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...
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-mediated 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 cotyledonal 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 cotyledonal node explants from young seedlings and imibed 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. Cotyledonal 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 cotyledonal nodes. In general, cotyledonal 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 super-binary vector, and acetylsyringone 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 T1 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 cotyledonal 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 Marker | Selection Agent | References |
|-----------------------|---------|------------------|-----------------------------|-----------------|----------------|------------|
| **Agrobacterium**     | Cotyledonary explant | Peking, Maple Prest | A208 EHA105                | npt II          | kanamycin      | Hinchee et al. (1988) |
|                       | A2327   |                  | AGL1 LBA4404, EHA105       | npt II          | kanamycin      | Donaldson and Simmonds (2000) |
|                       | AC Colibri |                | EHA105                     | bar             | glufosinate    | Zhang et al. (1999) |
|                       | Bert    |                  | EHA101                     | bar             | phosphinothricin | Ohlhorst and Somers (2001) |
|                       | Williams 82 |              | EHA101                     | bar             | glufosinate    | Olhoft et al. (2003) |
|                       | Williams, Williams 79, Peking, Thorne |         | EHA101                     | bar             | glufosinate or bialaphos | Zhang et al. (2004) |
|                       | Thorne, Williams, Williams 79, Williams 82 |       | EHA101                     | bar             | glufosinate    | Donaldson and Simmonds (2000) |
|                       | Jungery |                  | EHA105                     | bar             | phosphinothricin | Divakaran et al. (2002) |
|                       | Kariyutaka |              | LBA4404                    | npt II          | kanamycin      | Zhang et al. (1999) |
|                       | Hefeng 25, Dongnong 42, Heinong 37, Jilin 39, Jiyu 58 | | EHA105                     | lpt              | hygromycin     | Liu et al. (2006) |
| **Somatic embryo**    | Peking, PI 283332 |           | LBA4404, EHA101             | npt II          | G418           | Parrott et al. (1989a) |
|                       | Chapman |                  | EHA105                     | lpt              | hygromycin     | Trick and Finer (1998) |
| **Embryonic tip**     | Hefeng 25, Hefeng 35, Hefeng 39, Heinong 37, Dongnong 42, Lefeng 39 | | KYRT1                      | lpt              | hygromycin     | Dang and Wei (2007) |
| **Particle bombardment** | Embryonic axis | Williams 82, Mandarin Ottawa, BR-16, Doko RC, BR-91, Conquista | –              | npt II         | Underfined | McCabe et al. (1988) |
|                       | Fayette |                  | –                          | atras           | imazapyr      | Aragao et al. (2000) |
|                       | Jack and its derivative line | | –                          | lpt              | hygromycin    | Finer and McMullen (1991) |
|                       | Fayette |                  | –                          | npt II          | G418          | Sato et al. (1993) |
|                       |          |                  |                            | lpt              | 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) |
| Seed protein | Target gene | Target tissue | Transformation method<sup>2</sup> | Soybean genotype | References |
|--------------|-------------|---------------|-----------------------------------|-----------------|------------|
| 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 | Denkens 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) |
| Gly m Bd 30K gene | soybean | seed | soybean α’-subunit of β-conglycinin | Accumulation of zein protein | AG | Williams 82 | Kim and Krishnan (2004) |
| 11-kDa δ-zein gene | maize | seed | soybean α’-subunit of β-conglycinin | Accumulation of zein protein | PB | Jack | Li et al. (2005) |
| K9 fimbrial subunit gene (famC) | Escherichia coli | seed | cauliflower mosaic virus (CaMV) 35S bFGF gene human seed | Accumulation of bFGF AG | Sichuan | Ding et al. (2006) |
| Basic fibroblast growth factor (bFGF) gene | human | seed | CaMV 35S or soybean glycinin (gy2) | Accumulation of bioactive peptides | AG | Thorne | Pilker et al. (2003) |
| Modified β-conglycinin α’-subunit gene containing bioactive peptide (Novokinin, LPP, PR, Rubiscoin) | modified materials from soybean | seed | soybean α’-subunit of β-conglycinin | Accumulation of bioactive peptides | Whisker | Jack | Yamada et al. (2008) |
| Human growth hormone gene (hgh) | soybean | seed | soybean α’-subunit of β-conglycinin | Accumulation of mature form of hGH PB | BR-16 | Cunha et al. (2011) |
| Δ12 fatty acid desaturase gene (FAD2-1), Palmoyl-thioesterase gene (ForB) | soybean | seed | common bean β-phaseolin or soybean β-conglycinin | Increase of oelic acid and decrease of saturated fatty acid | AG | A3237, Thorne | Bulte 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 | Montiel de a lipina 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) |
| Δ6 desaturase gene (MpdESA), Δ6 elongase gene (MpeEO1), Δ5 desaturase gene (MpDES) | Marchantia polymorpha | seed | soybean α’-subunit of β-conglycinin | Production of CLA-Δ9,12 (long-chain polyunsaturated fatty acids) | PB | Jack | Kajiwara et al. (2008) |
| Δ12 fatty acid desaturase gene (GmFAD2-1), Sphingolipid compensation gene (SCL1) | Umbilopsis rambamiana | seed | soybean α’-subunit of β-conglycinin | Production of oil content AG | Undefined | Lardizabal et al. (2008) |
| Fatty acid Δ6 desaturase gene (FAD2), Acyl-CoA carrier protein in oleic acid 2 genes (FAAT-4 and 5), Diacylglycerol acyltransferase gene (DGAT1), Dihydricapillic acid synthase gene (DACS), High-solome protein gene (BHLAH), truncated cysteine synthase gene (CGS) | soybean | seed | common bean phaselodin | Increase of oelic acid | AG | Heining44 | Wang and Xu (2008) |
| Δ12 fatty acid desaturase gene (GmFAD2-1) | Saccharomyces cerevisiae | seed | common bean phaselodin | Increase of oelic acid | PB | Jack | Rao and Hildebrand (2009) |
| Δ6 fatty acid Δ6 desaturase gene (FAD2), Palmitoyl-thioesterase gene (FatB) | Vigna unguiculata | seed | soybean | Accumulation of bioactive peptides | AG | Undefined | Lardizabal et al. (2008) |
| Δ12 fatty acid desaturase gene (GmFAD2-1), Sphingolipid compensation gene (SCL1) | Umbilopsis rambamiana | seed | soybean α’-subunit of β-conglycinin | Production of oil content AG | Undefined | Lardizabal et al. (2008) |
| Δ6 fatty acid Δ6 desaturase gene (FAD2), Palmitoyl-thioesterase gene (FatB) | Vigna unguiculata | seed | soybean | Increase of oelic acid | AG | Heining44 | Wang and Xu (2008) |
| Δ6 fatty acid Δ6 desaturase gene (FAD2), Palmitoyl-thioesterase gene (FatB) | Vigna unguiculata | seed | soybean | Increase of oil content | PB | Jack | Rao and Hildebrand (2009) |
| Δ12 fatty acid desaturase gene (GmFAD2-1), Sphingolipid compensation gene (SCL1) | Umbilopsis rambamiana | seed | soybean α’-subunit of β-conglycinin | Production of oil content AG | Undefined | Lardizabal et al. (2008) |
| Δ6 fatty acid Δ6 desaturase gene (FAD2), Palmitoyl-thioesterase gene (FatB) | Vigna unguiculata | seed | soybean | Increase of oelic acid | AG | Heining44 | Wang and Xu (2008) |
| Δ6 fatty acid Δ6 desaturase gene (FAD2), Palmitoyl-thioesterase gene (FatB) | Vigna unguiculata | seed | soybean | Increase of oil content | PB | Jack | Rao and Hildebrand (2009) |
| Δ12 fatty acid desaturase gene (GmFAD2-1), Sphingolipid compensation gene (SCL1) | Umbilopsis rambamiana | seed | soybean α’-subunit of β-conglycinin | Production of oil content AG | Undefined | Lardizabal et al. (2008) |
| Target traits | Target gene | Origin of target gene | Target tissue | Promoter | Effect | Transformation method<sup>10</sup> | Soybean genotype | References |
|---------------|-------------|-----------------------|---------------|----------|--------|-------------------------------|-----------------|------------|
| Amino acid    | Mutated aspartokinase gene (lys-M44), Dihydrodipicolinic acid synthase gene (dapA) | *E. coli* (lys-M44), *Corynebacterium* (dapA) | seed | common bean β-phaseolin | Increase of free lysine | PB | A2396, A2242, A5403 | Farko et al. (1995) |
|               | Mutated anthranilate synthase gene (OASA1D) | rice | seed | CaMV 35S or soybean gyc2 | Increase of free tryptophan | PB | Jack | Ishitomo et al. (2010) |
|               | Mutated anthranilate synthase gene (OASA1D) | rice | seed | soybean gyc2 | Increase of free tryptophan | PB | JQ1, JQ7, Jack | Kita et al. (2010) |
|               | Mutated aspartate kinase genes (Xa4K_E257K and Xa4K_F359) | *Xenorhabdus bovienii* | seed | soybean 7Sα or *Vicia faba* US999 | Increase of threonine | AG | A3525 | Qi et al. (2011) |
| Secondary compound | 2-methyl-6-phytylbenzoquinol methyltransferase gene | *A. thaliana* | seed | soybean α′ subunit of β-conglycinin | Changes in tocophenol composition | AG | Undefined | Van Eenennaam et al. (2003) |
|               | Transcription factor gene CRC (C/R chimeric gene), Flavonone 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 | Reduction of phytate content | PB | Jack | Chien et al. (2004) |
|               | γ-tocopherol methyl transferase gene | *A. thaliana* | seed | CaMV 35S | Increase of α-tocopherol content | AG | Pungannamul-keong, Alchankong | Kim et al. (2005) |
|               | γ-tocopherol methyl transferase gene (GmMP51) | soybean | seed | CaMV 35S | Reduction of phytate content | PB | Conquista | Nanes et al. (2006) |
|               | Multidrug resistance-associated protein (MRP) gene | rice | germinating seed | rice globulin or CaMV 35S | Accumulation of tocotrienol | AG | Iksarnammukong | Kim et al. (2011) |
|               | Multidrug resistance-associated protein (MRP) gene | *Perilla frutescens* | seed | pea vicilin | Increase of α-tocopherol content | PB | Jack | Tanva et al. (2007) |
|               | Multidrug resistance-associated protein (MRP) gene | soybean (CBS5, IF5S), bean (PAL5) | seed | soybean lectin | Reduction of isoﬂavone | PB | Jack | Zerna et al. (2009) |
|               | Homogentisate geranylgeranyl transferase gene (GmHGGT) | *A. thaliana* | seed | CaMV 35S | Reduction of phytate content | PB | Jack | Shi et al. (2007) |
|               | β-aminor synthase gene (GamBAS1) | soybean | seed | soybean KTI3 | Reduction of phytate content | PB | Jack | Shi et al. (2007) |
|               | *Bacillus thuringiensis* | *B. thuringienis* | whole plant | CaMV 35S | Resistance to *B. thuringienis* | PB | F376 (pregony of Peking × Masahokudamono 502) | Parrott et al. (1994) |
| Insect resistance | *B. thuringienis* | *B. thuringienis* | whole plant | CaMV 35S | Resistance to velvetbean caterpillar | PB | Jack | Stewart et al. (1996) |
|               | *Nicotiana tabacum* | *N. tabacum* | whole plant | CaMV 35S | Resistance to velvetbean caterpillar | PB | Jack | Dufourmantel et al. (2005) |
|               | *Pinellia ternata* | *P. ternata* (pta), *P. ternata* (cryIAc), *B. thuringienis* (cryIAc) | whole plant | CaMV 35S | Resistance to cotton bollworm | AG | Multiple strains of *P. ternata* | Yang and Wei (2007) |
|               | *Beta procombs* | *Beta procombs* | root | (ocs-UAS)(mosaic-UAS-mos-P) | Resistance to soybean cyst nematode | PB | Westag | McLean et al. (2007) |
| Nematode resistance | *Beta procombs* | *Beta procombs* | root | (ocs-UAS)(mosaic-UAS-mos-P) | Resistance to soybean cyst nematode | PB | Westag | McLean et al. (2007) |
| Virus resistance | Bean pod mottle virus (BMV): *Fusarium oxysporum* | *B. thuringienis* | whole plant | CaMV 35S | Resistance to BMV | AG | Fayette | Di et al. (1996) |
|               | Soybean mosaic virus (SMV): *Beta vulgaris* | *B. thuringienis* | whole plant | CaMV 35S | Resistance to BMV | AG | Fayette | Reddy et al. (2001) |
|               | Soybean mosaic virus (SMV): *Beta vulgaris* | *B. thuringienis* | whole plant | CaMV 35S | Resistance to SMV | PB | 9341 | Wang et al. (2001) |
|               | Soybean mosaic virus (SMV): *Beta vulgaris* | *B. thuringienis* | whole plant | CaMV 35S | Resistance to SMV | PB | Jack | Furusani et al. (2006) |
|               | Soybean dwarf virus (SdDV): *Beta vulgaris* | *B. thuringienis* | whole plant | CaMV 35S | Resistance to SdDV | PB | Jack | Tougu et al. (2006, 2007) |
Table 2. (continued)

| Target traits          | Target gene                        | Origin of target gene | Target tissue | Promoter | Effect                                      | Transformation method | Soybean genotype | References                      |
|------------------------|-----------------------------------|-----------------------|---------------|----------|---------------------------------------------|-----------------------|------------------|---------------------------------|
| Fungus resistance      | Oxalate oxidase gene (gf-2.8)     | Wheat                 | Whole plant   | CaMV 35S | Resistance to white mould                   | AG                    | AC Colibri       | Donaldson et al. (2005)         |
|                        | 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         |                                   |                       |               |          |                                             |                       |                  |                                 |
|                        |                                  |                       |               |          |                                             |                       |                  |                                 |
| Iron deficiency stress |                                   |                       |               |          |                                             |                       |                  |                                 |
| Herbicide resistance   |                                   |                       |               |          |                                             |                       |                  |                                 |
| Mutated 5-enolpyruvylshikimic acid 3-phosphate (EPSP) synthase gene | petunia               | whole plant           | CaMV 35S     | Glyphosate tolerance | AG                    | Poking, Maple Prest | Hindhee et al. (1988)          |
| 5-enolpyruvoylshikimic acid 3-phosphate synthase gene (CP4-EPSP) | Agrobacterium sp. Strain CP4 | whole plant           | CaMV 35S     | Glyphosate tolerance | PB                    | A5403            | Pedgate et al. (1995)           |
| Acetohydroxyacid synthase gene (ahs) | A. thaliana | whole plant           | CaMV 35S     | Glyphosate tolerance | PB                    | BR-16, Doko RC, BR-9, Conquista | Aragao et al. (2000)       |
| 4-hydroxyphenylpyruvate dioxygenase gene (hpd) | Pseudomonas fluorescens | whole plant | N. tabacum | 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 AB2533 (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 VSPa and VSPj                  | AG                    | Augrow 3237      | Starwark et al. (2001)          |
| Feedback-insensitive anthranilate synthase (AS) to-subunit gene (ASL1) | tabacoo               | whole plant           | CaMV 35S     | Increase of free tryptophan                  | PB                    | Jack             | Imbha et al. (2007)            |
| A transposase gene     | maize                             | whole plant           | CaMV 35S     | Induction of transposition of Ds element     | AG                    | Bert, Thome      | Mathieu et al. (2009)          |
| GmTFL1b (TERMINAL FLOWER1b) for Dt1 | soybean                  | whole plant           | CaMV 35S     | Complemention of the stem growth habit       | AG                    | KA                | Liu et al. (2010)              |
| Dicer-like genes (DCL4a and DCL4b) | soybean                  | whole plant           | CaMV 35S     | Targeted mutagenesis                         | A. thaliana          | Bert, Thome      | 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 (Khalaflalla 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 glycycin, as carriers for bioactive peptides (Nishizawa et al. 2008, Yamada et al. 2008). A bioactive hexa-peptide, novo-kinin, 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 glycmin 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 toco-
(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 lepideroptan pests in a transgenic line expressing Bt cryIA 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. Hs1pro-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 P3CR, encoding 1-Δ1-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-phosphonomethyl-glycine; 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 study the 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 transformatants 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 over-expression, suppression, or complementation testing in the appropriate genetic background.

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