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Transgenic Azuki Bean Approaches

Mutasim M. Khalafalla[1*] • Hany A. El-Shemy[2]

[1] Commission for Biotechnology and Genetic Engineering, P.O. Box 2404 Khartoum, Sudan
[2] Biochemistry Department, Faculty of Agriculture, Cairo University, 12613 Giza, Egypt
Corresponding author: * **Mutasim@myway.com**

ABSTRACT

The first transgenic plants were reported in 1983. Since then, many recombinant proteins have been expressed in several important agronomic species of plants including tobacco, corn, tomato, potato, banana, alfalfa and canola. The choice of plant system was initially driven by convenience and ability to develop high frequency, routine and reproducible regeneration and genetic transformation systems. For this reason, azuki bean [*Vigna angularis* (Willd.) Ohwi & Ohashi] recently emerged as important transgenic grain legume crop. Azuki bean genetic transformation has taken rapid strides since the first transgenic azuki bean plant was produced 10 years ago. During the last 5 years, tremendous progress has been made to develop a high frequency, routine, and reproducible genetic transformation protocol for azuki bean through *Agrobacterium*-mediated transformation technology. This technology has been applied to produce azuki bean plants that withstand several a biotic stresses, as well as to gain tolerance against various pests and diseases. In addition, quality improving and increased nutritional value traits have also been introduced into azuki bean. Most of these gains were not possible through conventional breeding technologies. Moreover, using genetic transformation technology, azuki bean could be emerged as an important leguminous model plant providing the framework within which the molecular mechanisms that underlie the grain legume-specific character can be clarified.This review is an attempt to summarize the progress in transgenic azuki bean technology, with particular emphasis on agronomic and nutritional traits.

Keywords: genetic transformation, grain legumes, regeneration, pluses, *Vigna angularis*
Abbreviations: α AI, α-amylase inhibitor gene; **BA**, 6-Benzylaminopurine; **CaMV35S**, Cauliflower Mosaic Virus promoter; **dap**, dihydrodipicolinate synthase; **gfp**, green fluorescent protein; **gus**, β-glucuronidase; **hpt**, hygromycin phosphotransferase; **MS medium**, Murashige and Skoog medium; **nptII**, neomycin phosphotransferase II; **Trp**, tryptophan

CONTENTS

INTRODUCTION

Among the grain crops, grain legumes (also known as pulses) rank third behind cereals and oilseeds in world production, but constitute an important dietary constituent for humans and animals (Popelka *et al*. 2004). Grain legumes play a crucial role in the sustainability of agricultural systems and in food protein supply in developing countries.

Azuki bean [*Vigna angularis* (Willd.) Ohwi & Ohashi] is an edible grain legume grown and used for centuries primarily in the East Asian countries of Japan, Korea, China, and Taiwan where it is a vegetable protein source and is of importance in the traditional cultures of the region. Seeds are cooked and mixed with varying portions of sugar and other ingredients to produce *an*, which is used as pastry filling in traditional Oriental confections (Breene and Hardman 1989; Hang *et al*. 1993; Lumpkin *et al*. 1993; Ru-Qiang *et al*. 2000).

A number of biotic and a biotic stresses are severely affecting full realization of the yield potential of azuki bean. The crop is susceptible to many pathogens including several viral, fungal and bacterial pathogens and its production often hampered by insect attacks (Yamada *et al*. 2001). These constraints that limit crop production or quality have been addressed by conventional breeding and enhanced management, but genetic improvement of azuki bean through classical breeding is limited (Angenon *et al*. 1999) and there are situations where the existing germplasm lacks the required traits.

In recent years biotechnology is emerging as one of the latest tools of agricultural research could help provide solutions to certain constraints and contribute to produce cultivars resistant to biotic and a biotic stresses and have better protein quality and quantity, thus improving food security by increasing productivity and enhance the nutritional value of this pulse crop in developing countries.

In concert with traditional plant breeding practices, biotechnology is contributing towards the development of

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Invited Mini-Review

novel methods to genetically alter and control plant development, plant performance and plant products. Conventional breeding utilizes domestic crop cultivars and related genera as a source of genes for improvement of existing cultivars, and this process involves the transfer of a set of genes from the donor to the recipient. In contrast, biotechnological approaches can transfer defined genes from any organism, thereby increase the gene pool available for improvement.

One of the areas of plant biotechnology called transgenic technologies involves the delivery, integration and expression of defined genes into plant cells, which can be grown in artificial culture media to regenerate plants. Thus biotechnological approaches have the potential to complement conventional methods of breeding by reducing the time taken to produce cultivars with improved characteristics.

Transgenic crops represent a promising technology that can make a vital contribution to global food and feed, fiber security and to the alleviation of poverty, particularly in Developing countries. These crops can minimize damage through disease and pest-resistant varieties, reduce the use of chemicals and enhance stress tolerance in crops, thereby permitting economically productive farming on unproductive lands.

Azuki bean is a member of the tribe Phaseoleae, which includes many economically important legume crops, and it is closely related taxonomically to soybean (*Glycine max*), common bean (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*), and mung bean (*Vigna radiata*). Among the tribe *Phaseoleae*, azuki bean is not one of the cosmopolitan crops but it shows promise with regard to its competence for transformation and regeneration with the use of *Agrobacterium* for gene transfer and with kanamycin or hygromycin B for selection (Ishimoto *et al*. 1996; Yamada *et al*. 2001; El-Shemy *et al*. 2002: Khalafalla *et al*. 2005; Yamada *et al*. 2005). Thus the availability of the regeneration and genetic transformation systems paves the way for agronomic improvements through genetic engineering.

This review is an attempt to summarize the studies on regeneration and genetic transformation in azuki bean and to identify the hurdles being faced in the efficient recovery of transgenic plants.

TISSUE CULTURE AND REGENERATION SYSTEM

The use of modern techniques of cell, tissue and organ culture is central to many crop improvement programmes in both industrialized and developing countries. The regeneration of complete plants via tissue culture has made it possible to introduce foreign genes into plant cells and recover transgenic plants. Morphogenesis could occur directly from the explant or indirectly via the formation of a dedifferentiated callus. However the different pathways of regeneration, viz. organogenesis from callus, organogenesis directly from explants and embryogenesis from explants in a direct mode vary in their amenability to different gene delivery techniques (Chandra and Deepak 2003). The limiting step to the successful development of transgenic plants of the major edible crops has not been transgene insertion itself, but rather the regeneration of viable plants from the transgenic explant material (Murphy 2003). Therefore regeneration of plants from cell culture is a critical step in the production of novel varieties of plants (Nishimura *et al*. 2005).

Legumes are the most important group of crop plants next to cereals and much effort has been devoted to develop efficient *in vitro* regeneration systems because of their recalcitrance to tissue culture regeneration (Anurdaha *et al*. 2006). Among legumes, azuki bean is one of the few species that are amenable to *in vitro* culture therefore, relatively rapid and efficient regeneration protocols have been developed (Sato *et al*. 1990; Yamada *et al*. 2001; El-Shemy *et al*. 2002, 2004; Khalafalla *et al*. 2005).

In Azuki bean, *in vitro* morphogenesis has been tried using various explants. However, etiolated epicotyls from *in vitro* seedling have been most responsive explant for the induction of multiple shoots via organogenesis (Sato *et al*. 1990; Yamada *et al*. 2001; El-Shemy *et al*. 2002; Khalafalla *et al*. 2005). Although morphogenesis of adventitious shoots occurs directly or via callus formation from epicotyl explants of several azuki bean cultivars (Sato *et al*. 1990), but the regeneration efficiency and optimal concentrations of plant growth regulators for adventitious shoot formation is genotype-dependent (Yamada *et al*. 2001). For analyzing adventitious shoot formation from epicotyl explants and selecting azuki bean genotype that efficiently regenerated, Yamada and coworkers 2001 found that adventitious shoots and regenerated plants were obtained from all major Japanese cultivars tested on MS medium supplemented with 1 mgl^{-1} BA. Furthermore, they reported that, shoots regenerated from epicotyl explants were obtained within 30 days from beginning of culture and were easily rooted on MS medium without growth regulators.

GENETIC TRANSFORMATION

Plant genetic transformation is the science of direct gene transfer and integration, from one plant to another or from a microorganism to a plant, to create plants with altered genetic make-ups to achieve specific crop production goals. The altered plants are generally termed transgenic (Mulwa and Mwanza 2006).

Since its initial application to plants more than three decades ago, genetic transformation has become an indispensable tool in plant molecular biology and functional genomics research. Transformation is currently used for genetic manipulation of more than 144 plant species, representing almost all major phylogenetic lineages of the plant kingdom (Busov *et al*. 2005).

Although many different techniques (electroporation of intact tissues, silicone carbide whiskers, etc.) have been tested for gene delivery to plant cells, two major methods, namely *Agrobacterium*-mediated and particle bombardment, have been extensively employed for genetic transformation of crop plants.

Agrobacterium is a naturally-occurring soil bacterium that causes tumors, or galls on plants. For a long time it was widely considered that *Agrobacterium* is the only bacterial genus capable of transferring genes to plants. The discovery that this gall formation is due to the integration of bacterial DNA into the plant genome laid the foundations of plant biotechnology. The *Agrobacterium* ability to transfer genes from bacteria to plants has been widely exploited by researchers for genetic engineering and plant improvement, its simple and less fragmented pattern of gene insertions facilitates inheritance studies and, thus, interpretation of gene-phenotype relationships (Busov *et al*. 2005).

Agrobacterium-mediated transformation offers several advantages over direct gene transfer methodologies (particle bombardment, electroporation, etc), such as the possibility to transfer only one or few copies of DNA fragments carrying the genes of interest at higher efficiencies with lower cost and the transfer of very large DNA fragments with minimal rearrangement (Gheysen *et al*. 1998; Hansen and Wright 1999; Shibata and Liu 2000). The majority of legume transformation studies have favored the use of *Agrobacterium tumefaciens* to generate transgenic soybeans (Hinchee *et al*. 1988; Chee *et al*. 1989), chickpeas (Fontana *et al*. 1993) and pea (Puonti-Kaerlas *et al*. 1990; de Kathen and Jacobsen 1990; Zubko *et al*. 1990; Puonti-Kaerlas *et al*. 1992; Davies *et al*. 1993; Schroeder *et al*. 1993; Shade *et al*. 1994).

Among the tribe *Phaseoleae*, azuki bean is ranked behind soybean, common bean; cowpea and mungbean in production and economic importance, but its amenability to *in vitro* morphogenesis accelerated gene transfer and manipulation by genetic engineering. Azuki bean transformation systems have been developed based on cocultivation of epicotyl explant with *A. tumefaciens*. Useful transgenic plants

Table1 *Agrobacterium*-mediated transformation of azuki bean.

Target tissue	Expressed gene (s)	Selectable marker	Reporter gene (s)	Reference	Comment
Epicotyl	*α AI*, *npt*II	Kanamycin	*gus*	Ishimoto *et al*. 1996	Stable transformation and expression of α amylase inhibitor in the seeds result in complete block of bruchid development
Epicotyl	*npt*II, *hpt*,	Kanamycin	*gus*, *gfp*	Yamada *et al*. 2001	Stable transformation and generation of flowering transgenic plant in 6-8 months
Epicotyl	*npt*II, *hpt*	hygromycin	*gfp*	El-Shemy *et al*. 2002	Stable transformation and expression of *gfp* gene in the leaf
Epicotyl	*hpt*, *dnap*	hygromycin	*gfp*	El-Shemy *et al*. 2004	Stable transformation, production of transgenic, azuki bean seeds with elevated lysine level
Epicotyl	*hpt*, *bar*	Hygromycin, bialaphos	*gfp*	Khalafalla *et al*. 2005	Stable transformation and production of transgenic azuki bean leaves expressing *gfp* and *bar* genes
Epicotyl	*α AI*, *α AII*, *npt*II	hygromycin	*gus*	Yamada *et al*. 2005	Expression of new type of bean *αAI* gene that does not require post-translational processing for activation
Epicotyl	*OSA1D*, *hpt*	hygromycin	*gfp*	Hanafy *et al*. 2006	Stable transformation with *OASA1D* resulted in a 6.5–16.5-fold increase in free Trp content and 1.3–2.1-fold increase in total Trp content of azuki bean seeds

were obtained (Ishimoto *et al*. 1996). However, this method has not come into general use as the transformation frequency was very low and transgenic plants are not readily reproducible. Effort to increase the efficiency of *A*. *tumefaciens*-mediated transformation using the same explant have included the usage of high concentration of BA coupled with acetosyringone (Yamada *et al*. 2001), use of hygromycine as selectable marker (Shemy *et al*. 2002) and incorporation of GFP as a reporter gene (Shemy *et al*. 2004; Khalafalla *et al*. 2005). Azuki bean epicotyl explants contain cells that are competent for regeneration and hence emerged as a useful target for gene delivery via *Agrobacterium*-mediated transformation (**Table 1**).

Once efficient protocols for tissue culture and genetic transformation are developed, the production of transgenic plants with different genes is fairly routine (Sharma *et al*. 2000). Numerous factors are reported to influence the success of azuki bean genetic transformation. Yamada *et al*. (2001), aiming to optimize a previous azuki bean transformation system (Ishimoto *et al*. 1996), and using epicotyl explants, showed that 6-Benzylaminopurine (BA), at a high concentration (10 $mg.l^{-1}$), coupled with acetosyringone (AS) during co-cultivation with *A*. *tumefaciens* played important roles in improving transformation efficiency. However, addition of AS alone had no obvious effect on transient GUS expression. The frequency of GUS expression by *A*. *tumefaciens* was increased by more than 10 times when combined with AS and a high concentration of BA compared to the original co-cultivation medium.

The (negative) selection regimes for transformed cells are based on the expression of a gene termed the selectable marker producing an enzyme that confers resistance to a cytotoxic substance, often an antibiotic or herbicide. The most commonly used selection marker in azuki bean transformation is the antibiotic resistance markers. Yamada *et al*. 2001 used the bacterial neomycin phosphotransferase II (*npt*II) gene providing resistance to aminoglycoside antibiotics in azuki bean transformation. The hygromycin phosphotransferase (*hpt*) gene, widely used in rice transformation, has also been reported as an efficient selectable marker for achieving stable genetic transformation in azuki bean. El-Shemy *et al*. (2002), using the epicotyl explant successfully obtained azuki bean transgenic plants expressing the *hpt* gene marker in the leaves and molecular analysis indicated the successful integration of the transgenes and proved that *hpt* gene worked as efficiently as the *npt*II gene.

Improving transformation frequency remains the most important factor in plant transgene technology (Gheysen *et al*. 1998). The optimization of selection and identification systems is crucial for improving transformation efficiency (Somer *et al*. 2003). In azuki bean, development of a selection system based on hygromycin phosphotransferase (*hpt*) gene greatly increased transgenic plant production and reduced both the number of non-transformed escapes and time in culture. The improved system for azuki bean transformation had efficiency of about 14% (Khalafalla *et al*. 2005) up from 2% as reported by Yamada *et al*. (2001), whose selection system was based on the *npt*II gene.

Plant genetic transformation involves delivery of agene cassette into recipient cells followed by analysis of the expression of delivered gene. The gene delivery can be detected by assaying the expression of a reporter gene introduced into plant cell cultures or intact tissues. The reporter genes produce a visible effect, directly or indirectly, due to their activity in the transformed cells. Analysis of reporter gene expression does not require the integration of the transgene into the host genome and is commonly used to test promoter and gene functions (Patnaik and Khurana 2001).

The β-glucuronidase (*gus*) gene from *E*. *coli* has emerged as the most widely used scorable marker in plant transformation. *gus* enzyme hydrolyzes β glucuronide compounds and gives reaction products that can be quantified spectrophotometric or spectrofluorometrically (Jefferson *et al*. 1987). The use of the *gus* gene for the detection of transgenic azuki bean plants was reported by Ishimoto *et al*. (1996) and Yamada *et al*. (2001). The *gus* reporter gene system is extremely useful for optimization of parameters for genetic transformation, due to the availability of a simple histochemical detection procedure. One of the major limitations of the *gus* reporter gene system, however, is the destructive nature of its assay. Thus, to study the fate of introduced transgenes in living cells, vital reporter genes encoding for anthocyanin biosynthesis, green fluorescentprotein, and firefly luciferase have been used successfully (Patnaik and Khurana 2001).

A synthetic, spectrally modified, version of green fluorescent protein (*gfp*) from the jellyfish, *Aqueorea busovia* (Chiu *et al*. 1996) has also been used as a vital marker in azuki bean transformation (El-Shemy *et al*. 2001, 2004; Khalafalla *et al*. 2005). Recently Hanafy *et al*. (2006) reported the use of a modified *gfp* as a visible marker for the detection of transgenic azuki bean plants on the basis of *gfp* expression. The modified versions of *gfp* permit nondestructive analysis of transgene activity ease to follow the fate of introduced transgenes in individual tissue samples, facilitate rapid assessment and comparison of different transformation procedures, and provide valuable insight into the conditions influencing the efficiency of DNA integration and stable expression.

GENETIC TRANSFORMATION OF AZUKI BEAN FOR SELECTED TRAITS

Nutritional improvement

Azuki is one of the twelve most important grain legumes in the world (Lumpkin *et al*. 1993). However, its protein qua-

lity as in other leguminous seeds does not reach the same level as in animal products. This is due to various factors, among them the well known is the unbalanced amino acid composition for example, the content of sulphur containing amino acids in pulses tends to be lower (Baudoin and Maquet 1999).

One of the goals of plant genetic engineering has been to create crops that are tailored to provide better nutrition for humans and their domestic animals. A major target has been the improvement of the amino acid composition of seed proteins, because animals, including humans, are incapable of synthesizing 10 of the 20 amino acids needed for protein synthesis, and these "essential" amino acids must therefore be obtained from the diet (Chakraborty *et al*. 2000).

The essential amino acids that limit the nutritive value of azuki bean protein are the methionine, lysine, cystine and tryptophan. Therefore attempts to overcome this imbalance are nutritionally relevant, and the concept of azuki bean seed protein quality has been extended to technological and health-oriented aspects. Hanafy *et al*. (2006) introduced *OASA1D*, a modified rice gene into azuki bean in order to manipulate the total tryptophan (Trp) content and improve the nutritional quality of this grain legume. The *OASA1D* subunit is highly insensitive to Trp inhibition, and transformation with *OASA1D* resulted in a 6.5- to 16.5-fold free Trp content and a 1.3- to 2.1-fold total Trp content of seeds compared with the values for wild-type seeds. All of the transgenic azuki bean lines expressing *OASA1D* under the control of the 35S promoter of CaMV were fertile and advanced to the T2 generation and five independent lines fixed for the transgenes were developed. Furthermore, El-Shemy *et al*. (2004) have reported the production of transgenic, azuki bean seeds with elevated lysine level made by reducing the sensitivity of the key enzyme in lysine biosynthesis (dihydrodipicolinate synthase), to feedback inhibition by lysine and showed that the introduction of a feedback insensitive rice gene (*dap*) involved in biosynthesis of aspartate family amino acids led to the lysine content.

Storage pest tolerance

Production of azuki bean has remained constantly low because of its susceptibility to several pathogens and insect pests. Among the insect pests, bruchids cause substantial loss during storage. The cowpea weevil (*Callosobruchus maculatus*) and azuki bean weevil (*C. chinensis*) infest azuki bean seeds heavily and causes severe damage to seeds during storage. Since seeds are used for consumption, use of chemicals to protect the seeds is not recommended. Hence the transfer of resistance factors to susceptible plant varieties by conventional breeding or genetic engineering might thus be a promising approach to increase resistance to insect pests and thereby to reduce seed loss during storage (Yamada *et al*. 2005).

Bruchid species exhibit differences in sensitivity to secondary metabolites, suggesting that certain compounds play a more important role than others in resistance to insects. The α-amylase inhibitor is a seed protein contained in the common bean (*Phaseolus vulgaris* L.) and its linkage with certain amylases forming inactive complex provide seed protection against several bruchid beetles (Shade *et al*. 1994; Schroeder *et al*. 1995). However, other species of bruchids (*Zabrotes subfasciatus* Boh. and *Acanthoscelides obtectus* Say) have a protective mechanism to inactivate α-amylase (Ishimoto *et al*. 1996). The α-amylase in the midguts of these insects is inhibited by the α-amylase inhibitor (αAI) present in common bean seeds. Proof of concept has been obtained by transferring αAI gene driven by the promoter of phytohemagglutinin results in high levels of αAI in the seeds and the complete block of bruchid development on the seeds (Ishimoto *et al*. 1996). Moreover, α-amylase inhibitor gene (*αAI-Pa1*) isolated from the seeds of tepary bean (Phaseolus acutifolius A. Gray) has been introduced into azuki bean through *Agrobacterium*-mediated transformation showed the ability of the gene to inhibit growth of bruchid weevil and proofed that aAI-Pa1 is a new type of bean αAI that does not require post-translational processing for activation (Yamada *et al*. 2005).

Since α-amylase inhibitor is easily inactivated by cooking, introducing this gene into host plants can be regarded as a safe strategy (Ignacimuthu and Prakash 2006).

Herbicide resistance

Herbicide treatment in crop plantings has allowed economically viable weed control and increased productivity. The most preferred herbicides today are those that combine weed killing potency with low- or no environmental persistence. However, the very effective broad spectrum herbicides available also lack selectivity, thus limiting their use in some cropping operations. On the other hand, the continuous use of the few available selective herbicides is speeding up the development of herbicide resistance in weeds; hence making it difficult to achieve effective control in some crops (Mulwa and Mwanza 2006).

Herbicide tolerance is the most common trait in comercial transgenic crops, being part of 82% of all transgenic crops in the year 2003 (James 2003).

Azuki bean is a poor competitor against weeds because of its early slow growth. For this reason, the introduction of gene encoding for herbicide tolerance will be very helpful for its improvement. In order to achieve this objective, the *bar* gene encoding the enzyme, phosphinothricin acetyltransferase which directly inactivates the herbicides phosphinothricin and confers resistance to the commercial herbicides, bialaphos was introduced in azuki bean by *A. tumefaciens*-mediated transformation (Khalafalla *et al*. 2005). The presence of transgenes in transformed azuki bean plants was confirmed by polymerase chain reaction (PCR) and southern blot analysis. Transcription of the *bar* gene was assessed by reverse transcription polymerase chain reaction (RT-PCR) analysis. Transgenic plants exhibited functional expression of the *bar* gene as determined by assaying for resistance to bialaphos applied directly to leaves.

CONCLUSION

It is obvious that azuki bean transformation system through *Agrobacterium* has become a routine procedure in many laboratories. This system represents a significantly important tool for understanding gene functions and provides the initial impetus for the genetic modification of azuki bean. The new tool of biotechnology not only have the potential for increasing the effectiveness and efficiency of azuki bean breeding programs, but will also provide insights into the genetic control of key traits to be used for genetic manipulation. Application of biotechnology will thus contribute greatly to improving yield stability by generating plants with improved resistance to biotic and a biotic stresses rather than raising the overall yield. The coming years will undoubtedly witness an increasing application of biotechnology for the genetic improvement of azuki bean and hence its value and productivity it might be further improved and could be emerged as an important leguminous model plant to provide the framework within which the molecular mechanisms that underlie the grain legume-specific character can be clarified and leguminous crops in general can be improved for the production of transgenic plants. The next challenges for azuki bean improvement via genetic engineering are to prevent large harvest losses by pods shattering and shortening maturity period.

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