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Review

Recent advances in soybean transformation and their application to molecular breeding and genomic analysis

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Herbicide-resistant transgenic soybean plants hold a leading market share in the USA and other countries, but soybean has been regarded as recalcitrant to transformation for many years. The cumulative and, at times, exponential advances in genetic manipulation have made possible further choices for soybean transformation. The most widely and routinely used transformation systems are cotyledonary node–*Agrobacterium*-mediated transformation and somatic embryo–particle-bombardment-mediated transformation. These ready systems enable us to improve seed qualities and agronomic characteristics by transgenic approaches. In addition, with the accumulation of soybean genomic resources, convenient or promising approaches will be requisite for the determination and use of gene function in soybean. In this article, we describe recent advances in and problems of soybean transformation, and survey the current transgenic approaches for applied and basic research in Japan.

Key Words: Soybean [*Glycine max* (L.) Merrill], transformation, *Agrobacterium tumefaciens*, particle bombardment.

Introduction

Soybean [*Glycine max* (L.) Merrill] is an important crop, with food, nutritional, industrial, and pharmaceutical uses. Soybean seeds contain about 40% protein and about 20% oil. They are also abundant in physiologically active metabolites such as isoflavones, lecithins, tocopherols and saponins, in addition to functional proteins and are used as an affordable source of foods that promote and maintain health (Sugano 2005). Soybean production has increased the most among major crops in response to recent increases in demand for vegetable protein, oil and other constituents (Hartman *et al.* 2011). Therefore, soybean improvement is crucial to meeting demand.

The genomic era is now under way for soybean, as for other many crops. Recently, a soybean genomics database has been developed from the whole genome sequence (Schmutz *et al.* 2010), and a large number of genomic, transcriptional, and functional annotated sequences can be retrieved from Phytozome (http://www.phytozome.net/search.php). In addition to efforts to sequence the whole genome, several resources have been developed, including an expressed sequence tag (EST) database, full-length cDNAs and cDNA microarrays (Stacey *et al.* 2004, Umezawa *et al.* 2008). These resources provide a range of opportunities for soybean improvement by marker-assisted breeding and transgenic approaches, and for understanding gene function by map-based cloning and reverse genetic approaches. An efficient and stable transformation system is essential to these goals.

Roundup Ready soybean cultivars are an example of transgenic soybean (Padgette *et al.* 1995), and have been planted on the majority of soybean fields in the world since 2004 (ISAAA, http://www.isaaa.org/). However, soybean remains recalcitrant to routine genetic transformation. The first fertile transgenic soybeans were produced nearly simultaneously by *Agrobacterium tumefaciens* infection with cotyledonary node plant regeneration (Hinchee *et al.* 1988), and by particle bombardment of meristems of immature soybean seeds (McCabe *et al.* 1988). The system was successfully adapted to embryogenic suspension cultures for the regeneration of fertile transgenic soybeans (Finer and McMullen 1991). Since then, these two methods have continued to be improved and have produced most transgenic soybeans to date.

In this review, we describe recent advances in and problems of soybean transformation, with a focus on the methods

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that generate fertile transgenic plants (Table 1). We discuss the convenience and prospects of transgenic approaches for the identification of gene function and the improvement of agronomic characteristics (Table 2), and survey the recent transgenic research in Japan.

Two common platforms for soybean transformation

1. Cotyledonary node–Agrobacterium-mediated transformation

A biological vector, *Agrobacterium tumefaciens*, is used to transfer desirable genes placed in the T-DNA region into a host plant genome (Beijersbergen *et al.* 1992, Horsch *et al.* 1985). The advantages of *Agrobacterium*-meditated transformation include its straightforward methodology, familiarity to researchers, minimal equipment cost and reliable insertion of a single transgene, or a low copy number (Hansen and Wright 1999). *Agrobacterium*-mediated transformation of soybean in co-cultivation has been followed by organogenesis from cotyledonary nodes (Hinchee *et al.* 1988), immature cotyledons (Parrott *et al.* 1989a, 1994), and embryogenic suspension cultures (Trick and Finer 1998). Originally the method relied on a soybean genotype that conferred susceptibility to *A. tumefaciens* infection and on the availability of plant regeneration (Delzer *et al.* 1990, Hinchee *et al.* 1988, Owens and Cress 1985). However, recent advances, as described below, overcome some of these shortcomings (Dinkins and Collins 2008, Olhoft and Somers 2007, Somers *et al.* 2003).

The successful and repeatable production of transgenic soybean has been achieved by using cotyledonary node explants from young seedlings and imbibed mature seeds (Donaldson and Simmonds 2000, Hinchee *et al.* 1988, Olhoft *et al.* 2003, Paz *et al.* 2006, Zhang *et al.* 1999) for *Agrobacterium*-mediated transformation. Cotyledonary node regions contain axillary meristems at the junction between cotyledon and hypocotyl. The axillary meristems proliferate and regenerate through the formation of multiple adventitious shoots on culture medium containing the cytokinin benzylaminopurine. The degree of shoot formation depends on the genotype of an explant, most types of which can form adventitious shoots at the cotyledonary nodes. In general, cotyledonary nodes are pre-wounded mechanically with a scalpel (Olhoft *et al.* 2001) or a small needle (Xue *et al.* 2006), but it requires practiced skill to prepare enough target tissue for bacterial infection (Zhang *et al.* 1999). In contrast, scratching with a stainless steel microbrush enables any technician to wound the tissues easily and uniformly, regardless of skill (Yamada *et al.* 2010).

The addition of reducing agents such as L-cysteine and thiol compounds in the solidified co-cultivation medium significantly increases the efficiency of transformation of cotyledonary node cells (Olhoft *et al.* 2001, Olhoft and Somers 2001) and the production of fertile transgenic plants (Olhoft *et al.* 2003). The reducing agents seem to inhibit wound- and pathogen-induced responses, thereby increasing the capacity for *Agrobacterium*-mediated transformation (Olhoft *et al.* 2001). The combination of the reducing agents, a superbinary vector, and acetosyringone has increased transformation efficiencies and the competency of soybean genotypes for transformation (Dang and Wei 2007, Liu *et al.* 2008, Sato *et al.* 2007). The first transgenic soybeans were produced using the *nptII* gene, which detoxifies kanamycin as a selectable marker (Hinchee *et al.* 1988). Now transgenic cells are selected exclusively by the combination of the *bar* gene and the herbicide phosphinothricin (glufosinate) (Zeng *et al.* 2004, Zhang *et al.* 1999). The concentration of the selection agent greatly affects the transformation frequency (Zeng *et al.* 2004), so the appropriate selection schemes are varied among soybean genotypes.

These improved protocols have been widely applied to several Japanese soybean cultivars, including Kariyutaka, Kinusayaka, Tamahomare, and Suzuyutaka (Sato *et al.* 2007, Sayama *et al.* unpublished data). Kariyutaka, with an early maturity genotype, produces a small number of T_1 seeds about 5 months after co-cultivation with *A. tumefaciens* (Sato *et al.* 2007). Its short life span might be useful in the rapid development of transgenic soybean lines. Transformation frequencies range from 0.2% to around 10% (Olhoft *et al.* 2003, Paz *et al.* 2004, 2006, Zeng *et al.* 2004), indicating that the transformation efficiency still relies on the skill of the practitioner and on the soybean genotype. The frequency of transformation is still low in comparison with somatic embryo–particle-bombardment-mediated transformation.

In the USA, public facilities, including the Plant Transformation Facility at Iowa State University and the Plant Transformation Core Facility at the University of Missouri, provide transgenic plants for public research, mainly by cotyledonary node–*Agrobacterium*-mediated transformation. A similar facility needs to be launched in Japan.

2. Somatic embryo–particle-bombardment-mediated transformation

Particle bombardment, otherwise known as gene gun or biolistic technology, directs small tungsten or gold particles coated with the desired genes toward the target plant cells (Christou *et al.* 1988). Since an electrical-discharge gene gun was first used in soybean (McCabe *et al.* 1988), transformation by particle bombardment has been achieved in immature seed meristem (McCabe *et al.* 1988), somatic embryogenic tissue (Finer and McMullen 1991), and apical meristem (Aragão *et al.* 2000).

Somatic embryos were initially used as a target for *Agrobacterium*-mediated transformation (Parrott *et al.* 1989a), and later found to be amenable to transformation by particle bombardment (Finer and McMullen 1991, Maughan *et al.* 1999, Sato *et al.* 1993). Somatic embryogenesis in soybean was first reported by Christianson *et al.* (1983). Somatic embryos are induced from immature cotyledons cultured on medium containing moderately high concentrations of an auxin such as 2,4-dichlorophenoxyacetic acid (2,4-D), and are used to generate proliferative embryogenic cultures and

Table 1. Summary of representative soybean transformation systems

Transformation method	Explant	Soybean genotype	Strain of *A. tumefaciens*	Selection		References
				Marker	Agent	
Agrobacterium	Cotyledonary explant	Peking, Maple Prest	A208	*npt II*	kanamycin	Hinchee *et al.* (1988)
		A3237	EHA105	*bar*	glufosinate	Zhang *et al.* (1999)
		AC Colibri	EHA105	*npt II*	kanamycin	Donaldson and Simmonds (2000)
		Bert	AGL1	*bar*	phosphinothricin	Olhoft and Somers (2001)
		Bert	LBA4404, EHA105	*hpt*	hygromycin	Olhoft *et al.* (2003)
		Williams 82	EHA101	*bar*	glufosinate	Zeng *et al.* (2004)
		Williams, Williams 79, Peking, Thorne	EHA101	*bar*	glufosinate or bialaphos	Paz *et al.* (2004)
		Thorne, Williams, Williams 79, Williams 82	EHA101	*bar*	glufosinate	Paz *et al.* (2006)
		Jungery	LBA4404	*bar*	phosphinothricin	Xue *et al.* (2006)
		Kariyutaka	EHA105	*bar*	glufosinate	Sato *et al.* (2007), Yamada *et al.* (2010)
		Hefeng 25, Dongnong 42, Heinong 37, Jilin 39, Jiyu 58	EHA105	*hpt*	hygromycin	Liu *et al.* (2008)
	Somatic embryo	Peking, PI 283332	LBA4404, EHA101	*npt II*	G418	Parrott *et al.* (1989a)
		Chapman	EHA105	*hpt*	hygromycin	Trick and Finer (1998)
	Embryonic tip	Hefeng 25, Hefeng 35, Hefeng 39, Heinong 37, Heinong 43, Dongnong 42, Lefeng 39	KYRT1	*bar*	phosphinothricin	Dang and Wei (2007)
Particle bombardment	Embryonic axis	Williams 82, Mandarin Ottawa	–	*npt II*	Undefined	McCabe *et al.* (1988)
		BR-16, Doko RC, BR-91, Conquista	–	*ahas*	imazapyr	Arãgao *et al.* (2000)
	Somatic embryo	Fayette	–	*hpt*	hygromycin	Finer and McMullen (1991)
		Fayette	–	*npt II*	G418	Sato *et al.* (1993)
		Jack and its derivative line	–	*hpt*	hygromycin	Parrott *et al.* (1994), Stewart *et al.* (1996), Maughan *et al.* (1999), Reddy *et al.* (2003), El-Shemy *et al.* (2004), Furutani and Hidaka (2004), Khalafalla *et al.* (2005), Kita *et al.* (2007) etc.

Table 2. Summary of transgenic approaches for improvement of seed components, agronomic traits, and functional genomics in soybean

Target traits	Target gene	Origin of target gene	Target tissue	Promoter	Effect	Transformation method[1)]	Soybean genotype	References
Seed component								
Seed protein	β-casein gene	bovine	seed	soybean lectin	Accumulation of β-casein protein	PB	Jack	Maughan *et al.* (1999)
	15-kDa zein gene	maize	seed	common bean β-phaseolin	Accumulation of zein protein	PB & AG	Jack, F173	Dinkins *et al.* (2001), Reddy *et al.* (2003)
	Gly m Bd 30K gene	soybean	seed	soybean α-subunit of β-conglycinin	Reduction of allergen (Gly m Bd 30K protein)	PB	Jack	Herman *et al.* (2003)
	Modified glycinin gene (*V3-1*)	soybean	seed	soybean glycinin (*gy2*)	Accumulation of V3-1 protein	PB	Jack	El-Shemy *et al.* (2004)
	11-kDa δ-zein gene	maize	seed	soybean α′ subunit of β-conglycinin	Accumulation of zein protein	AG	Williams 82	Kim and Krishnan (2004)
	27-kDa γ-zein gene	maize	seed	soybean α′ subunit of β-conglycinin	Accumulation of zein protein	PB	Jack	Li *et al.* (2005)
	K99 fimbrial subunit gene (*fanC*)	*Escherichia coli*	seed	cauliflower mosaic virus (CaMV) 35S	Accumulation of FanC	AG	Thorne	Piller *et al.* (2005)
	Basic fibroblast growth factor (*bFGF*) gene	human	seed	CaMV 35S or soybean glycinin (*gy1*)	Accumulation of bFGF	AG	Sichuan	Ding *et al.* (2006)
	Modified β-conglycinin α′ subunit gene containing bioactive peptide (Novokinin, LPYPR, Rubiscolin)	modified materials from soybean	seed	soybean α′ subunit of β-conglycinin	Accumulation of bioactive peptides	PB	Jack	Nishizawa *et al.* (2008)
	Modified β-conglycinin α′ subunit gene (*4novokinin-α′*)	modified materials from soybean	seed	soybean α′ subunit of β-conglycinin	Accumulation of bioactive peptides	Whisker	Jack	Yamada *et al.* (2008)
	β-conglycinin α′ subunit gene	soybean	seed	soybean glycinin (*gy2*)	Reduction of β-conglycinin	PB	Jack	Nishizawa *et al.* (2010)
	Human growth hormone gene (*hgh*)	human	seed	soybean α′ subunit of β-conglycinin	Accumulation of mature form of hGH	PB	BR-16	Cunha *et al.* (2011)
Oil	Δ12 fatty acid desaturase gene (*FAD2-1*), Palmitoyl-thioesterase gene (*Fat*B)	soybean	seed	common bean β-phaseolin or soybean β-conglycinin	Increase of oleic acid and decrease of saturated fatty acid	AG	A3237, Thorne	Buhr *et al.* (2002)
	Δ6 desaturase gene	*Arabidopsis thaliana*	seed	soybean β-conglycinin	Production of γ-linolenic acid (GLA) and stearidonic acid (STA)	AG	A3237, Thorne, NE3001	Sato *et al.* (2004)
	Δ5 desaturase gene, Δ6 desaturase gene, GLELO elongase gene, Δ15 desaturase gene	Mortierella alpina 1S-4 (Δ5 and 6 desaturase,GLELO), soybean (Δ15 desaturase)	seed	soybean α′ subunit of β-conglycinin	Production of arachidonic acid	PB	Jack	Chen *et al.* (2006)
	Δ6 desaturase gene, Δ15 desaturase gene (*fad3*)	*B.officinalis* (Δ6 desaturase gene), *A. thaliana* (*fad3*)	seed	soybean β-conglycinin	High accumulation of stearidonic acid (STA)	AG	Thorne, NE3001, 420-5	Eckert *et al.* (2006)
	ω-3 fatty acid desaturase gene (*GmFAD3*)	soybean	seed	soybean glycinin	Reduction of α-linolenic acids (18:3)	AG	Jack	Flores *et al.* (2008)
	Δ6 desaturase gene (*MpDES6*), Δ6 elongase gene (*MpELO1*), Δ5 desaturase gene (*MpDES5*)	*Marchantia polymorpha*	seed	soybean α′ subunit of β-conglycinin	Production of of C20-LCPUFAs (long-chain polyunsaturated fatty acids)	PB	Jack	Kajikawa *et al.* (2008)
	Diacylglycerol acyltransferase 2A gene (*UrDGAT2A*)	*Umbelopsis ramanniana*	seed	soybean α′ subunit of β-conglycinin	Increase of oil content	AG	Undefined	Lardizabal *et al.* (2008)
	Δ12 fatty acid desaturase gene (*GmFAD2-1*)	soybean	seed	soybean lectin	Increase of oleic acid	AG	Heinong44	Wang and Xu (2008)
	Sphingolipid compensation gene (*SLC1*)	*Saccharomyces cerevisiae*	seed	common bean phaseolin	Increase of oil content	PB	Jack	Rao and Hildebrand (2009)
	Fatty acid ϖ-6 desaturase 2 gene (*FAD2*), Acyl-acyl carrier protein thioesterase 2 genes (*FATB-4* and *FATB-5*), Diacylglycerol acyltransferase gene (*DGAT1*), Dihydrodipicolinate synthetase gene (*DHPS*), High-lysine protein gene (*BHL8*), truncated cysteine synthase gene (*CGS*)	soybean (*FAD2*, *FATB4* and *5*, *CGS*), *Yarrowia lipolytica* (*DGAT1*), *Corynebacterium glutamicum* (*DHPS*), barley (*BHL8*),	seed	soybean KTI3 (Kunitz trypsin inhibitor 3)	Improvement of oil content and composition	PB	Undefined	Li Z. *et al.* (2010)
	Epoxygenase gene (*SIEPX*), Diacylglycerol acyltransferase genes (*VgDGAT1* and *VgDGAT2*)	*Stokesia laevis* (*SIEPX*), *Vernonia galamensis* (*VgDGAT1and 2*)	seed	common bean phaseolin	Increase of epoxy fatty acid	PB	Jack	Li R. *et al.* (2010)

Table 2. (continued)

Target traits	Target gene	Origin of target gene	Target tissue	Promoter	Effect	Transformation method[1)]	Soybean genotype	References
Amino acid	Mutated aspartokinase gene (*lysC*-M4), Dihydrodipicolinic acid synthase gene (*dapA*)	*E. coli* (*lysC*-M4), *Corynebacterium* (*dapA*)	seed	common bean β-phaseolin	Increase of free lysine	PB	A2396, A2242, A5403	Falco *et al.* (1995)
	Mutated anthranilate synthase gene (*OASA1D*)	rice	seed	CaMV 35S or soybean *gy2*	Increase of free tryptophan	PB	Jack	Ishimoto *et al.* (2010)
	Mutated anthranilate synthase gene (*OASA1D*)	rice	seed	soybean *gy2*	Increase of free tryptophan	PB	JQ1, JQ7, Jack	Kita *et al.* (2010)
	Mutated aspartate kinase genes (*XbAK_E257K* and *XbAK_T359I*)	*Xenorhabdus bovienii*	seed	soybean 7Sα′ or *Vicia faba* USP99	Increase of threonine	AG	A3525	Qi *et al.* (2011)
Secondary compound	2-methyl-6-phytylbenzoquinol methyltransferase gene (*At-VTE3*), γ-tocopherol methyltransferase gene (*At-VTE4*)	*A. thaliana*	seed	soybean α′ subunit of β-conglycinin	Changes in tocophenol composition	AG	Undefined	Van Eenennaam *et al.* (2003)
	Transcription factor gene *CRC* (*C1/R* chimeric gene), Flavanone 3-hydroxylase gene (*F3H*)	maize (*CRC*), soybean (*F3H*)	seed	common bean β-phaseolin	Increase of isoflavones	PB	Jack	Yu *et al.* (2003)
	Phytase gene	soybean	seed	soybean α′ subunit of β-conglycinin	Reductiion of phytate content	PB	Jack	Chiera *et al.* (2004)
	γ-tocopherol methyl transferase gene	*A. thaliana*	seed	CaMV 35S	Increase of α-tocopherol content	AG	Pungsannamul-kong, Alchankong	Kim *et al.* (2005)
	myo-inositol-1-phosphate synthase gene (*GmMIPS1*)	soybean	seed	CaMV 35S	Reduction of phytate content	PB	Conquista	Nunes *et al.* (2006)
	Multidrug resistance-associated protein (MRP) gene	soybean	seed	soybean KTI3	Reduction of phytate content	PB	Jack	Shi *et al.* (2007)
	γ-tocopherol methyl transferase gene	*Perilla frutescens*	seed	pea vicilin	Increase of α-tocopherol content	PB	Jack	Tavva *et al.* (2007)
	Chalcone synthase gene (*CHS6*), Isofravone synthase gene (*IFS2*), Phenylalanine ammonia-lyase gene (*PAL5*)	soybean (*CHS6*, *IFS2*), bean (*PAL5*)	seed	soybean lectin	Reduction of isoflavone	PB	Jack	Zernova *et al.* (2009)
	Homogentisate geranylgeranyl transferase gene (*OsHGGT*)	rice	germinating seed	rice globulin or CaMV 35S	Accumulation of tocotrienol	AG	Iksannamulkong	Kim *et al.* (2011)
	β-amyrin synthase gene (*GmBAS1*)	soybean	seed	soybean α′ subunit of β-conglycinin	Reduction of seed saponin content	PB	Jack	Takagi *et al.* (2011)
Biotic resistance								
Insect resistance	Insecticidal crystal protein gene (*cry1Ab*)	*Bacillus thuringiensis*	whole plant	CaMV 35S with an alfalfa mosaic virus leader sequence	Resistance to velvetbean caterpillar	PB	F376 (progeny of Peking × Masshokutoukou 502)	Parrott *et al.* (1994)
	Insecticidal crystal protein gene (*cry1Ac*)	*B. thuringiensis*	whole plant	CaMV 35S	Resistance to coan earworm, soybean looper, tabacco budworm, velvetbean catepillar	PB	Jack	Stewart *et al.* (1996)
	Insecticidal crystal protoxin gene (*cry1Ab*)	*B. thuringiensis*	whole plant	*Nicotiana tabacum Prrn* (*np*)+bacteriophage T7 of gene 10L	Resistance to velvetbean caterpiller	PB	Jack	Dufourmantel *et al.* (2005)
	Pinellia ternata agglutinins gene (*pta*), Insecticidal crystal protein gene (*cryIAc*)	*Pinellia ternata* (*pta*), *B. thuringiensis* (*cryIAc*)	whole plant	CaMV 35S	Resistance to cotton bollworm	AG	Hefeng 25, Hefeng 35, Hefeng 39, Heinong 37, Heinong 43, Dongnong 42, Lefeng 39	Dang and Wei (2007)
Nematode resistance	Nematode resistance gene (*Hs1^{pro-1}*)	*Beta procumbens*	root	(*ocs*-UAS)$_3$(*mas*-UAS-*mas*-P)	Resistance to soybean cyst nematode	PB	Westag	McLean *et al.* (2007)
Virus resistance	Coat protein precusor gene (*CP-P*)	Bean pod mottle virus (BPMV)	whole plant	CaMV 35S	Resistance to BPMV	AG	Fayette	Di *et al.* (1996)
	Capsid polyprotein gene (*pCP*)	Bean pod mottle virus (BPMV)	whole plant	Figwort mosaic virus (FMV) 35S	Resistance to BPMV	PB	Jack	Reddy *et al.* (2001)
	Coat protein gene	soybean mosaic virus (SMV)	whole plant	CaMV 35S	Resistance to SMV	AG	9341	Wang *et al.* (2001)
	Coat protein gene	soybean mosaic virus (SMV) attenuated isolate (Aa15-M2)	whole plant	CaMV 35S	Resistance to SMV	PB	Jack	Furutani *et al.* (2006)
	Coat protein gene	soybean dwarf virus (SbDV)	whole plant	CaMV 35S	Resistance to SbDV	PB	Jack	Tougou *et al.* (2006, 2007)

Table 2. (continued)

Target traits	Target gene	Origin of target gene	Target tissue	Promoter	Effect	Transformation method[1)]	Soybean genotype	References
Fungus resisnance	Oxalate oxidase gene (*gf-2.8*)	Wheat	Whole plant	CaMV 35S	Resistance to white mould	AG	AC Colibri	Donaldson *et al.* (2001)
	Oxalate decarboxylase gene (*oxdc*)	*Flammulina* sp.	whole plant	CaMV 35S	Resistance to white mould	PB	BR-16	Cunha *et al.* (2010)
Abiotic tolerance								
Drought stress	L-Δ^1-Pyrroline-5-carboxylate reductase gene (*P5CR*)	*A. thaliana*	whole plant	soybean heat shock gene	Tolerance to heat/drought stress	AG	Ibis	De Ronde *et al.* (2004a, 2004b)
	Molecular chaperone BiP (binding protein) gene (*soyBiPD*)	soybean	whole plant	CaMV 35S	Tolerance to drought stress	PB	Conquista	Valente *et al.* (2009)
Iron deficiency stress	Ferric chelate reductase gene (*FRO2*)	*A. thaliana*	whole plant	CaMV 35S	Alleviation of iron deficiency chlorosis	AG	Thorne, A3237	Vasconcelos *et al.* (2006)
Herbicide resistance								
	Mutated 5-enolpyruvylshikimic acid 3-phosphate (EPSP) synthase gene	petunia	whole plant	CaMV 35S	Glyphosate tolerance	AG	Peking, Maple Prest	Hinchee *et al.* (1988)
	5-enolpyruvylshikimic acid 3-phosphate synthase gene (*CP4 EPSPS*)	*Agrobacterium* sp. Strain CP4	whole plant	CaMV 35S	Glyphosate tolerance	PB	A5403	Padgette *et al.* (1995)
	Acetohydroxyacid synthase gene (*ahas*)	*A. thaliana*	whole plant	*A. thaliana ahas*	Imazapyr tolerance	PB	BR-16, Doko RC, BR-91, Conquista	Arãgao *et al.* (2000)
	4-hydroxyphenylpyruvate dioxygenase gene (*hppd*)	*Pseudomonas fluorescens*	whole plant	*N. tabacum Prrn*(*np*)+bacteriophage T7 of gene 10L	Isoxaflutole tolerance	PB	Jack	Dufourmantel *et al.* (2007)
	Phosphinothricin (PPT) *N*-acetyltransferase genes (*mat* and *hpat*)	bialaphos-resistant soil bacteria *Streptomyces* sp. Strain AB3534 (*hpat*), *Nocardia* sp. strain AB2253 (*mat*)	whole plant	CaMV 35S	PPT tolerance	PB	Jack	Kita *et al.* (2009)
Others								
	Vegetative storage protein gene (*VspA*)	soybean	whole plant	CaMV 35S	Reduction of VSPα and VSPβ	AG	Asgrow 3237	Staswick *et al.* (2001)
	Feedback-insensitive anthranilate synthase (AS) α-subunit gene (*ASA2*)	tabacco	whole plant	CaMV 35S	Increase of free tryptophan	PB	Jack	Inaba *et al.* (2007)
	Ac transposase gene	maize	whole plant	CaMV 35S	Induction of transposition of Ds delineated element	AG	Bert, Thorne	Mathieu *et al.* (2009)
	GmTFL1b (*TERMINAL FLOWER1b*) for *Dt1*	soybean	whole plant	soybean *GmTFL1b* (*Dt1*)	Complementation of the stem growth habit in determinate line	AG	KA	Liu *et al.* (2010)
	DICER-LIKE genes (*DCL4a* and *DCL4b*)	soybean	whole plant	estrogen-inducible expression system (XVE system)	Targeted mutagenesis	*A. rhizogenes*	Bert	Curtin *et al.* (2011)

[1)]AG: *Agrobacterium*, PB: Particle bombardment.

to recover whole plants (Finer and Nagasawa 1988, Lazzeri *et al.* 1985, 1987, Parrott *et al.* 1988, Ranch *et al.* 1985). As the formation of proliferative embryogenic tissue depends on genotype, the use of transformation has been limited to a few soybean cultivars. On the basis of its capacity for induction of primary somatic embryos, proliferative embryogenic cultures, and recovery of whole plants, cultivar Jack has been recognized as a competent genotype for transformation and has been exclusively used to generate transgenic soybeans (Meurer *et al.* 2001, Stewart *et al.* 1996, Tomlin *et al.* 2002), because modification of tissue culture protocols have only partially overcome the effects of genotype (Bailey *et al.* 1993a, 1993b). The limitation has often precluded the functional analysis of transgenes in combination with a specific genotype, and the direct improvement of leading cultivars by transformation. Somatic embryogenesis is a heritable trait and can be improved by hybridization breeding (Parrott *et al.* 1989b); the competence for somatic embryogenesis was successfully transferred and combined in other genotypes (Kita *et al.* 2007, 2010).

Physical procedures for transformation tend to result in the integration of large complexes, fragmentation, and reconstitution of transgenes, which sometimes lead to the silencing of transgenes or homologous endogenous genes (El-Shemy *et al.* 2004, Kinney *et al.* 2001, Reddy *et al.* 2003). The use of a reporter gene such as *sGFP(S65T)* or *DsRed2* in addition to a selectable marker gene could help to reduce the problem of gene silencing associated with physical transformation systems and facilitate the recovery of transgenic plants that stably express the target gene between the two marker genes (El-Shemy *et al.* 2004, Nishizawa *et al.* 2006). As shown in rice transformation (Fu *et al.* 2000), linearized transgene constructs lacking vector backbone sequences might also generate transgenic soybean plants with a low transgene copy number by the simple integration of the constructs.

Soybean somatic embryos have attracted additional attention as a model of zygotic embryos. Proliferative somatic embryos can retain regenerative properties for more than a year, with differentiation and development being readily induced when required (Finer and Nagasawa 1988, Parrott *et al.* 1988). Mature somatic embryos accumulate seed storage proteins with the same temporal and spatial regulation as developing seeds (Dahmer *et al.* 1992, Nishizawa and Ishimoto 2009), and their fatty acid composition is similar to that of seeds (Dahmer *et al.* 1991, Shoemaker and Hammond 1988). Transgenic embryos have usually been obtained within 7 weeks after the introduction of exogenous genes by particle bombardment (Khalafalla *et al.* 2005), and homogeneous masses of transgenic embryos can be readily and repeatedly induced to differentiate. Somatic embryos have therefore been used to assess transgenic seed traits before recovery of whole plants, and then selected clones are recovered as whole transgenic plants (Cahoon *et al.* 2000, 2002, Chen *et al.* 2006, Herman *et al.* 2003, Nishizawa *et al.* 2010).

The improved and refined protocols for somatic embryo–particle-bombardment-mediated transformation are widely reproducible across laboratories, even though there are still some limitations as previously noted (El-Shemy *et al.* 2004, Furutani and Hidaka 2004, Furutani *et al.* 2006, 2007, Ishimoto *et al.* 2010, Khalafalla *et al.* 2005, Kita *et al.* 2009, 2010, Nishizawa *et al.* 2008, Takagi *et al.* 2011, Tougou *et al.* 2006, 2007, Yamada *et al.* 2008). The RIKEN Plant Science Center has supported the Transformation Network Consortium (TRANSNET) to enhance both basic and applied research in plant biology in Japan since 2008. Under a collaborative research agreement, staff at the National Agricultural Research Center for Hokkaido Region will create transgenic soybeans by particle-bombardment-mediated transformation on request from academic researchers in Japan.

Transgenic approaches to improvement of seed components and agronomic traits

1. Modification of seed components

1-1. Protein and amino acid compositions: The abundant proteins and oil in soybean seeds are attractive targets for improvement by transformation. Soybean protein is the nutritional equivalent of meat and eggs except for its deficiency of sulfur amino acids, especially methionine (FAO/WHO 1990, Young 1991). High-methionine proteins such as bovine β-casein and maize zein were induced to accumulate in soybean seed under the regulation of seed-expression promoters (Dinkins *et al.* 2001, Kim and Krishnan 2004, Li *et al.* 2005, Maughan *et al.* 1999), but not enough for nutritional improvement. The accumulation of these methionine-rich proteins may be limited by the absence of the proper maturation process in soybean or by the availability of sulfur-containing amino acids or of sulfur itself. Although there is no information about the increase of free sulfur-containing amino acids in soybean, three other essential amino acids, lysine, tryptophan and threonine, substantially increased in soybean seeds by the expression of genes for feedback-insensitive enzymes involved in their synthesis (Falco *et al.* 1995, Ishimoto *et al.* 2010, Kita *et al.* 2010, Qi *et al.* 2011). Improvement of the pool of soluble amino acids would seem to be a reliable approach to improving the nutritional quality of soybean.

Soybean is also considered one of the most efficient protein bioreactors for plant molecular farming. Pharmaceutical proteins such as human growth hormone, fibroblast growth factor, and an edible vaccine were accumulated in stable transgenic soybean seeds (Cunha *et al.* 2011, Ding *et al.* 2006, Piller *et al.* 2005). Although bioactive proteins comprised up to 3% of the total seed protein content, the content of pharmaceutical proteins is nowhere near the content of endogenous storage proteins. Instead, another strategy was devised to use the major storage proteins, β-conglycinin and glycinin, as carriers for bioactive peptides (Nishizawa *et al.* 2008, Yamada *et al.* 2008). A bioactive hexa-peptide, novokinin, was incorporated into the α′ subunit of β-conglycinin

at four sites by minimum replacement of amino acids constituting analogous sequences, and transgenic soybean seeds accumulated the modified protein with the intended properties (Yamada *et al.* 2008). So far, however, the levels of modified storage proteins have not come close to the amount of the original protein. Mutant lines lacking all subunits of glycinin and β-conglycinin may prove more amenable to the accumulation of modified storage proteins, and of foreign proteins (Kita *et al.* 2007, Takahashi *et al.* 2003), since a decrease in the abundance of the endogenous storage proteins prolamine and globulin in rice was compensated for by the enrichment of foreign proteins, resulting in an almost equivalent total amount of seed storage proteins (Tada *et al.* 2003).

Although soy proteins are highly nutritious, some are recognized as allergens in some people (Ogawa *et al.* 2000). Among them, Gly m Bd 30K, also called P34, is regarded as the major or immunodominant allergen in soybean seed. Transgene-induced gene silencing (co-suppression) could be used to remove allergens from soybean seeds without any compositional, developmental, or structural changes (Herman *et al.* 2003).

1-2. Oil composition: Almost three-fourths of global vegetable oil production comes from oil palm, soybean, rapeseed and sunflower, in that order. Soybean oil is widely used in food and in industry in printing ink, lubricants and biodiesel. Improvement of the oil content and its composition has been a goal in the use of transformation technology. As vegetable oil is stored in seeds in the triacylglycerol form, exotic acyltransferase genes were introduced into soybean to enhance the biosynthesis of triacylglycerol, resulting in a maximum increase of 3.2% (by weight) in seed oil content in mature seeds (Lardizabal *et al.* 2008, Li Z. *et al.* 2010, Rao and Hildebrand 2009).

Oil composition determines the performance of an oil. Transgenic approaches could provide many options to tailor soybean oil for specific uses. Typically, soybean oil is composed of palmitic, stearic, oleic, linoleic and linolenic acids (Yadav 1996). The high level of polyunsaturated fatty acids in natural soybean oil renders the oil unstable and thus susceptible to the development of disagreeable odors and flavors. Therefore, soybean oil with decreased polyunsaturated fatty acids would be ideal for use in food. Down-regulation of the desaturation of fatty acids by ribozyme termination of RNA transcripts or RNA interference (RNAi) gene silencing (see Kasai and Kanazawa 2012) decreased the content of polyunsaturated fatty acids or increased that of oleic acid (Buhr *et al.* 2002, Flores *et al.* 2008, Li R. *et al.* 2010, Wang and Xu 2008). On the other hand, ectopic expression of heterogeneous genes involved in fatty acid modification could generate other fatty acids such as γ-linolenic, stearidonic, arachidonic, eicosapentaenoic and vernolic acids, which are undetectable or minor fatty acids in non-transgenic soybean seeds (Chen *et al.* 2006, Eckert *et al.* 2006, Kajikawa *et al.* 2008, Li R. *et al.* 2010, Sato *et al.* 2004).

The vitamin E family comprises tocopherols and tocotrienols (α, β, γ and δ forms). All isoforms possess lipid antioxidant activity, and α-tocopherol possesses the highest vitamin E activity in mammals (Bramley *et al.* 2000, Herbers 2003). Vitamin E is widely used as an antioxidant in foods and oils, as a nutrient additive in poultry and cattle feeds and as a supplement in the human diet to help prevent diseases. In soybean processing, tocopherols are extracted with the oil. Their content is only about 1.5% of the total oil component, yet they are critical for oxidative stability of the oil (Hoppe and Krennrich 2000). Enhancing the key step in the conversion of γ-tocopherol to α-tocopherol elevated the α-tocopherol content to 95% of the total tocopherol content in transgenic soybean seeds (Kim *et al.* 2005, Tavva *et al.* 2007, Van Eenennaam *et al.* 2003).

1-3. Other compounds: Isoflavones are an important group of compounds that are synthesized in legumes. In addition to their role in the mediation of plant–microbe interactions (Ebel 1986, Rivera-Vargas *et al.* 1993, Subramanian *et al.* 2005, van Rhijn and Vanderleyden 1995), isoflavones are known as phytoestrogens and biologically active substances associated with human health benefits such as anti-cancer effects and decreased risk of coronary heart disease (Setchell 1998). The soybean isoflavones daidzein, genistein, and glycitein are synthesized through the phenylpropanoid pathway, modified by legume-specific enzymes, and stored in the vacuole as glycosidic conjugates (Graham 1991). Activation of the phenylpropanoid pathway by the maize C1 and R transcription factors combined with blockage of the competing pathway by co-suppression of flavanone 3-hydroxylase increased isoflavone accumulation by up to four times that in wild-type seed (Yu *et al.* 2003). In contrast, transgenic soybeans containing three gene cassettes encoding chalcone synthase, isoflavone synthase, and phenylalanine ammonia lyase produced seeds with 70% less isoflavone (Zernova *et al.* 2009). These results indicate that regulation of the expression of genes for phenylpropanoid biosynthesis enzymes and isoflavone-specific enzymes can alter the content and composition of isoflavones.

Saponins are a group of structurally diverse molecules that include glycosylated triterpenic or steroidal compounds, and are widely distributed among plant species. In soybean, a number of triterpenoid saponins have been identified, and have been classified into four groups (A, B, E and DDMP) on the basis of the chemical structure of the aglycone (Kudou *et al.* 1992, Shiraiwa *et al.* 1991a, 1991b). Soybean saponins have various pharmacological effects such as antilipidemic effects (Topping *et al.* 1980) and antiproliferative effects against human colon cancer cells (Ellington *et al.* 2005, 2006). On the other hand, they are considered unwanted components in foods, because they are the main cause of undesirable flavors and of foaming in tofu production. The biosynthesis of saponins in transgenic seeds was almost completely suppressed by RNAi silencing of β-amyrin synthase, a key enzyme in the synthesis of a common aglycone of soybean saponins (Takagi *et al.* 2011).

Soybean seeds contain large quantities of phytic acid

(phytate), which releases phosphorus (P) and myoinositol during seed germination. Monogastric animals lack phytase, the digestive enzyme required to remove phosphate from the inositol in phytate, and therefore P in phytate is not available to them. Fertile transgenic soybean plants containing phytase showed a nearly threefold increase in P availability as well as a reduction of phytate (Chiera *et al.* 2004). Myoinositol-1-phosphate is synthesized from glucose 6-phosphate in a reaction catalyzed by myoinositol-1-phosphate synthase, and then converted into phytate. RNAi gene silencing drastically reduced phytate and inhibited seed development (Nunes *et al.* 2006). Suppressing a multidrug-resistance–associated protein (MRP) ATP-binding cassette (ABC) transporter gene in maize and soybean generated low-phytic-acid seed (Shi *et al.* 2007).

2. Enhancement of biotic and abiotic resistance

2-1. Insect and nematode resistance: Insecticidal crystal proteins (cry proteins or δ-endotoxins) are an active component of *Bacillus thuringiensis* (Bt) toxin, a biological insecticide (Tabashnik 1994). Expression of the Bt *cry* gene in soybean has proven highly effective for controlling insect pests (Dufourmantel *et al.* 2005, Miklos *et al.* 2007, Parrott *et al.* 1994, Stewart *et al.* 1996), and the resistance to lepidopteran pests in a transgenic line expressing Bt *cry1A* was confirmed under field conditions (Walker *et al.* 2000). However, the discovery that insects can adapt to Bt cry proteins raises concerns about long-term or high-dose use (McGaughey and Whalon 1992). Strategies suggested for managing the development of resistance to Bt cry proteins include the combination of the Bt *cry* gene and defoliating insect resistance QTLs or other insecticidal proteins (Macrae *et al.* 2005, Walker *et al.* 2002, Zhu *et al.* 2008).

Soybean cyst nematode (SCN; *Heterodera glycines* Ichinohe) is a primary pest of soybean production. Effective management of SCN relies on the combination of resistant cultivars and crop rotation. Resistance to SCN is controlled by multiple loci, but diverse nematode populations have broken down the elaborate resistance. Therefore, other strategies for SCN resistance are needed. *Hs1*$^{pro\text{-}1}$, a gene from wild beet for resistance to the closely related beet cyst nematode, enhanced SCN resistance in soybean (McLean *et al.* 2007).

2-2. Disease resistance: Soybean mosaic virus (SMV) is endemic in virtually all regions where soybeans are grown in the presence of vector insects. SMV can cause serious yield losses (Ross 1969), so virus resistance is an essential trait for introduction. There have been some efforts to improve virus resistance in soybean by transgenic approaches. Overexpression of a coat protein gene and the 3-UTR region from SMV resulted in high resistance to SMV in transgenic soybean plants (Furutani *et al.* 2006, Wang *et al.* 2001). In addition, resistance to bean pod mottle virus and soybean dwarf virus has been introduced into susceptible soybean by transgenic approaches (Di *et al.* 1996, Reddy *et al.* 2001, Tougou *et al.* 2006, 2007).

Sclerotinia stem rot (white mould) is serious fungal disease of soybean. As oxalic acid is an important pathogenicity factor of the fungus (Godoy *et al.* 1990), the introduction of a gene to degrade oxalic acid would provide an effective defense against the fungus in soybean. Overexpression of heterogeneous genes encoding oxalate oxidase or oxalate decarboxylase reduced disease progression and lesion length after inoculation of leaves and stems with the fungus (Cunha *et al.* 2010, Donaldson *et al.* 2001).

2-3. Abiotic stress tolerance: Drought stress is one of the major environmental limitations on crop production. Transgenic soybean expressing *P5CR*, encoding L-Δ^1-pyrroline-5-carboxylate reductase, which catalyzes the final step in proline biosynthesis, under the control of an inducible heat shock promoter was more tolerant to drought and high temperature than non-transgenic plants (De Ronde *et al.* 2004a, 2004b). Furthermore, overexpression of an endogenous gene encoding ER-resistant molecular chaperon binding protein from soybean (*soyBiPD*) delayed leaf senescence during drought (Valente *et al.* 2009).

Iron is abundant in soil, but its availability is sometimes limited in aerated soil. Ectopic expression of the *Arabidopsis* ferric chelate reductase gene conferred tolerance to iron deficiency chlorosis, but constitutive expression decreased productivity (Vasconcelos *et al.* 2006).

2-4. Herbicide resistance: The most successful transgenic trait introduced into soybean is resistance to the non-selective herbicide glyphosate (*N*-phosphonomethylglycine; Roundup) (Padgette *et al.* 1995). Roundup Ready soybean cultivars were introduced into commercial production in 1996 and have been planted on most soybean fields since 2004 (ISAAA, http://www.isaaa.org/). Glyphosate binds to and blocks the activity of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), an enzyme of the shikimic acid pathway, which produces aromatic amino acids. A glyphosate-tolerant EPSPS was introduced into soybean to confer a high level of glyphosate tolerance (Hinchee *et al.* 1988, Padgette *et al.* 1995). In addition, the introduction of genes for acetohydroxyacid synthase (AHAS) from Arabidopsis, 4-hydroxyphenylpyruvate dioxygenase (HPPD) from *Pseudomonas fluorescens*, and phosphinothricin *N*-acetyltransferase (PAT) from bialaphos-resistant soil bacteria conferred tolerance to, respectively, imazapyr, isoxaflutole and phosphinothricin (Aragão *et al.* 2000, Dufourmantel *et al.* 2007, Kita *et al.* 2009). These herbicide resistance genes are also used as markers to allow the selection of transgenic soybeans (Rech *et al.* 2008).

Transgenic approaches to soybean genomics research

Soybean genes have often been evaluated for their function in heterogeneous plants such as *A. thaliana* or tobacco because soybean has remained recalcitrant to routine transformation. However, they should also be evaluated in the genetic background of a soybean with a null mutant or recessive allele for the target gene. Therefore, the functional

analysis of target genes requires the transformation of a wide range of soybean genotypes. *Agrobacterium*-mediated transformation has now been successfully used in a wide range of soybean genotypes and been simplified (Table 1). This transformation system could provide a sophisticated method of gene functional analysis for soybean genomics research. There is one example of the complementation of an isolated gene by the transgenic approach. The habit of stem growth is an important agronomic trait. A recessive allele, *dt1*, decreases plant height and number of nodes. The *Dt1* gene of soybean was isolated as a *TFL1* orthologue of *A. thaliana* (Liu *et al.* 2010). The genomic region of the *Dt1* allele was introduced into the genetic background of the *dt1* allele by *Agrobacterium*-mediated transformation to complement the *dt1* allele (Liu *et al.* 2010), revealing that the *Dt1* locus exactly controls stem growth habit in soybean.

Agrobacterium tumefaciens is commonly used for DNA delivery. An alternative system using *Agrobacterium rhizogenes* is termed hairy root transformation. This system, which inserts the T-DNA region into the genome of host plant root cells (Chilton *et al.* 1982), has been optimized to the study of symbiotic and pathogenic interactions in roots (Kereszt *et al.* 2007). Hairy root transformation offers the advantage over *A. tumefaciens*-mediated transformation that as every transgenic root represents an independent transformation event, high numbers of transformants can be obtained and analyzed in a relatively short period of time. This system has contributed to elucidating the molecular mechanism of nodulation in soybean root (Indrasumunar *et al.* 2011, Kasai and Kanazawa 2012, Yang *et al.* 2010).

The process of soybean transformation is sometimes integrated into systems of gene-tagging or mutagenesis. Transformation mediated by *A. tumefaciens* or *A. rhizogenes* has been used to develop gene-tagging by transposon elements or site-direct mutagenesis using zinc-finger nucleases (Curtin *et al.* 2011, Mathieu *et al.* 2009). These combination systems are appropriate for soybean genomics research.

Concluding remarks

Transformation procedures have been simplified and optimized for various soybean genotypes. The techniques provide soybean breeders and researchers with opportunities to use transgenic plants for the improvement of agronomic traits as well as the analysis of gene function. Indeed, herbicide-resistant transgenic soybeans have been successfully released and planted in many countries. If a transgenic soybean were developed with agronomically important traits such as high yielding ability and multiple stress resistance which could not be achieved by current genetic resources, transgenic approaches might be more widely accepted in soybean breeding. In addition, transformation is an essential approach for genomics research in many crops, not only soybean. Target genes are readily isolated by map-based cloning or database information through well-organized genomic resources, which provide information on a large number of genomic, transcriptional, and functionally annotated sequences in soybean. Transgenic approaches are likely to become routine for the elucidation of gene function by overexpression, suppression, or complementation testing in the appropriate genetic background.

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