cDNA and the derived aminoacid sequence of the predicated polypeptide. Aminoacids (Single-letter code) are indicated above the first base of each condon. Possible glycosylation sites are overlined. A poly (A) tract was not found although ploy (A) addition signals were present and underlined Anjana Raina and Asis Datta 1992).

closely matches the values recommended by WHO making it more important nutritionally.

To summarize, AmA1 is a novel seed specific protein, because of its high nutritional value. The gene encoding this protein may have potential for compensating for amino acid deficiencies of many seed proteins once it is genetically engineered into target plants.

Molecular Cloning of Oxalate Decarboxylase Gene to Remove Nutritional Stress (Oxalic Acid) from Plants

Oxalate is distributed ubiquitously in animal and plant kingdoms. In humans, oxalic acid and its salts occur in urine, blood, kidney etc. Although minute amounts are synthesized by mammals, much of the oxalate of animals originates from the oxalate ingested with plant material. Oxalic acid accumulates in a number of biological systems as a non metabolic pool. Earliest interest in toxicity of oxalates arose because of instances of severe human poisoning following consumption of large quantities of oxalate rich leaves of certain plants. Some green leafy vegetables (e.g. *Amaranthus* Spinach, rhubarb) are rich sources of vitamins and minerals, but they contain oxalic acid as a nutritional stress factor. The lethal dose of oxalic acid for humans has been reported as 2-5g (Proceedings of 2nd Amaranth Conference). On the basis of that 100 g of fresh oxalate rich vegetables (spinach, amaranth etc.) could be consumed at a meal, the total amount of oxalate consumed will be 1.6g and if it is taken twice daily, 3.2 g will be consumed. Such plants, when consumed in large amounts, become toxic to humans because oxalate chelates calcium, and precipitation of calcium oxalate in the kidney leads to hyporoxaluria and destruction of renal tissues (Decastro 1988, Hodgkinson 1970). Apart from this, at least two other instances can be cited where oxalic acid is involved in an indirect manner. In one case, production of oxalic acid is an important attacking mechanism utilized by (Sclerotinia) *Whetzelinia sclerotiorum* during pathogenesis. This fungus infects sunflower causing serious damage to crops. Oxalic acid is formed in *W. sclerotiorum* from oxaloacetate hydrolysis catalyzed by oxaloacetate acetyl hydrolase or from glyoxalate by the enzyme glyoxalate: NAD oxidoreductase. Oxalic acid accumulates in the infected tissues early in pathogenesis and its concentration increases during the time the pathogen is colonizing the host tissues. Concurrent with this increase in oxalate there is a lowering of *p*H to 4.5, especially at margins of lesions. The oxalic acid appears to enter the vascular stream and moves into foliage. The accumulation of oxalic acid in leaves causes symptoms of wilting and eventually leaf death. Thus, oxalic acid functions as a mobile toxin that moves from the base of stems to xylem sap and leaves (Maxwell 1973).

In another case, oxalic acid is an important substrate which is involved in neurotoxin biosynthesis in the protein rich legume, *Lathyrus sativus*. Consumption of *Lathyrus* sativus (chickling vetch) causes neurolathyrism, which is characterized by spasticity of leg muscles, lower limb paralysis, convulsions, and death. *L. sativus* is a protein-rich hardy legume that grows under extreme conditions such as draught and water-logging and does not require complex management practices. The neurotoxin is an amino acid derivative, β-oxalyl aminoalanine (BOAA) and β-N-oxalyl-L-α, β-diaminopropionic acid (ODAP) is present in various parts of the plant. ODAP synthesis is a two-step reaction in which oxalic acid is an essential starting substrate. ODAP acts as a metabolic antagonist of glutamic acid, which is involved in transmission of nerve impulses in the brain. Hence, despite its rich protein

content, the legume cannot be used as a food source (Mickelsen et al. 1973).

The study on oxalate degrading enzyme could provide a useful tool to be employed in alleviating the stress conditions created by oxalic acid in many cases. Thus, the isolation of a new useful gene will serve as a tool to degrade oxalic acid in plants where oxalic acid accumulates as such or is a substrate in the synthesis of neurotoxin or is a medium for pathogenesis. This can be achieved by effecting single gene transfer to these plants. Moreover, oxalic acid-depleted transgenic plants would be useful to study the role of oxalic acid in the plant.

Of the known oxalic acid degrading enzymes, oxalate decarboxylase synthesized by the basidiomycete *Collybia velutipes* is of interest because the earlier report on crude extract of the enzyme (Shimazono & Hayaishi 1957) showed the single step breakdown oxalic acid by the enzyme in absence of any cofactor/prosthetic group. It acted specifically on oxalic acid and produced stoichiometric quantities of carbon dioxide and formic acid. Thus the enzyme oxalate decarboxylase was purified to homogeneity and the properties of the enzyme were studied. The subunit molecular mass of the glycosylated enzyme was 64 kDa, whereas the mass of the deglycosylated protein was 55 kDa. The cDNA encoding the enzyme was obtained by immunoscreening a lambda gt11 expression library (Mehta & Datta 1991). To summarize, this novel gene will be used to develop transgenic plants with low oxalic acid content.

Conclusions

With the advent of Recombinant DNA technology, it has become possible to isolate new genes and to add DNA sequences that regulate their temporal and spatial expression within the transgenic plant. There are several efficient transformation methods to introduce foreign DNA e.g. Agrobacterium mediated gene transfer, DNA uptake into isolated protoplasts and microprojectile bombardment are known. Using these methods it has already been possible to introduce into crop plants, genes from a wide range of donor organisms to modify a number of different plant characters. However, the application of methods is frequently not straight forward in some crop plants. In our laboratory, efforts will be made to develop transgenic plants using these two genes encoding seed protein and oxalate decarboxylase.

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