Chapter 9
Plant Transformation Methods and Applications

Young Hee Joung, Pil-Son Choi, Suk-Yoon Kwon, and Chee Hark Harn

Abstract Conventional plant breeding uses crossing, mutagenesis, and somatic hybridization for genome modification to improve crop traits by introducing new beneficial alleles from crossable species. However, because of crossing barriers and linkage drag, conventional plant breeding methods are time-consuming and require several generations of breeding and selection. To feed the several billion people living on this planet, the main aim of breeders is to increase agricultural production. Hence, new technologies need to be developed to accelerate breeding through improving genotyping and phenotyping methods. Molecular breeding technologies and their applications are discussed in some of the previous chapters. In this chapter, genetic modification technologies are introduced, including the protocols that have been used for the genetic transformation for ten major crops. Two other genetic modification methods are also introduced: (1) cisgenesis by which only beneficial alleles from crossable species are transferred into a recipient plant to enhance the use of existing gene alleles; and (2) reverse breeding to increase the available genetic diversity in breeding germplasm by blocking chromosome recombination during cell division.

Author contributed equally with all other contributors.

Y.H. Joung
School of Biological Sciences & Technology, Chonnam National University, Gwangju, Republic of Korea
e-mail: yhjoung@jnu.ac.kr

P.-S. Choi
Oriental Pharmaceutical Development, Nambu University, Gwangju, Republic of Korea
e-mail: cps6546@hanmail.net

S.-Y. Kwon
Korea Research Institute of Bioscience and Biotechnology, Daejeon, Republic of Korea
e-mail: sykwon@kribb.re.kr

C.H. Harn (✉)
Nongwoo Bio Co., Yeoju, Republic of Korea
e-mail: chharn@nongwoobio.co.kr

H.-J. Koh et al. (eds.), *Current Technologies in Plant Molecular Breeding*,
DOI 10.1007/978-94-017-9996-6_9

9.1 Principles of Gene Transfer

9.1.1 Transformation Using Agrobacterium

9.1.1.1 Background

Over the last 30 years, DNA transfer has played a dominant role in the production of transgenic plants and in gene function analysis studies. Most transgenic plants were obtained using *Agrobacterium*-mediated approaches. *Agrobacterium tumefaciens* is a plant pathogenic soil bacterium that infects wounded cells and causes a plant disease called crown galls in many dicotyledons and some monocotyledons (Zhu et al. 2003). Chilton et al. (1977) devised a plant transformation method using T-DNA of the *A. tumefaciens* Ti-plasmid that is stably integrated into the genome of plant cells where it modifies the genomic characteristics. Plant transformation methods using *Agrobacterium* are more effective and economical than other methods. Therefore, it is currently the most commonly used method because it provides a simple way of inserting a foreign gene into a plant genome. The recent development of various binary vector systems, selection markers, and advances in plant tissue-culture techniques not only have improved the efficiency of the plant transformation method using *Agrobacterium*, but also have enlarged the range of *Agrobacterium*-applicable hosts. Indeed, the transformation efficiency of plants has been far enhanced by the increasing infectiousness of *Agrobacterium*. To understand the mutual mechanisms between *Agrobacterium* and their plant hosts, it is important to identify the transformation-related regulatory factors involved and to determine their functions. The current understanding of these mechanisms is described in this chapter (Nandakumar et al. 2011; Zhao et al. 2014).

9.1.1.2 T-DNA Insertion by *Agrobacterium*

Attachment of *Agrobacterium* to Plant Cells When plant tissues are wounded, they exude organic acids, amino acids, saccharides, and other small molecules that can invoke chemotaxis in *Agrobacterium* and boost the secretion of acetylated acidic polysaccharides. Subsequently, *Agrobacterium* cells adhere onto the surface of plant cells. The attachment process in which cellulose fibers are synthesized and secreted is regulated by *attR* and *cel* genes in the *Agrobacterium* genome, resulting in solid adhesion of the bacteria to the surface of plant cells. The attachment process of *Agrobacterium* is known to be related indirectly with *chvA, chvB,* and *pscA* genes in the bacterial genome, as well as with arabinogalactan proteins, cellulose synthase-like proteins, and cell wall proteins in host plants (Zhu et al. 2003; Zhao et al. 2014).

Activation of Virulence (*vir*) Genes To recognize plant cells, *Agrobacterium* uses a two-way signaling system, which consists of the VirA protein and the VirG:VirA protein pair that directly perceive phenolic compounds like acetosyringone secreted

from wounded plant cells. These compounds induce autophosphorylation of a VirA domain. The phosphate group of the VirA is then transferred to VirG, which binds to the enhancement elements of *vir* genes in the Ti-plasmid and regulates their transcription (Zhu et al. 2003; Zhao et al. 2014).

T-DNA Processing and T-Strand Formation VirD2 is a nuclear localization signal binding protein that is covalently bound to the 5′ end of the T-strand. VirD2 recognizes the left and right borders of T-DNA sequences, makes a nick between the third and fourth bases of antisense T-DNA border sequences, and forms covalent bonds at the 5′ end of single-stranded DNA to form the T-strand. The VirD1 protein can change the structure of T-DNA, which alleviates the tension and helps to stimulate T-strand formation by VirD2 (Filichkin and Gelvin 1993; Zhao et al. 2014).

Transport of T-strand and Vir Proteins and T-complex Formation VirD2 protein transfers the T-strand into plant cells through a VirB channel, which consists of VirB and VirD4 proteins. The VirB channel is a filamentous pilus, which connects the *Agrobacterium* and host plant cell that functions as a transporter complex through cell membranes. VirE2, which is transferred into the cytoplasm of the infected plant cell, combines with the single stranded T-DNA, to form a T-strand/protein polymer called the T-complex. The T-complex protects the T-strand from the deoxyribonucleases that exist in the plant cytoplasm and is an ideal structure to transport the large T-strand to the nucleus of a plant cell (Zhao et al. 2014).

T-complex Transfer to a Nucleus of a Plant Cell The T-complex is larger than the nuclear pores in the nuclear membrane of plant cells and is transferred to the plant nucleus by active transport. VirE2, which surrounds the T-complex, and plant-derived importin-α proteins, which specifically recognize nuclear localization sequences (NLS) in the VirD2 protein play important roles in the active transport. In *Arabidopsis thaliana*, VirD2 has been shown to conjugate specifically with NLS of AtKAPα, a member of the karyopherin-α family, and is then transferred to the plant nucleus (Zhao et al. 2014). VirE2 is essential for T-DNA transport into the plant cell nucleus. VirE2 does not combine with AtKAPα, but with the plant-derived proteins, VIP1 (VirE2 interacting protein) and VIP2, then its transfer to the nucleus is mediated by karyopherin-α. The over-expression of the *VIP1* gene particularly increases the import of T-DNA to the nucleus, and as a result the transformation efficiency is correspondingly enhanced (Tzfira and Citovsky 2000; Zhao et al. 2014).

Insertion of T-DNA into Plant Genomes VirE2 is not involved in insertion of T-DNA, but it is needed to protect the T-DNA from plant deoxyribonucleases. VirE2 secures the integrity of the T-DNA during its transportation from the cytoplasm to the nucleus, and regulates its integration into the plant chromosome. Before the T-DNA is inserted into the plant chromosome, the VirE2 surrounding the T-strand has to be removed. VirF is a defining factor of host-specificity in *Agrobacterium*. It functions as an F-box protein and shows target protein specificity in the proteolysis-related Skp1p–cullin–F-box protein (SCF) complex. When transferred into plant

Table 9.1 Functions of virulence (*Vir*) genes in T-strand insertion into plant genomes

Gene	Functions
VirA, VirG	Perception of phenolic compounds from plant wounds/induction of virulence (*Vir*) gene expression
VirD2	Endonuclease cutting T-DNA border to initiate T-strand synthesis
VirD1	DNA topoisomerase processing T-DNA
VirD2	Attached to 5′ of T-strand/ formation of T-DNA complex/ transport of T-DNA complex through nuclear pores
VirC	Combined to overdrive region to induce effective T-strand synthesis
VirE2	Single-strand DNA binding protein protecting T-strand from nuclease
VirE1	Plays the role of chaperone to stabilize VirE2 in *Agrobacterium*
VirB, VirD4	Components of T-DNA transference

cells, VirF was found to be involved in the proteolysis of VirE2 and VIP1 in the nucleus. VirD2 is known to be related to the precise insertion of T-DNA into plant genomes. Because T-DNA insertion into plant genomes is an illegitimate recombination, the host DNA repair and recombination-related genes are expected to influence the insertion of the T-DNA. In recent studies, *rat5* (resistance to *Agrobacterium* transformation) mutants were isolated and analyzed, and proteins like histone H2A, which are related to chromosome structure regulation, were found to affect directly the insertion of T-DNA into chromosomes (Mysore et al. 2000; Zhao et al. 2014) (Table 9.1).

9.1.2 Direct Gene Transfer

9.1.2.1 Particle Bombardment

Particle bombardment systems are used to introduce gold powder or tungsten-coated DNA directly into plant genomes by acceleration. This technique was applied to gramineous crops, and large numbers of transformants were developed. Particularly, insect-resistant corns in which a *Bacillus thuringiensis* (Bt) toxin gene was introduced were commercialized and the technique was used on a commercial scale (Gahakwa et al. 2000). Before a foreign gene was introduced into plant cells, the DNA was coated with tungsten or gold, and then particle bombardment was performed. As a direct gene-introduction technique, shooting the foreign gene into target plant tissues at high speed was a very important step. Regardless of the insertion of a foreign gene into a plant genome, this method has wide applicability because it can stabilize transformant production for studies on, for example, transient expression, pathogenicity, and defense mechanisms of viruses. This technique is different from the *Agrobacterium*-mediated method because it is free from biological limitations, is applicable to various plant materials, and can introduce multiple genes simultaneously. Success or failure for this technique depends on the optimization of transformation systems such as their form, preparation, acceleration

of particles, selection of target tissues, and balance between number and size of particles, damage degree of plant tissue, and quantity of DNA transferred into target cells. If the quantity of DNA is low, the efficiency of transformation is also low. However, if the quantity of DNA is too high, the number of introduced genes will be so high that recombination phenomenon is evoked, resulting in gene silencing of the introduced gene. The bombardment procedure is described below.

Micro-carrier Coating Gold or tungsten particles are normally used as micro-carriers. The particles are pre-processed by sterilizing with ethanol and then washing with sterilized water. Plasmid DNA is coated with micro-carrier in 2.5 M $CaCl_2$ and 0.1 M spermidine solution, and cleared with alcohol. Micro-carrier coated with DNA is loaded onto a membrane and the alcohol is evaporated.

Target Tissues of Plant Materials Mature or immature embryos, embryogenic calli, and leaf discs are mostly used as target tissues. The plant tissue is prepared and preprocessed by placing it in the center of media with high osmotic density for about 4 h. (The time is varied according to the plant tissue being used.) High concentrations of maltose or mannitol are mainly used to obtain high osmotic densities and to reduce the damage to plant tissue caused by particle impact.

Conditions of Bombardment Prepared plant tissues are placed in a vacuum chamber (chamber pressure 27 mmHg) and the distance between the micro-carrier and the stopping plate is adjusted to about 13 cm. Gold- or tungsten-coated DNA is dispensed onto a micro-carrier membrane and transferred into a bombardment machine under vacuum. At 1,100 psi, micro-carriers are shot at the plant target tissue.

Selection of Transformed Plants and Regeneration The day after bombardment, the plant target tissues are cultivated on organogenesis or embryogenesis media according to the tissue type. To obtain young plantlets, shoots that survive on selection media are then transferred to root-induction media. Plantlets are passed through acclimation procedures to become mature plants. Leaves or tissues of the mature plants are subjected to molecular analysis to check whether they have been transformed.

9.1.2.2 Polyethylene Glycol (PEG)-Mediated Transformation

Foreign DNA can be introduced to protoplasts (cells that lack plant cell walls) by adding a di-cation (Ca^{2+}) and PEG, which destabilize plant protoplasts and allow the foreign DNA to enter the protoplasts. The foreign DNA then migrates to the plant nucleus and is inserted into the genome. Because the manipulation and cultivation of plant protoplasts is difficult, PEG-mediated transformation is not an efficient transformation method. In addition, the foreign DNA in the target tissue is easily broken down during the transformation process, and recombination events always occur. Because of these problems, this method is used only in some special cases.

9.1.2.3 Electroporation-Mediated Transformation

Electric shocks can be used to introduce foreign genes into plant cells or protoplasts. Plant tissues are cultured in a buffer mixture containing foreign DNA, and electric shocks are applied. As a result, DNA is introduced through pores in the protoplasts and can then be inserted into the plant genome. Protoplasts were usually used in the early studies, but cells, callus tissues, immature embryos, and immature inflorescence have also been used. This technique is mainly applied to gramineous crops such as rice, wheat, and corn, and has a strong point that tissues from recalcitrant crops can be cultured and subjected to electric shocks. Transformation efficiency varies depending on the condition of the plant tissues, field strength, and treatment of the target tissues. Linear DNA rather than supercoiled DNA, and buffer mixture containing spermidine, which induce condensation of DNA, can increase transformation efficiency.

9.1.2.4 Transformation Using Silicon Carbide Fibers

Transformation using silicon carbide fibers is a simple technique that does not need any particular equipment. Plant tissues such as suspended cultured cells, embryos, and embryogenic calli are stirred with DNA in a buffer mixture containing silicon carbide fibers. The silicon carbide fibers penetrate plant cell walls and cell membranes and consequently foreign genes can flow into the cells. Transformation efficiency is affected by the size of fibers, stirring conditions, shape of the container, plant materials, and characteristics of the plant cells (thickness of the cell wall). This technique is simple, inexpensive, and applicable to many kinds of plant tissues. However, transformation efficiency is quite low, and careful operation is required when using this technique because of the riskiness of the silicon carbide fibers. This method was successfully applied to soft corn calli (Frame et al. 2000), but for hard corn calli, the transformation frequency was low and has rarely been used.

9.1.2.5 Transformation by Micro-injection

Glass micro-capillary injection pipettes are used to introduce foreign DNA into the nucleus or cytoplasm of cells. The micro-injection method has been used for animal cells, which have large cell sizes, but has not been applied successfully in plant cells because the cell wall, which consists of polysaccharides like lignin and cellulose, hinders the insertion of glass micro-capillary pipettes into the cells. Foreign genes can be introduced into plant protoplasts by micro-injection; however, preparing and manipulating protoplasts is a difficult task, and is costly and time consuming. Nevertheless, a major characteristic of the micro-injection method is that it can be used to introduce chromosomes into plant cells in addition to plasmid DNA. Potentially, this technique could be used in functional studies of plant cells and physiological research of plastids.

9.2 Transformation Protocols Applied to Crops

9.2.1 Rice (Oryza sativa)

9.2.1.1 Background

The direct and indirect introduction of DNA into protoplasts mediated by particle bombardment (also called gene gun) and *Agrobacterium* has been used for rice transformation. But *Agrobacterium*-mediated transformation is now the favored method because complicated processes for plant regeneration from protoplasts can be skipped, and low copy numbers and the ease of foreign gene fixation reduce gene silencing. However, the genotype-dependent problem has not been resolved, which means that *Agrobacterium*-mediated transformation is confined to a few specific cultivars. A commonly used protocol for rice *Agrobacterium*-mediated transformation is described in Sect. 9.2.1.2 below.

9.2.1.2 *Agrobacterium*-Mediated Transformation

① Plant material: Brown rice without seedcoats are prepared for transformation. The naked seeds are sterilized once in ethanol for 10 min, then in 2–3 % sodium hypochlorite solution for 10 min three times, and washed with sterilized water more than three times.

② Callus induction: 10–20 sterilized seeds are placed on medium containing 20–25 mL of callus induction medium (2N6 + 2,4-D 2-mg/L) and cultured at 27 °C in dark for 3–4 weeks.

③ Callus selection and proliferation: Pale yellow-colored calli are selected from various types of calli after the callus induction stage and preprocessed on 2N6 medium for 3 days to stabilize the calli.

④ Preparation of *Agrobacterium* and transformation: *Agrobacterium*-carrying expression vectors are spread on AB or YEP medium and cultured at 27 °C in dark for 3–5 days. Although various strains of *Agrobacterium* could be used, strains LBA4404 and EHA101 are widely used. Glycerol stocks of *Agrobacterium* carrying preferred plasmids are made in 0.5- or 1.5-mL Eppendorf tubes and stored long-term at −80 °C. For transformation, *Agrobacterium* is spread directly onto an AB medium plate. An *Agrobacterium* colony is picked using a sterilized loop or spatula, inoculated in 15-mL AAM (100 mM acetosyringone supplemented) medium contained in a 50-mL falcon tube, and grown in dark for 2 days. The 2 day-grown *Agrobacterium* suspension is diluted to 1.5–3.0 of the OD_{650} value for final suspension and co-cultivation.

⑤ Co-cultivation of *Agrobacterium*: Pale yellow calli 1–2 mm in diameter are placed in a petri-dish with 20 mL of *Agrobacterium* suspension, and shaken well for 30 min. The suspension is removed, and the infected calli are placed onto sterilized filter paper to remove the rest of the suspension. They are then transferred onto 2N6-AS medium, and co-cultured in dark for 3 days.

⑥ Selection of transformed calli: Three days later, the calli are washed three times with cefotaxime solution (250 mg/L) to remove *Agrobacterium*, and completely dried on sterilized filter paper. The calli are placed on callus selection medium (2N6-CP) and resistant calli are obtained after 2–3 weeks of cultivation at 27 °C in dark. A selection marker gene, *hpt* (hygromycin phosphotransferase) or *bar* (bialaphos resistant) is usually used and the applicable concentration for selection is 50 and 6 mg/L, respectively. Well-grown callus colonies are sub-cultured 2–3 times on selection medium, then carefully selected and proliferated.

⑦ Plant regeneration from selected calli: Surviving calli are selected on MSR-CP medium containing 3-mg/L PPT (when the *bar* gene is the selection marker) at 25 °C in light for 4 weeks. In general, a non-transformed callus will take about 4 weeks to generate plants, but a transformed callus will take 1–2 weeks more. Shoots differentiated from calli are transferred on MS medium (the basal medium for tissue-culture of crops) without any plant hormones to induce roots. When the regenerated plants are acclimated in 1 % HYPONEX solution for 7–10 days, they are transplanted into pots for seeds. The plants are analyzed by PCR, southern blot, and northern blot.

9.2.2 Barley (Hordeum vulgare)

9.2.2.1 Background

Barley is used worldwide for food, beer, and forage. Barley, which contains β-glucan and vitamin B1, has been recognized as a healthy food. However, compared with rice, corn, and other monocotyledonous crop plants, transformation of barley is known to be recalcitrant. Barley plants can be regenerated easily from the embryonic disk of immature embryos. Various transformation techniques such as particle bombardment, PEG-mediated, and electroporation-mediated have been used in barley. *Agrobacterium*-mediated transformation was attempted in this crop (Tingay et al. 1997); however, almost no regeneration was achieved from anthers or immature embryos of Korean barley varieties. Barley transformation using the particle bombardment and *Agrobacterium*-mediated methods are described in Sects. 9.2.2.2 and 9.2.2.3 below.

9.2.2.2 Particle Bombardment Method

① Sterilization: Immature seeds (0.5 mm) from mature plants are submerged in 70 % ethanol for 1 min, surface sterilized with 1.25 % NaOCl solution, washed three times with aseptic water for 10 min, and dried on sterilized filter papers.

② Pre-cultivation: Inside a sterile-air hood, the sterilized immature seeds are peeled off, and the immature embryos are cultured on PL medium containing 2.5-mg/L 2,4-D. At this stage, embryonic disks cut from the immature embryos are

collected on the center of the medium, 40 embryonic disks per plate, and culture at 25 ± 1 °C in dark for 3 days.

③ Preparation of M10 particle bombardment: 66.7-mg tungsten of 0.7 μm in size is prepared in a 1-mL tube using micro-carrier, mixed with 70 % ethanol to sterilize. Then the supernatant is discarded, and the metal powder is washed two times with aseptic water. Finally, the particles are put into a test tube containing 1 mL of 50 % glycerol and used for 2 weeks by storing in a refrigerator.

④ DNA coating: 45-μL M10 prepared in step ③, 10-μL DNA, 50-μl 2.5 M $CaCl_2$, and 20-μL 100 mM spermidine are added into a 1-mL tube orderly, and vortexed 2–3 min followed by centrifugation. The supernatant is poured off, and the pellet is washed gently, first with 140-μL 70 % ethanol and then with 140-μL 100 % ethanol, without any disturbance. Finally, 240 μL of 100 % ethanol is added, and the solution is vortexed, and 5 μL is dispensed for each bombardment.

⑤ Callus induction: The micro-carrier coated with the gene of interest is shot into pre-cultured immature barley embryos by the particle bombardment method (see step ④). One day later, the bombarded immature embryos are transferred onto PL medium and cultured at 25 ± 1 °C in dark for callus induction.

⑥ Selection: 2–3 days after particle bombardment, the transformed immature embryos are transferred to MS medium with 2–2.5-mg/L 2,4-D (selection marker is treated moderately according to the vector used) at 25 ± 1 °C in dark for 3–4 weeks.

⑦ Regeneration and growth of plants: Calli that grew well on the selection medium show resistance and are proliferated and transferred onto MS medium containing 1-mg/L BAP for shoot induction. To grow complete plantlets, the primary shoots are then transferred to 1/2 MS medium and cultivated at 25 ± 1 °C with a 16-h photoperiod. After 3 weeks, the plantlets are moved to pots to mature.

9.2.2.3 *Agrobacterium*-Mediated Transformation

① Preparation of *Agrobacterium* solution: One day before co-cultivation, 200 μL of 2-day-cultured bacterial suspension is added to a 5-mL falcon tube and suspended at 28 °C, 150 rpm for 1 day. When the OD_{600} value reaches 1.4–1.7, the culture is used as the final co-cultivation solution.

② Preparation of immature embryos: Immature embryos (1.5 mm) are isolated from well-grown barley grains, as is done in the particle bombardment method. The seeds are kept as fresh as possible in a sealed tube or petri-dish. The immature embryos are removed from the immature seeds using sharp scalpels, and placed with the embryonic disks face upward.

③ Co-cultivation: 200–500 μL of *Agrobacterium* solution is poured onto the immature embryos in each plate and the plates are incubated for 20–30 min. After incubation, the immature embryos are cultured at 28 °C in dark for 2–3 days.

④ Selection of callus: The immature embryos are cultured or sub-cultured on BCI medium (Wan and Lemaux 1994) containing selection agent for three times at 2-week intervals, until a callus begins to grow.

⑤ Plant regeneration: Prosperously growing calli are transferred to FHG medium (Olsen 1987) containing 0.5-mg/L BAP, 1.0 mg/L IAA, and 50 mg/L hygromycin, cultured at 25 °C in light, and then sub-cultured every 2 weeks for four times to induce shoots.

⑥ Induction of whole plants: Regenerating shoots induced on the selection medium are moved to $^{1}/_{2}$ BCI rooting medium (Shrawat et al. 2007) at 25 °C in light for 2 weeks. Plantlets are transplanted into soil after suitable acclimating to obtain mature plants. Leaf disks are collected from the surviving plants, and checked for insertion and expression of target genes by PCR, southern blot, and northern blot.

9.2.3 Corn (Zea mays)

9.2.3.1 Background

Corn is the most cultivated crop worldwide, greater than rice and wheat, and is an important forage and diet crop. Corn has also been used to produce ethanol as a bioenergy source. Thereby, the development of new corn cultivars using molecular breeding methods is actively in process. In early studies of corn, transformation was done by both *Agrobacterium*-mediated and particle bombardment methods. Later, researchers preferred to use *Agrobacterium*-mediated transformation methods on immature embryos or embryogenic calli because of various advantages. This strategy applied to explants of immature embryos and embryogenic calli of corn are described in Sect. 9.2.3.2 below (Carbone 2013).

9.2.3.2 *Agrobacterium*-Mediated Transformation

① Preparation of immature embryos and embryogenic calli: Hi-II cultivar plants, which have been proved to be the best choice for transformation, are planted in a greenhouse. During the flowering stage, pollen is collected to pollinate the ears at 9–10 am. Nine to 10 days post-pollination, immature embryos of 2 mm in size are used as the material. Freshly isolated immature embryos are inoculated on MS medium containing MS salt, Eriksson vitamins, sucrose, MES, L-proline, myo-inositol, casamino acid, BBL agar, $AgNO_3$, thiamine-HCl, and 2,4-D (pH 5.8) to induce embryogenic calli. The selected embryogenic calli are sub-cultured on the same medium to proliferate type II calli. Immature embryos and embryogenic calli are used for *Agrobacterium*-mediated transformation.

② Inoculation and co-cultivation: Immature embryos or embryogenic calli are inoculated in an *Agrobacterium* suspension (C58CI) for 5 min, then the suspension is removed with pipettes, and the tissues are dried on filter paper. The infected immature embryos or embryogenic embryos are transferred to solid co-cultivation medium and cultured at 24 °C in dark for 2 days.

③ Selection: After co-cultivation, the immature embryos or embryogenic calli are activated on delay medium without selectable agent for 6–7 days. For immature

embryos, the embryo axes are removed before the transfer. The embryonic calli are transferred directly to selection medium. Prosperously growing calli are selected while sub-culturing every 2 weeks for four times.

④ Induction of somatic embryos and regeneration of plants: Prosperously growing calli are transferred from the selection medium to 1st regeneration medium for 7 days in dark to induce somatic embryos. Then, the somatic embryos are moved to 2nd regeneration medium in light to obtain whole plantlets. Acclimated plantlets are transplanted into soil and cultivated in a greenhouse to obtain seeds for the next generation of corn.

9.2.4 Wheat (Triticum aestivum)

9.2.4.1 Background

The study of *Agrobacterium*-mediated transformation in wheat started in 1990, and some kanamycin-resistant transgenic wheat plants were obtained. For transformation, mainly immature embryos known for their high totipotency are used. *Agrobacterium*-mediated transformation as applied to wheat is described in Sect. 9.2.4.2 below (Zhou et al. 1995; Cheng et al. 1997; Zhang et al. 2014).

9.2.4.2 *Agrobacterium*-Mediated Transformation

① Preparation of immature embryos and embryogenic calli: Wheat variety 'Bobwhite' (highly recommended for its high regeneration rate) is sowed in a greenhouse. About 14 days post-pollination, immature grains of wheat are sampled, surface-sterilized with 70 % ethanol for more than three times, and peeled off on a clean bench. The immature embryos are used as materials for *Agrobacterium*-mediated transformation. Embryogenic calli are cultured and selected from the immature embryos on CM4C medium for 10–25 days in the dark. Then, the embryogenic calli are chopped into 2-mm pieces and used for co-cultivation.

② Preparation of *Agrobacterium* suspension: The C58 strain harboring a vector carrying *nptII* selectable marker and *β-glucuronidase* (GUS) reporter gene is applied. The strain is inoculated in LB medium with appropriate antibiotics in shaker, and used for co-cultivation when the OD_{660} reaches 1–2.

③ Co-cultivation: *Agrobacterium* suspension is added to the prepared wheat immature embryos or embryogenic calli at 23–24 °C in dark for 3 h. After inoculation, the suspension is removed with pipettes and the infected tissues are transferred to aseptic filter paper to completely remove the suspension. The inoculated immature embryos or embryogenic calli are transferred onto MS medium with 10-g/L glucose and 200-μM acetosyringone or CM4C medium, and co-cultivate at 24–26 °C in the dark for 2–3 days.

④ Selection: After co-cultivation, the immature embryos or embryogenic calli are cultured by resting on CM4C solid medium containing 250-mg/L carbenicillin for 2–5 days. Then, newly growing calli are selected and induced on CM4C medium containing 25-mg/L G418 and 250-mg/L carbenicillin.
⑤ Transformant induction: Carefully selected embryogenic calli are transferred onto 1st regeneration medium of MMS0.2C containing 1.95-g/L MES, 0.2-mg/L 2,4-D, 100-mg/L ascorbic acid, 40-g/L maltose, 2-g/L gelrite, 25-mg/L G418, and 250-mg/L carbenicillin as well as MS salts and vitamins to induce green shoots. Next, young shoots are moved to the 2nd regeneration medium of MMS0C, which is the same as the 1st regeneration medium except that it does not contain 2,4-D. Plants are moved into soil when they are more than 3 cm in height.
⑥ Transformants check: Young leaves are taken from the healthy plants developed on 2nd regeneration medium and judged for transformants by GUS assay.

9.2.5 Onion (Allium cepa)

9.2.5.1 Background

Onion is the fifth most widely grown vegetable in the world, and its global yield has gradually increased. The recognition of onion as an important health food plus the development of processed foods, many of which contain onion, have increased the consumption of onion. To our knowledge, it may take more than 10 years to breed a new onion variety, longer than any other crop. Therefore, the development of new breeding technologies for onion is in high demand. Several research teams have used biotechnology approaches to try to improve onion varieties. For example, the development of a regeneration system and insect-resistant transformants for onion has been reported by Colin Eady (1995), while a transgenic onion with an insect-resistant gene was successfully developed by Holland (Zheng et al. 2001). However, more research is still needed to achieve stable gene expression, progeny production, and transformant tests. Recently, *Agrobacterium*-mediated transformation has been used in onion as described in Sect. 9.2.5.2 below (Aswath et al. 2007).

9.2.5.2 *Agrobacterium*-Mediated Transformation

① Preparation of onion materials: Onion seeds are submerged in 70 % ethanol for 30 s, surface-sterilized in 2 % sodium hypochlorite solution, washed with aseptic water more than twice, and dried on filter paper. To isolate the mature embryos more easily, the onion seeds are submerged in aseptic water for about 24 h in a refrigerator before sterilization.
② Callus induction: MS basal medium supplemented with 5-μM 2,4-D is used for callus induction from the mature embryos of onion. After cultivating the mature

embryos for 6 weeks, hard and yellowish embryogenic calli are selected, and sub-cultured on the same medium for proliferation. Finely chopped embryogenic calli are used for co-cultivation with *Agrobacterium*.

③ Preparation of *Agrobacterium* suspension: *Agrobacterium* with transformed expression vector is spread on YEP medium and cultured at 27 °C in dark for 3–5 days. Various strains of *A. tumefaciens* like LBA4404 and EHA101 have been used for onion. Even though different selection agents could be applied according to the expression vectors that are used, in this protocol, mannose is used as the selection chemical. To prepare the *Agrobacterium* suspension, an *Agrobacterium* colony is inoculated using sterilized loops in YEP liquid medium supplemented with appropriate antibiotics, and cultured for 2 days in a shaker until the OD_{650} reaches 1.5.

④ Co-cultivation: Embryogenic callus clumps of onion are co-cultivated with the prepared *Agrobacterium* suspension for 30 min. After infection, the cultures are centrifuged briefly, and supernatant is removed completely by pipette and dried on aseptic filter paper. Then, the infected embryogenic calli are cultured on MS basal medium containing 5-μM 2,4-D, 20-g/L sucrose, and 100 μM acetosyringone at 28 °C in dark for 3 days.

⑤ Selection and induction of somatic embryos: After co-cultivation, embryogenic calli are selected on MS medium containing 5-μM 2,4-D, 10-g/L sucrose, 10-g/L mannose, and 300-mg/L cefotaxime, and sub-cultured every 2–3 weeks for proliferation. To induce somatic embryos from well-grown embryogenic calli, the embryogenic calli that show mannose resistance are cultured on the medium with 5-μM kinetin, 1-μM Abscisic acid, and 10-g/L mannose for more than 3 weeks in light.

⑥ Plant regeneration: Soft and green calli that emerge on the surface of the embryogenic calli are transferred onto the next selection medium consisting of MS basal components and 10-g/L mannose but no hormones for further development, and followed by the induction of germination on 1/2 MS medium containing 10-g/L mannose. Acclimated plantlets are moved to soil. Genomic DNA is isolated from the leaves of the surviving putative transgenic plants, and identified by PCR, southern blot, or northern blot.

9.2.6 Soybean (Glycine max)

9.2.6.1 Background

Both *Agrobacterium*-mediated transformation and particle bombardment strategies have been used for soybean. In the early days, mainly particle bombardment was used with somatic embryos, and genetically modified soybeans with herbicide-resistance and insect-resistance were developed using this method. However, the copy number of the inserted genes in these transgenic plants was high and the stability of the inserted genes was disturbed by gene silencing and rearrangement in the

progeny (Vaucheret et al. 1998; Dai et al. 2001). Also, mainly somatic embryos were used, and to obtain somatic embryos, immature seeds are needed; therefore, securing the right material all year-round was difficult. Further, genotypes from which somatic embryos can be isolated are limited.

Recently a cotyledon node-based *Agrobacterium*-mediated transformation method has been reported (Fig. 9.1). It is known that cotyledonary nodes, the portion that includes tissues of the growing point, are not appropriate targets for transformation in other crops. If the growing point is included, effective selection is difficult because of the rapid growing rate, and the frequency of chimera plants generation is very high when differentiated cells are present. However, in soybeans, a portion that includes the growing point is used as a target of the transformation, because the extent of responsiveness of other tissues has been found to be very low, and direct organogenesis scarcely occurred (Zhang et al. 1999). To enhance the efficiency of the *Agrobacterium*-mediated transformation method for soybean, various treatments have been added to the primary method. The most infective treatment is the addition of an antioxidant such as L-cysteine, sodium thiosulfate, and DTT. The effect of antioxidant addition has been elucidated in other plants including grape, sugarcane, and rice. It has been reported that the optimum composition and concentration of antioxidant in soybean suppresses the activity of two oxidases, polyphenol oxidase (PPO) and pyranose oxidase (POD), inducing browning of the inside of plant cells, consequently contributing to the formation of a primary leaf and an increase of transformation efficiency (Olhoft and Somers 2001; Olhoft et al. 2003). Another treatment that has been used to increase the transformation rate in soybean

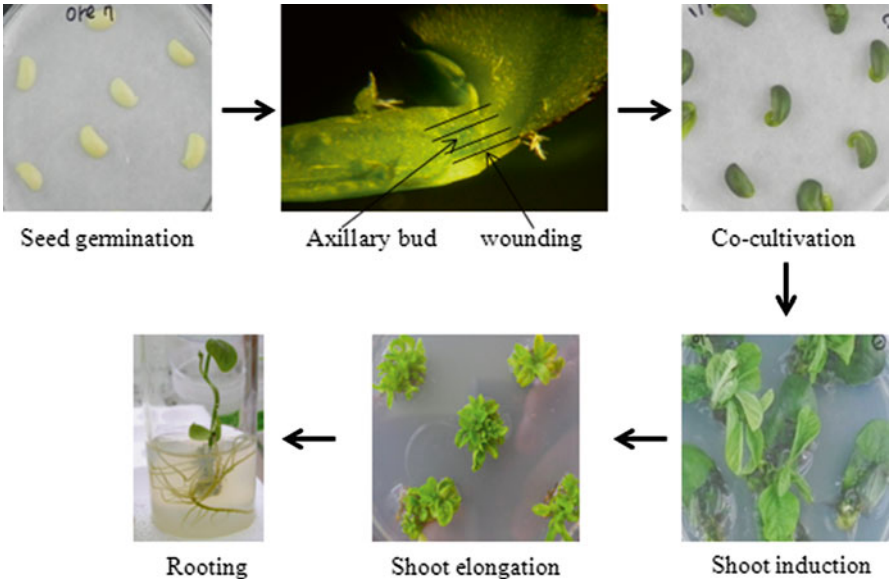

Fig. 9.1 Scheme for genetic transformation of soybean (*Glycine max* (L.) Merrill) cotyledonary nodes

is cutting the explants at an exact point. In soybean transformation experiments that had high efficiency (more than 10 % stable transformation), the explant was cut very precisely at an extremely narrow region between the axillary bud and the hypocotyl (Paz et al. 2006). Hypocotyl-based *Agrobacterium*-mediated transformation has also been developed and this method produced explants more easily than the cotyledonary node-based approach. Specially, it has been reported that the addition of silver nitrate together with the antioxidant enhances the efficiency of the antioxidation effect (Wang and Xu 2008). The cotyledonary node-based *Agrobacterium*-mediated transformation method is described in Sect. 9.2.6.2 below.

9.2.6.2 *Agrobacterium*-Mediated Transformation

Plant Materials Soybean seeds are surface-sterilized in 70 % ethanol for 30 s, then sterilizes in 1 % sodium hypochlorite with shaking for 30 min and washed with sterilized water for 10 min three times. The seeds are sowed on germination medium (Table 9.2) and germinated at 24 °C in an incubation room under 16/8 h of light/dark for 7 days. The size of true leaves similar to that of the cotyledons is usually used for inoculation. The appropriate time for the inoculation step for each variety is determined by observing the germination state after sowing.

***Agrobacterium* Cultivation** *Agrobacterium* is cultured at 25 °C at 250 rpm with shaking until the OD_{600} reaches 1.0. The pellet is harvested and re-suspended with liquid co-culture medium containing an antioxidant like L-cysteine, sodium thiosulfate, or DDT to $OD_{600}=0.5$. (Culture compounds and hormones are added on the day of inoculation.)

***Agrobacterium* Infection and Co-cultivation** Cut 1 cm of the hypocotyl located under the cotyledon of germinated seedlings. Vertically cut the hypocotyl by inserting a scalpel between the two cotyledons, and remove the true leaves. Make wounds with a scalpel between the axillary bud and cotyledonary node 10 times under a microscope. The scalpel is first daubed with a concentrated *Agrobacterium* culture ($OD_{600}=2$). Put about 50 explants into 25-mL co-culture medium (Table 9.2), sonicate for 10 s, vacuum for 30 s, inoculate for 30 min, and then remove the *Agrobacterium* solution on the surface of the explants with sterilized filter papers. Place a sheet of filter paper on co-culture medium, put the adaxial side of the explants face down on the filter paper, and culture at 25 °C for 5 days in dark.

Selection and Regeneration After 5 days of co-cultivation, remove the *Agrobacterium* by washing the explants lightly with 1/2 shoot induction medium (SIM) (Table 9.2). After removing the water, seven explants per plate are set at a right angle with SIM without selectable antibiotics. After 2 weeks, all portions except the shoots are removed. The shoots are then transferred to SIM with selectable antibiotics placing the shoots with the adaxial side down. After 2 weeks, browned shoots/shoot pads are cut out with a scalpel and placed in shoot elongation medium (SEM) (Table 9.2) with selectable antibiotics. Every 2 weeks, the shoots

Table 9.2 Composition of media mentioned in this chapter

GM	CCM	SIM	SEM	RM
B5 or MS salt	B5 or MS salt	B5 or MS salt	B5 or MS salt	B5 or MS salt
B5 vitamins	B5 vitamins	B5 vitamins	B5 vitamins	B5 vitamins
MES 3 mM	MES 20 mM	MES 3 mM	MES 3 mM	MES 3 mM
	BAP 7.5 mM	BAP 7.5 mM	GA 1.5 mM	IBA 5 mM
	GA 0.7 mM	Cefotaxime 500 ppm	Cefotaxime 500 ppm	Cefotaxime 500 ppm
	Acetosyringone 0.2 mM			
	L-cysteine* 3.3 mM			
	Sodium-thiosulfate 1.0 ppm			
	DDT 1.0 mM			
Sucrose 3 %	Sucrose 3 %	Sucrose 3 %	Sucrose 3 %	Sucrose 3 %
Agar 0.8 %	Agar 0.8 %	Agar 0.8 %	Agar 0.8 %	Agar 0.8 %
pH 5.8	pH 5.4	pH 5.6	pH 5.6	pH 5.6

*L-cysteine should be kept in foil because they are sensitive to light
Abbreviates: *GM* germination medium, *CCM* co-culture medium, *SIM* shoot induction medium, *SEM* shoot elongation medium, *RM* rooting medium, *B5* Gamborg' B-5 medium, *MS* Murashige and Skoog medium, *MES* 2-(N-morpholino)ethanesulfonic acid, *BAP* 6-Benzylaminopurine, *GA* Gibberellic acid, *IBA* Indole-3-butyric acid, *DDT* 1,1,1-trichloro-2,2-bis-(4′-chlorophenyl)ethane

are transferred to new SEM with selectable antibiotics and the shoot pad is cut out continuously. After 8–10 weeks of inoculation, the shoots that have elongated to about 4 cm are transferred to rooting medium.

Acclimation Rooting is induced when the shoots are 4–5 cm and have 5–6 leaves (Fig. 9.1). To stimulate root induction, the bases of the shoots are dipped in 1-mg/L IBA solution before transfer to the rooting medium. Transfer shoots with roots to soil and acclimate, afterwards, transfer to pots for seed fructification.

9.2.7 Potato (Solanum tuberosum)

9.2.7.1 Background

Potato is known to be relatively easy to tissue-culture and regenerate, and has been widely used for genetic transformation. *Agrobacterium*-mediated transformation is most commonly used for the potato transformation and the process for gene introduction involves callus induction, genesis of shoots from induced callus, and healthy growth of generated shoots. Callus formation was reported to be stimulated by appropriate concentrations of cytokinin and auxin, shoot genesis was accelerated by removal of auxin, and continual development of shoots was facilitated by addition of gibberellin (Gaspar et al. 1996). Regeneration methods have been categorized as one-step, two-step, or three-step methods. One-step methods use one kind of culture medium from callus formation to shoot development; two-step methods use

different media for callus formation, and for shoot induction and development stage; and three-step methods use different media for each of the three stages. Regeneration efficiency changes mainly according to which method is applied and optimal hormone combinations and concentrations. Even in the same crops, there are differences according to the varieties that are used.

9.2.7.2 *Agrobacterium*-Mediated Transformation

Plant Materials For transformation, *in vitro* cultured plantlets for the cultivar of interest can be used; however, if no *in vitro* cultured plants are available, transformation can be started from a tuber. Because it is difficult to germinate from the true seeds of potato, potato has to be propagated in various ways. Good quality, virus- or pathogen-free potato should be used. Shoots that have sprouted from dormant potato can be cleanly broken off, washed with running water, surface sterilized with 70 % ethanol for 5 min, and washed with distilled water. The potato shoots are submerged in 10 % sodium hypochlorite solution for 10–15 min (15 min is adequate for healthy shoots), then washed with sterilized water more than three times to remove the sodium hypochlorite. From here, all the work should be done on a clean bench. After completely drying the sample, the stems are proliferated on MS medium. Culturing conditions are 16 h of light period (4,000 lux light intensity) and 23 °C. The required numbers of *in vitro* cultured stems can be secured by sub-cultivation over a certain period. For transformation, leaves, stems, and tubers have been used, but leaf disks are most generally used. For stems, the internodes are used but particular attention must be paid to obtaining internodes as far as possible from nodes so as not to include growing points. It is important to note that there is a high chance of obtaining fake plants when leaves are used. The transformation of many different cultivars has been reported, but different conditions from those described here need to be used for red potato, which is known to be difficult to transform (Dale and Hampson 1995).

***Agrobacterium* Cultivation** The virulence of *Agrobacterium* is different for different crops; therefore, it is advisable to choose the strain with the highest efficiency in the crop of interest. The most commonly used strain is LBA4404 because it is known to have high transformation efficiency in potato (Ooms et al. 1987). Antibiotics are selected according to the strain and vector for transformation. *Agrobacterium* is streaked onto YEP solid medium and cultured at 28 °C for 36–48 h. A single colony is inoculated to YEP liquid medium supplemented with antibiotics (addition of acetosyringone is optional) and cultured until $OD_{600} = 0.5–0.6$ for co-cultivation with explants. The OD_{600} should not exceed 1.0.

Pre-treatment of Explants Pre-processing of potato leaves for co-cultivation with *Agrobacterium* can increase the regeneration efficiency. Various medium compositions can be used, but floating the leaves on MS liquid medium with 80-mg/L NH_4NO_3, 14.7-mg/L $CaCl_2$, 5.0-ppm NAA, and 5.0-ppm BAP for a day is the strategy. Floating the potato leaves before placing them on the callus formation medium improved both the callus induction rate and shoot generation regardless of the cultivars.

Co-cultivation When the *Agrobacterium* culture is ready, wounds are made in the *in vitro* cultured leaves for co-cultivation. Excessive wounding like shattering cells when cutting leaves from a stem and large wounded regions should be avoided. Larger wounding regions could produce large callus formations, but exorbitant energy is needed for the wounds to recover, which often makes it difficult to obtain healthy transformants. Enough bacterial suspension is poured to submerge the leaf disks. For cultivars that show high regeneration rates like *S.tuberosum* cv. Desiree, acetosyringone need not be added, but when the regeneration rate is low, acetosyringone could be helpful. In our laboratory, shaking is not applied during the co-cultivation step, but shaking has been applied in other laboratories. After submerging for about 10–15 min, the leaf disks are dried completely on sterilized filter paper. The leaf disks should be treated gently, not with tweezers, to avoid withering of leaf disks.

Regeneration Induction While in most other crops, the regenerated entity is induced directly from a cutting plane, in potato the shoot formation is indirect because a callus is induced from a cutting plane and shoot induction occurs from the callus. So after co-cultivation, the leaf disks are placed first on MS medium containing 2-mg/L 2,4-D for callus formation. To improve the chance for insertion of the target T-DNA gene into the plant genome, antibiotics commonly used as selection pressure are not contained in the medium. After 2 days, the leaf disks are transferred to regeneration medium for shoot induction. Because the regeneration rate differs according to the concentrations of the hormone treatment and the potato varieties, it is better to first determine the optimum regeneration conditions for the variety of interest. It has been reported previously that $AgNO_3$ can increase the regeneration rate. Regeneration medium is generally the MS medium supplemented with 0.01-mg/L NAA, 0.1-mg/L gibberellic acid, and 2.0-mg/L zeatin.

Transformant Selection The precise selection of transformed plants is important and depends on the selectable markers used in the vectors. Until now, the most commonly used vectors contain a kanamycin-resistant gene. For selection of transformants, the regeneration medium is supplemented with 500-mg/L carbenicillin to remove any bacteria that remain on the leaf disks after co-cultivation, and kanamycin for selectable pressure. The optimum concentration of kanamycin that least affects regeneration of the potato shoots and hinders growth of non-transformed shoots is used. Under these conditions, the Desiree variety formed only a few calli with 50-mg/L kanamycin, but transformed shoots could be selected because *S.tuberosum* cv. Sumi, cv. Atlantic, and cv. Norland varieties have less resistance to kanamycin than cv. Desiree, which caused them to stop growing, and also caused withering of the non-transformed cv. Desiree calli or plants. Nevertheless, non-transformed regenerated plants could survive on selection medium by obtaining tolerance to antibiotics during long-term cultivation of over 8 weeks, so an effective way of reducing negative plants is to use 100-mg/L kanamycin when multi shoots are formed by potato calli. The shoot for which the roots are grown completely on medium containing antibiotics is more likely the transformed plant.

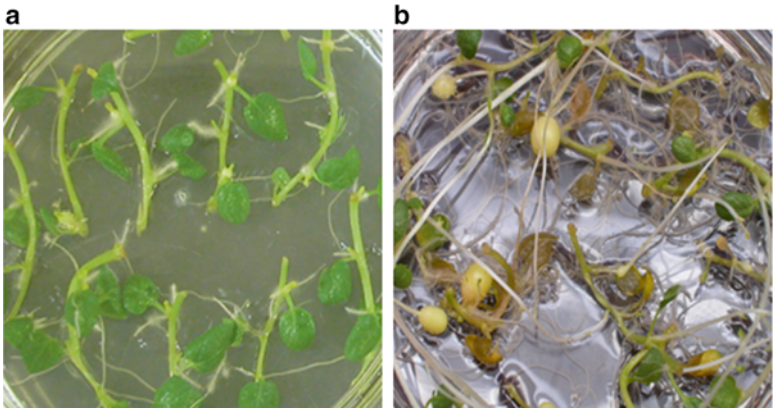

Fig. 9.2 (**a**) Propagation of transgenic potato shoots, (**b**) Tuber induction from the transgenic shoots

Fixation and Maintenance of Transformants Shoots that have grown into independent entities after 10 weeks of cultivation on the medium with antibiotics are proliferated on MS medium with kanamycin and no hormones and analyzed to select confirmed transformed plants (Fig. 9.2). Because vegetatively propagated plants like potato can be maintained by *in vitro* cultivation, it is relatively convenient to fix lines rather than seed propagated plants. However, loss of genes or variations in genes has been reported to occur by continuous cultivation; therefore, consecutive cultivation on selection medium and gene re-analysis should be done periodically.

9.2.8 Tomato (Solanum lycopersicum)

9.2.8.1 Background

Tomato is another important solanaceous crop and the genetics and genomics of tomato have been studied more than other plant crops, so tomato is used widely for cultivar development using transformation techniques and gene functional studies. Tomato transformation is usually done using *Agrobacterium*-mediated transformation method, and young leaf, hypocotyl, or cotyledon have been used as explants. It has been reported that cotyledon produces the highest efficiency. Transformation of tomato using mannose as a selectable marker instead of antibiotic-resistant genes (Sigareva et al. 2004) has been reported. Tomato plastid transformation (Nugent et al. 2005) also has been reported. Micro-Tom is a cherry tomato that has been identified as a model plant in molecular biological, and gene analysis through transformation has been widely studied. Dan et al. (2006) reported mass scale

Micro-Tom transformations that showed an average 56 % transformation rate using kanamycin and glyphosate selection. Transformation efficiency differs according to the genotype, but cotyledon-based transformations have been shown to transform plants with a large variety of genotype (Ellul et al. 2003a; Park et al. 2003).

9.2.8.2 *Agrobacterium*-Mediated Transformation

Plant Materials Seeds are sterilized for transformation generally with 70 % ethanol for 30 s, washed three times with distilled water, submerged in 25 % commercial Clorox and Tween-20, then washed three times with distilled water and germinated on 1/2 MS medium containing 2 % sucrose at 25 °C for about 7 days. When the cotyledon emerges from the seed, it is cut, and cultured on pre-culture medium (1-mg/L BA and 0.1-mg/L NAA) in dark for 1 day.

***Agrobacterium* Cultivation and Co-cultivation** The tomato strains LBA4404, EHA101, and EHA105 have no special differences, but it is easier to remove *Agrobacterium* from strain LBA4404 rather than the EHA strains after co-cultivation. *Agrobacterium* is streaked on YEP solid medium, and a single colony is re-inoculated after cultivation at 28 °C, for 36–48 h. Inoculate to 20 mL of YEP liquid medium containing 50-mg/L rifampicin, 50-mg/L kanamycin, 200-μM acetosyringone, and the suspension is cultured until the OD_{600} reaches 0.7. The cell pellet is harvested and re-suspended to 20-mL of 1/2 MS medium (2.2-g/LMS salt, 2 % sucrose, pH 6.0), and cultured with stirring. The virulence-induced bacterial solution and the cotyledon fragments are then shaken together at 22 °C for 10 min. The cotyledon fragments are placed on sterilized tissues to absorb and remove the bacterial solution. The cotyledon fragments are then placed on co-cultivation medium with the back of the cotyledon facing the medium and cultured in dark for 48 h.

Selection and Shoot Elongation After co-cultivation, the explants are cultured on selection medium, B5 MS salt containing 3 % sucrose, 2.0-mg/L zeatin, 0.2- mg/L IAA, 100-mg/L Km, and 300-mg/L Cb for 1–2 months. Callus formation is observed on the selection medium after about 5 weeks. Calli are isolated from the explants and transferred to shoot elongation medium; in some cases, the explants turned yellow-brown and died. Tomato transformants are usually selected from shoots generated from calli (indirect shoot formation). Shoot genesis can be observed after a week of transferring the calli to shoot medium, B5 MS salt containing 3 % sucrose, 2.0-mg/L zeatin, 0.2-mg/L IAA, 100-mg/L Km, and 300-mg/L Cb, and the shoots begin to grow after 2–3 weeks. When the shoots are about 1–2 cm in height (after about 2–3 months of cultivation), they are transferred to root medium. It is difficult to generate shoots from cherry tomato calli when the calli enters the auxes is stage, finally aging and becoming dark brown. Generally, tomato calli with lots of moisture do not give rise to shoots and consequently become white or dark brown.

Rooting and Acclimation Process The cultured shoots are isolated from calli on rooting medium, 1/2 MS containing 2 % sucrose, 20-mg/L Km, and 300-mg/L Cb

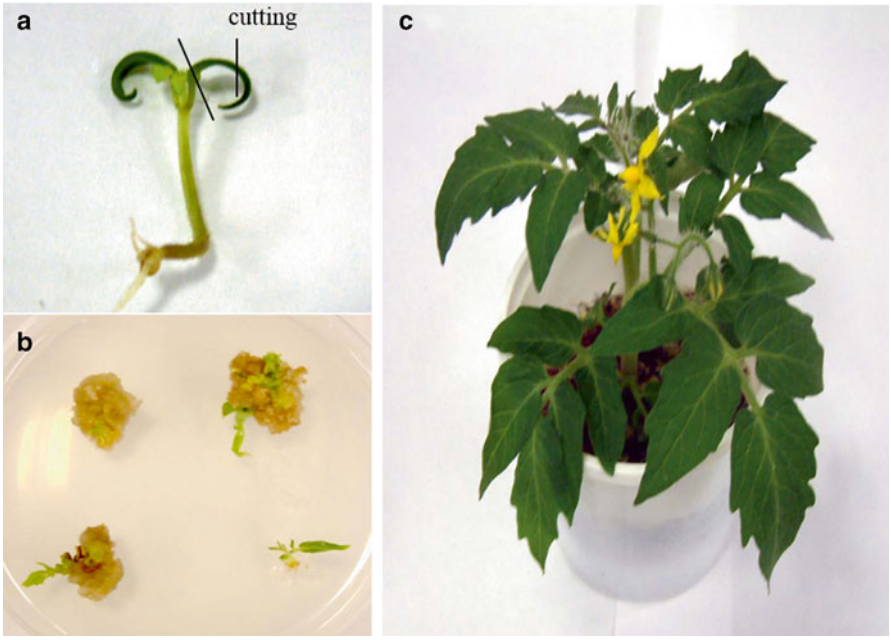

Fig. 9.3 (**a**) Cotyledon of tomato seedlings for experimental materials, (**b**) Shoot induction, (**c**) transformed tomato plant

for about 5–6 weeks. When transferring the shoots, the shoots including some of the callus are cut and placed on root medium. After about 2 weeks on the rooting medium, root induction is observed, and the roots grow enough to acclimate at about 5–6 weeks. When the induced roots reach 5–10 cm in height, the plants can be transferred to Jiffy pots (www.jiffygroup.com) and when the roots extend out of the Jiffypots, the plants are transferred to bigger pots (Fig. 9.3).

9.2.9 Pepper (Capsicum annuum)

9.2.9.1 Background

Pepper is a high value vegetable crop, which ranks seventh in vegetable cultivation area worldwide, and is a commercially important crop that is used as food, dye, and medicine by approximately 5 billion people. Pepper transformation is difficult and lags behind the other major cereal and vegetable crops, and although pepper transformation has been tried in many laboratories, it has usually failed. Integrating the results of the few successful studies indicates that the efficiency of pepper transformation depends on the virulence degree of the *Agrobacterium* strain, the genotypes of the pepper, and selection of the marker in the vector system. Therefore, a methodical and reproducible system for pepper transformation is urgently required.

Generating pepper transformants from explants using the direct shoot formation method is very difficult, so generating shoots from calli is the preferred method. Here, two methods for generating shoots, callus-mediated shoot formation (CMSF) from calli and callus-induced transformation after callus formation based on Lee et al. (2004, 2009) are described below.

9.2.9.2 *Agrobacterium*-Mediated Transformation

Plant Materials Any pepper variety can be used for transformation; however, if the aim is to develop a particular variety for further breeding, the corresponding line should be chosen. Generally, the seeds are sterilized with 95 % ethanol for 30 s, washed three times with distilled water, submerged in 50 % commercial Clorox for about 10 min, then washed three times with distilled water and germinated on 1/2 MS medium at 25 °C in an incubation room. Cotyledons and hypocotyls from 8 to 10 day-old plants are used as explants.

***Agrobacterium* Cultivation** *Agrobacterium* is cultured on YEP liquid medium to which antibiotics and acetosyringone have been added. The *Agrobacterium* is cultured until the OD_{600} reaches 0.3–0.8, and does not exceed $OD_{600} = 1.0$. The cells are harvested and re-suspended in 1/2 MS medium (2.2-g/LMS salt, 1.5 % sucrose, pH 5.7) up to OD_{600} 0.3–0.5. The virulence-induced bacterial solution and cotyledon fragments are shaken together at 22 °C for 10–20 min.

Correlation Between *Agrobacterium* Strains and Virulence The virulence of *Agrobacterium* and the degree of host infection differ in different crops; therefore, depending on the strain used for the transformation, the infection efficiency of *Agrobacterium* can change in different pepper varieties. For example, a strain of P915 line showed the highest regeneration rate, and EHA105, which is commonly used for transformation because of its high virulence, also showed a high regeneration rate.

Callus-Mediated Shoot Formation Method A protocol for pepper transformation using the CMSF method is described in Table 9.3. The most important point when using this method is the selection of transgenic plants, which means that large numbers of directly grown shoots are discarded during the process of regeneration and calli that can give shoots are selected carefully.

Callus-Induced Transformation Method The transformation efficiency of the CMSF method, which transforms explants and induces shoots from calli that are naturally formed on a cutting plane of the explants, depends on the induction rate of the naturally occurring calli. Thus inducing calli artificially and generating shoots from them could enhance the success rate of the whole transformation. During screening, the hormones for callus induction and the concentration of auxin should be chosen so that there is not only mass induction of calli but also that the induced calli have the ability to produce shoots.

Table 9.3 Pepper transformation method using callus-mediated shoot formation

Procedure	Medium and buffer	Duration
Germination	1/2MS (MS* 2.2 g L^{-1}, 1.5 % sucrose, 0.8 % agar, pH 5.7)	8–10 days
Pre-culture	MS (MS 4.4 g L^{-1}, 3.0 % sucrose, 0.8 % agar, pH 5.7)	2–36 h
	2.0-ppm zeatin, 0.05-ppm NAA (or 0.1-ppm IAA)	
Co-culture	MS + 2.0-ppm zeatin, 0.05-ppm NAA	38–96 h in dark
	Washing buffer (1/2MS + 500–800-ppm cefotaxime)	
Selection	MS + 2.0-ppm zeatin, 0.05-ppm NAA + 80–100 mgL^{-1} kanamycin, 300-ppm cefotaxime	Callus formation: 4–5 weeks
		Callus development: 2–3 weeks
Shooting	MS + 2.0-mgL^{-1} zeatin, 0.01-mgL^{-1} NAA + 60–100-mgL^{-1} kanamycin, 300-mgL^{-1} cefotaxime	Shoot formation: 1–2 weeks
		Shoot elongation: 6–8 weeks
Rooting	MS + kanamycin 20–30 mgL^{-1}, cefotaxime 200 mgL^{-1}	Root formation: 4–5 weeks
		10 cm height: 2–3 weeks

**MS* Murashige and Skoog medium
Abbreviates: *NAA* 1-Naphthaleneacetic acid, *IAA* Indole-3-butyric acid

9.2.10 Watermelon (Citrullus lanatus)

9.2.10.1 Background

Watermelon, a horticultural crop that originated in tropical and subtropical areas, is an important crop of the Cucurbitaceae (cucumber) family. Watermelon is known to be difficult to transform. A watermelon transformation system was first reported by Choi et al. (1994), and more recently transformed plants generated from cotyledonary fragments through organogenesis have been reported (Ellul et al. 2003b; Gaba et al. 2004). However, a stable transformation system could not be established from those studies because limited selectable markers were used and success was dependent on a specific laboratory or cultivar. Selectable marker genes that have been used for watermelon are *npt*, and rarely *pmi* (phosphormannose isomerase) gene. The *bar* gene, a herbicide-resistance gene, which is known to be a highly effective selectable marker in a large number of crops including Cucurbitaceae, has not been used as a selectable marker in watermelon. For the development of a stable and highly efficient transformation system for crops, it is important to choose the *Agrobacterium* strain with the highest virulence and one in which the *bar* gene can be used as a selectable marker.

9.2.10.2 *Agrobacterium*-Mediated Transformation

Plant Materials Watermelon seeds (*C.lanatus* cv. Daesan) are surface sterilized with 70 % ethanol for 1 min and then with 0.4 % hypochlorite for 15 min. Ten seeds per petri dish are placed onto MS basal medium containing 2 % sucrose and 0.8 %

agar, and germinated at 25 °C in dark for 5 days. After 5 days, cotyledon fragments are excised carefully so as not to include shoot tips and used as explants for co-cultivation with *Agrobacterium*.

Agrobacterium *Agrobacterium* solution with $OD_{600} = 0.5–0.6$ is centrifuged at 4,000 g for 10 min. The pellet is harvested and re-suspended in co-cultivation solution. The prepared cotyledon fragments are submerged in the re-suspended bacterial solution and shaken at regular intervals. After 10 min, sterilized filter paper is placed on the surface of the co-cultivation medium (1/2 MS, 3 % sucrose, 20-mM ME, 3.2-ppm BAP, 0.5-mg/L IBA, 0.5 % phyto agar, 20-mM MES, 200-μM acetosyringone, and 100-ppm L-cysteine, pH 5.4). Then 10–15 fragments per plate are placed on the filter paper and cultured at 24 °C in light.

Selection of Transformed Plants After 2 days of co-cultivation, the explants are transferred to shoot induction medium containing antibiotics and cultured in light, then sub-cultured every 2 weeks. Next, six explants are arranged on one petri dish with the base of the cotyledon fragments submerged in the medium. After 2 weeks of cultivation, all shoots generated from the cotyledon fragments are sub-cultured in new medium, as before. The shoots are cultured for 8–10 weeks at 2-week-intervals. Green healthy shoots are isolated from the base of the cotyledon fragments, transferred to elongation medium, and sub-cultured every 2 weeks until they grow to more than 3 cm in height. Culture usually takes more than 4 weeks. When the *bar* gene is used as the selectable marker, the composition of the shoot induction medium is MS salt, 3 % sucrose, 3-mM MES, 0.8 % phyto agar, 3.2-ppm BA, 0.5-ppm IBA, 50-ppm vancomycin, 50-ppm ticarcillin, 50-ppm cefotaxime, and 5-ppm glufosinate. The shoot elongation and root induction medium contains MS salt, 2 % sucrose, 3-mM MES, 0.1-ppm IBA, 0.05-ppm gibberellic acid ¸0.8 % agar, 50-ppm vancomycin, 50-ppm ticarcillin, and 50-ppm cefotaxime, pH 5.8.

Acclimation to Soil When the plants grown in the incubator are 10 cm in height, they are transferred to aseptic soil, covered with plastic, and acclimated in an incubation room for about 7 days. For plants that are beginning to take root, holes are made in the plastic and the plants are acclimated for another 2–3 days in an incubation room. When the new leaf emerges, the plants are transferred to a greenhouse and grown for 2 weeks, then transferred to bigger pots.

9.3 Roadmap for Commercializing Genetically Modified Crops

9.3.1 Introduction

The number of hectares in which biotech crops (genetically modified (GM) crops) are grown exceeded 175 million hectares in 2013 and a record 18 million farmers in both large and small developing countries grew biotech crops (James 2014). The

global hectarage of biotech crops has increased more than 100-fold from 1.7 million hectares in 1996 to over 175 million hectares in 2013. This makes biotech crops the fastest adopted crop technology in recent history. This adoption rate speaks for itself in terms of there silience of biotech crops and the benefits they deliver to farmers and consumers. The global value of biotech seed alone was about USD 15.6 billion in 2013. This represents 22 % of the USD 71.5 billion global crop protection market in 2012, and 35 % of the approximately USD 45 billion commercial seed market.

To commercialize a GM crop, many processes must be considered. First, a GM crop that properly expresses a transgene should have commercial merit and marketing value. Second, the GM line should be prepared with other elite inbred lines, so that many different GM F_1 hybrids can be cultivated easily, like non-GM crops. Third, the GM crop should go through human health and environmental risk assessments and pass international biosafety government regulations. Fourth, the GM crop developer must identify the available GM market where farmers are satisfied with the production and expense of cultivating the GM F_1 hybrid. These processes take years and are expensive; therefore, it is important to prepare in advance all plans and steps necessary for the research and development, production, quality assurance, and marketing of a GM crop.

9.3.2 Gene Discovery

There is a limit to what breeders can do to develop a new variety using non-GM breeding methods such as crossing among the same species. GM breeding methods use a selected target gene that can be obtained from another organism, such as a microorganism, insect, another plant, animal, or human to develop new varieties. Finding a target gene that will become a transgene is difficult because the target should be applicable to all crop species and be commercially valuable. In the search for useful genes, generally about 1,000 genes with similar functions are screened to find one commercially useful target gene. Exceptions to this are *Bacillus thuringiensis* and herbicide-resistant genes because, for several decades, farmers have known about their effects and value. Therefore, farmers, scientists, breeders, consumers, and marketing scientists need to interact to identify characters that are necessary for the development of high value-added crops. Once this is decided, it will take at least 2–3 years to find a gene, depending on the targeted character.

9.3.3 Genetic Transformation and Validation

The efficiency of genetic transformation varies depending on the crop and the transformation of some crops has been protracted because of difficulties in *Agrobacterium*-mediated transformation. Therefore, transferring genes into resilient explants

requires effort, and a successful and reproducible transformation method needs to be in place. Usually, it takes about 1 year to produce a T_0 plant once the transformation technique is ready.

It is advisable to work with many T_0 plants at the beginning of selection. Usually, 50–100 T_0 plants are sufficient to self-cross and look for the next T_1 generation. Generally, about 100 plants of each T_1 line are screened for the presence or absence of the transgene and the phenotype of interest. Breeders then select the phenotype with the highest level of the desired characteristics and perform self-crosses and back-crosses. Continuous selection in the self-crossed and back-crossed generations can be performed in a large field or greenhouse to identify the best GM line. A GM line should maintain the target gene and have the proper characteristics and horticultural aspects. After selecting the best T_1 plants, validating the final choice(s) requires at least 2–3 years.

9.3.4 Selection of an Event

During the validation process, molecular evaluation among the selected candidate GM crops is conducted as follows: (1) only one copy of the transgene in the GM crop genome; (2) the transgene has been inserted into an intergenic site; (3) no disturbance in the DNA sequence 1-kb upstream and downstream of the insertion site (Fig. 9.4); and (4) no insertion of apart of the transgene (including promoter, terminator, or any region between the left and right borders) into other site in the genome. If the GM crop fulfills these conditions, it is called an event. An event is a prerequisite to conducting risk assessment research and breeding. A careful evaluation at the molecular level is necessary to verify the event line. At least three events are required for experimental repetition to conduct the risk assessment research.

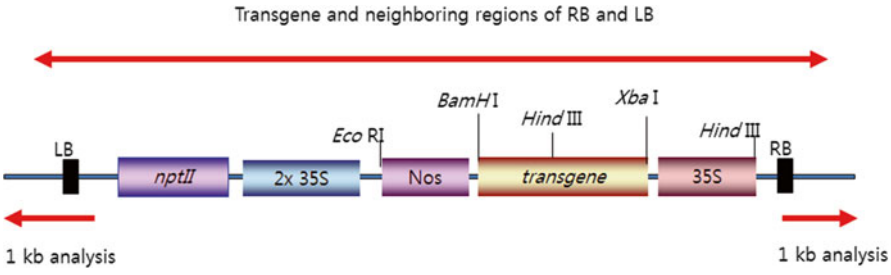

Fig. 9.4 Insertion site of a transgene in the genome of a genetically modified crop. Neighboring regions must be thoroughly investigated because the transgene is inserted randomly. At least 1 kb on both sides of the *left* and *right* borders as well as the internal region of the transgene should be sequenced to identify any DNA sequence disorder. In addition, to be considered an event, no part of the transgene should be repeated in the genome

9.3.5 Developing a GM Line and Establishing Regulatory Data

9.3.5.1 GM Line Development

Transformed crops usually reveal soma-clonal variations that occurred during tissue culture. These variations need to be corrected by self-crossing of several generations. In autogamous crops such as rice, maintaining the T generation by self-crossing and selecting among progeny is a common way to develop a GM line (Fig. 9.5).

However, continuous self-crossing of an allogamous crop causes self-depression and abnormal phenotypes (Fig. 9.6); therefore, continuous backcrossing to the recurrent line is important to stabilize the genome. Two ways of backcrossing have been used. In the first, the selected GM crop is crossed to a non-GM control line (the one that was used for genetic transformation) for the risk assessment. The GM crop should be backcrossed twice, because this should be sufficient to correct the genome for the field test. In the second, a GM breeding line can be constructed by backcrossing several times to the recurrent line of interest and continually self-crossing to stabilize the genome (Fig. 9.7). In this way, the breeder generates a new germplasm with the transgene. It can take up to 5–6 years to develop a GM line depending on the crop.

9.3.5.2 Risk Assessment Study

A human health and environmental risk assessment must be conducted. The criteria for the assessment vary depending on the crop. Table 9.4 lists the items that are necessary to submit to a government agency for a review request. This information

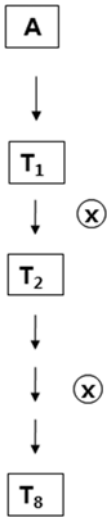

Fig. 9.5 Development of an autogamous crop line. Selection through the T_6–T_8 generations is sufficient to correct a disturbed genome

Fig. 9.6 Appearance of an abnormal phenotype caused by self-depression after continuous self-crossing over four generations. The GM (T_4) line did not develop properly because the shoot was weak

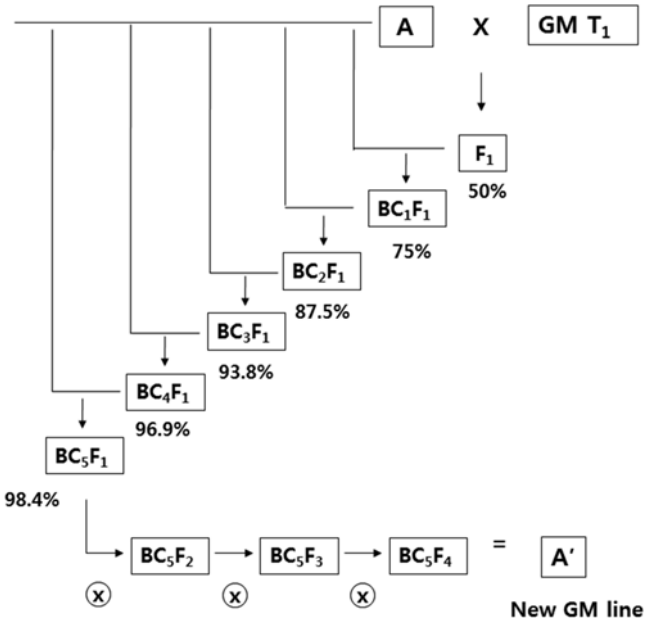

Fig. 9.7 Construction of a GM breeding line by backcrossing and self-crossing to stabilize the genome. The GM T_1 line can be crossed to an elite recurrent line by backcrossing (BC_5) and, consequently, self-crossing (F_4) to obtain a new genetic source (BC_5F_4)

is specific to Korea but the items are similar to what is generally required universally. All these items must be thoroughly worked through in the field and laboratory. In Korea, for example, it takes about 270 days to determine if a GM crop has passed the review. If the GM crop passes the review, the developer can cultivate the GM crop on land; however, the permission to cultivate is restricted to domestic land. If the developer wants to cultivate the GM crop in a foreign country, the GM crop has to go through an inspection review in that country. All countries have their own risk assessment regulations; thus, usually the developer needs to conduct assessment research in each country to obtain permission to cultivate and market a GM crop in that country.

Table 9.4 Items to be submitted to government agencies for risk assessment of GM crop cultivation

1. General data
1.1. Purpose of development
1.2. Benefits of development and uses
2. Data on host organism(s)
2.1. Taxonomic characteristics (e.g., scientific name, common name, and variety/line).
2.2. Distribution in nature
2.3. History of use (including use with the country and other countries, history of cultivation and development through breeding, etc.)
2.4. Biological characteristics (viability and reproduction/propagation capabilities in the natural environment or under test conditions simulating the natural environment)
2.4.1. Mode/cycle of reproduction/propagation and likelihood of cross-hybridization
2.4.2. Interactions with other species in the natural ecosystem and oceanic ecosystem (e.g., natural enemy, pathogenic organisms, competitors, and predators)
2.4.3. Mode of reproduction and reproductive compatibility with other species or relatives
2.4.4. Dissemination (dissemination characteristics of pollen/seed, environmental factors affecting dissemination)
2.5. Capability of producing hazardous materials (including the capability of relatives)
2.6. Reports on toxigenicity and allergenicity of the host and relatives
2.7. Contamination by pathogenic and foreign agents (e.g., viruses)
2.8. Centers of origin and genetic diversity
2.9. Other important physiological characteristics including parasitism
2.10. Research data on pathogenicity or relationship to known pathogens in the case of microorganisms
2.11. Potential to become a weed (become wild)
3. Data on donor organism(s)
3.1. Taxonomic status (e.g., scientific name, common name, and variety/line)
3.2. Distribution status in natural ecosystem and oceanic ecosystem
3.3. History of use by humans
3.4. Biological characteristics
3.5. Toxigenicity
3.6. Data on reported toxigenicity and allergenicity of the donor and relatives
3.7. Research data on pathogenicity or relationship to known pathogens in the case of microorganisms
4. Data on the vector
4.1. Name and source (e.g., GenBank accession number)
4.2. Molecular weight of DNA
4.3. Vector structure
4.4. Restriction map (including genetic elements in the vector, loci, orientation, and sequences)
4.5. Presence of hazardous sequences
4.6 Potential of vector being transferred to other cells or dependency on the host
4.7. Identification and functions of genetic elements in the intermediate host
4.8. Data on intermediate hosts (toxigenicity and allergenicity)

(continued)

Table 9.4 (continued)

5. Data on introduced gene(s)
5.1. Functions (e.g., GenBank accession numbers) and characteristics of introduced gene(s)
5.2. Sources and sequences of components in an introduced gene
5.2.1. Size, name and functions of the introduced gene
5.2.2. Regulators (promoters and terminators)
5.2.3. Selectable marker genes
5.2.4. Other promoters and other factors affecting the DNA functions
5.3. Modifications to the gene for use
5.4. Presence of hazardous sequences
5.5. Sites and orientation of gene sequences in the finalized vector
5.6. Presence of exogenous open reading frames and potential of transcription and expression thereof
6. Data on LMO (living modified organism; i.e., a living GMO) development
6.1. Genetic modification methods (transgenic methods)
6.2. Description on the development process for LMO (e.g., cultivation and breeding)
6.3. Stability of phenotypes of an inserted gene through multiple generations
7. Molecular characterization of LMO
7.1. Data for inserted gene in LMO
7.1.1. Identification results and composition for an inserted gene in LMO
7.1.2. Insertion site of a gene (chromosome or cellular organelle) and adjacent sequences
7.1.3. Number of copies of an inserted gene
7.1.4. Methods to detect and to verify the expression of an inserted gene
7.1.5. Data on stability of an inserted gene
7.1.5.1. Changes in the sequences and size of an introduced gene through multiple generations
7.1.5.2. Changes in expression sites, timing, and level of an introduced gene through multiple generations
7.1.6. Data proving that an inserted gene in the LMO genome does not encode toxins and allergens
7.2. Data on genetic product
7.2.1. Traits/characteristics of genetic product (e.g., proteins, non-coding RNA)
7.2.2. Post-translational modification of an inserted gene (variation after protein formation)
7.2.3. Expression level, timing and site of a marker protein due to an inserted gene or the insertion, and measurement methods and sensitivity thereof
7.2.4. Effects of genetic product on metabolic pathways
7.2.5. Structure and functions of the selectable marker gene and the mechanism of resistance and metabolite(s) thereof
7.2.6. LMO detection and identification methods
8. Comparison between LMO and non-LMO
8.1. Enhanced characteristics and traits after modification
8.2. Difference in viability and propagation between the host and the LMO
8.3. Differences from the host or the species of the host
8.3.1. Viability and reproduction/ propagation capabilities in the natural environment or under test conditions simulating the natural environment
8.3.2. Mode/cycle of reproduction/propagation and likelihood of cross-hybridization

(continued)

Table 9.4 (continued)

8.3.3. Interactions with other species in the natural ecosystem and oceanic ecosystem (e.g., natural enemy, pathogenic organisms, competitors, and predators)
8.3.4. Mode of reproduction and reproductive compatibility with other species or relatives
8.3.5. Dissemination (dissemination characteristics of pollen/seed, environmental factors affecting dissemination)
8.3.6. Production of hazardous materials and residual effects on the ecosystem
9. Detailed data on adverse effects
9.1. Generation of toxic materials (e.g., presence of toxic materials secreted by an organism)
9.2. Potential effects on organisms in the vicinity and the ecosystem
9.3. Potential to become a weed
9.4. Information on the environment to which the LMO is to be introduced
9.4.1. Distance from the production origin of the LMO
9.4.2. Geographic and climatic characteristics and ecological characteristics of flora in the vicinity
9.5. Data for toxicity of the LMO
9.5.1. Potential toxicity of gene product or metabolite of the LMO
9.5.2. Structural similarity between the gene product or metabolite of the LMO and protein toxin.
9.5.3. Average exposure of the LMO through uptake or eating
9.5.4. Biological similarity evaluation between the expressed protein from the LMO and the protein from non-LMO
9.5.5. Single dose toxicity test
9.5.6. Other toxicity test data if necessary
9.6. Data for allergenicity of the LMO
9.6.1. Potential allergenicity of the gene product
9.6.2. Structural similarity between the gene product or metabolite of the LMO and the allergen
9.7. Cross-response evaluation of the patient-specific IgE antibody when the structure of the gene product is similar to the one of allergen.
10. Data on environmental release, monitoring, and disposal of LMO
10.1. In the case of LMO for use as food/feed or for processing (data from production country)
10.1.1. Data on environmental release (duration and time)
10.1.2. Location of release (geographical and geological aspect, relation with biologically important area such as the preservation of the natural environment)
10.1.3. Ecosystem of release (distribution and kinds of species such as allied species)
10.1.4. Method and amount of release
10.1.5. Results and data of release
10.1.6. Information of the release permission (nation, date and number of permission)
10.1.7. Monitoring plan (methods, time, frequency)
10.1.8. LMO inactivation methods
10.1.9. Emergency plan (containment)
10.1.10. Treatment method of LMO waste
10.2. In the case of LMO for cultivation (information of domestic field and containment)
10.2.1. Time
10.2.2. Method

(continued)

Table 9.4 (continued)

10.2.3. Magnitude
10.2.4. Treatment method at the end of test
10.2.5. Results and data
10.2.6. Monitoring plan (method, time, frequency)
10.2.7. LMO inactivation methods
10.2.8. Management plan of emergency status (containment)
10.2.9. Treatment method of LMO waste
11. Approvals and uses in other countries
11.1. Name of country
11.2. Name of organization (agency, institution, firm)
11.3. Organization of risk assessment evaluation
11.4. Permission number
11.5. Permission data
11.6. Status of use
12. Submission of LMO
12.1. Inactivated LMO seed (50 seed) and LMO seed 1 kg
12.2. Inactivated vegetative mass (50 individual)
12.3. Micro-organism case, small volume of inactivated LMO culture
12.4. Information of DNA sequence of the transgene for detecting the LMO
12.5. Information of DNA sequence of the LMO genome data, 5′ and 3′ region of transgene, insertion region including 1 kb forward and backward.
12.6. Other genome information for development of the analysis method.

This list is applicable worldwide

9.3.6 Pre-farming and Performance Testing

To evaluate the performance of a GM crop, breeders usually conduct field tests by crossing the GM line to other lines to evaluate the various F_1 combinations. A performance evaluation is conducted to select commercially valuable F_1 combinations (Fig. 9.8). The selected F_1 hybrid is tested further in farms indifferent regions, and the observations and preferences of the farmers are considered when selecting the final combination. Testing of a hybrid combination may take 2 years or more depending on the variety.

9.3.7 Launching for Commercialization

Once the breeder receives permission from the government agency following the GM line or crop risk assessment and the F_1 hybrid for commercialization has been selected, the breeder determines where the seeds will be produced and cultivated. GM F_1 hybrid crops must pass the regulatory requirements of the country where the breeder aims to sell the F_1 hybrid and a pipeline is constructed to develop a

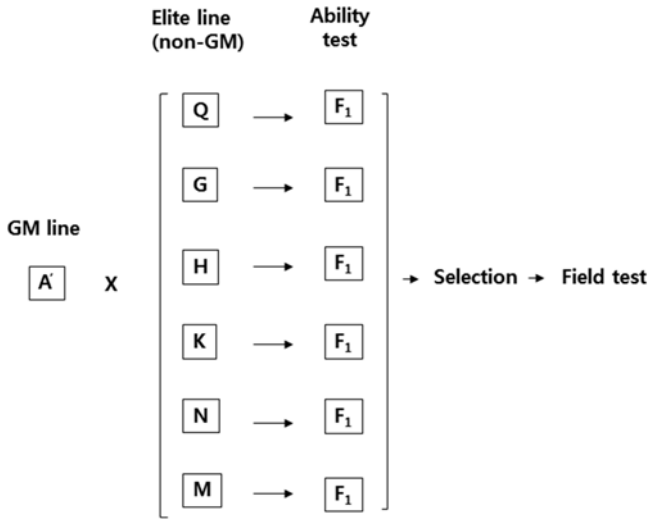

Fig. 9.8 Evaluating the performance of GM crops to select a commercially valuable F_1 hybrid combination. It is common to use a non-GM line as the female MS (male sterile) and the GM line as the male

commercial GM crop. It can take 13–15 years to commercialize a GM crop from the beginning (Fig. 9.9) but if the target gene is available and the GM breeding infrastructure is already in place, it may take 10 years or less. It takes a further 5–6 years to prepare the risk assessment data for exporting the GM crop, depending on the country, and to simultaneously conduct breeding trials in a local market. Therefore, establishing a pipeline to develop commercial GM seeds including a review request and risk assessment data is very important and should be prepared well for time and cost efficiency.

9.4 Development of New Genetic Sources Using GM Technology

9.4.1 Cisgenesis

9.4.1.1 Introduction

To transfer an elite gene or characteristic from a wild-type or open-pollinated crop (donor) to an ordinary line using non-GM breeding processes, the procedure is as follows: (1) male and female plants are crossed to produce the F_1; (2) the F_1 is back-crossed to the recurrent line (i.e., the ordinary line) generally for 5–6 generations (BC_5 or BC_6); and (3) BC_5 or BC_6 are self-crossed for three generations to obtain BC_5F_3 or BC_6F_3. In this way, the gene of interest can be transferred from the donor

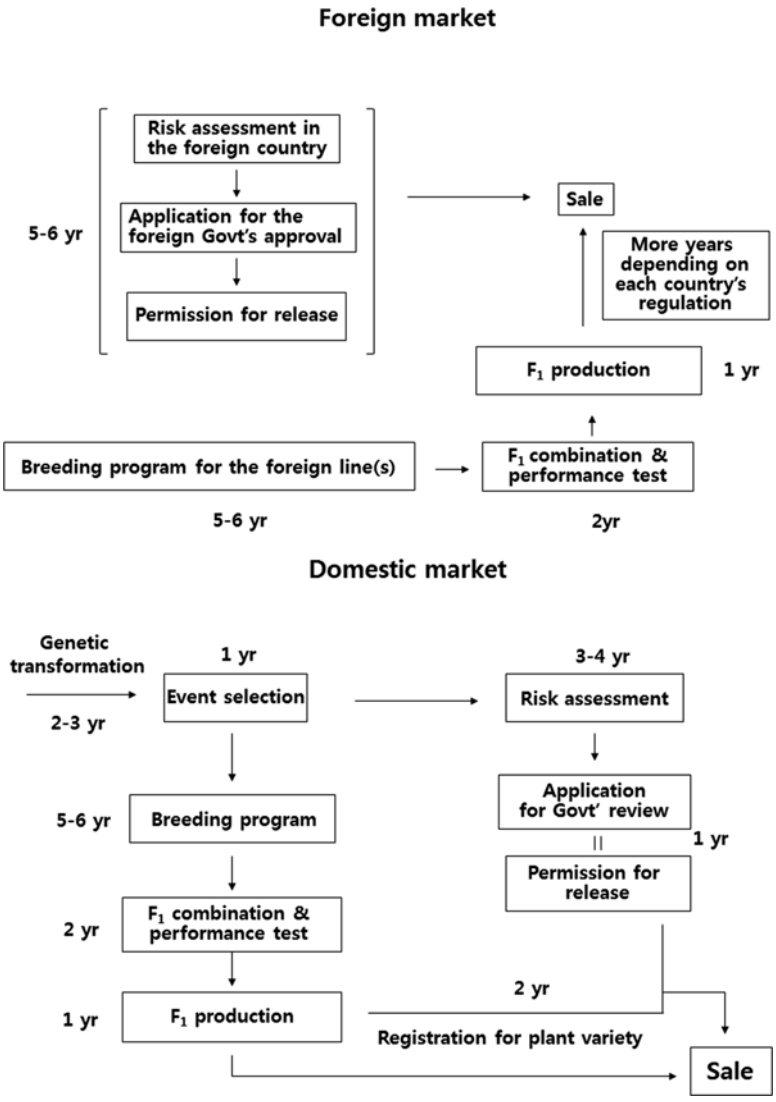

Fig. 9.9 Road map to the commercialization of GM seeds in domestic (Korea) and foreign (outside Korea) markets. The steps and durations may vary depending on each country's regulations but they are similar. The event line can be crossed to a non-GM line in the foreign country to develop a new GM line, and the GM F_1 would fit the local market

crop and the genome can be fixed, often not perfectly but reasonably. This method has traditionally been used worldwide in most crop breeding practices and breeders prefer to use this process even though it takes several years. However, this process may also transfer recessive genes of the donor to the recurrent line by linkage drag from the target gene. Cisgenesis can be used to transfer a gene not by crossing, but

by genetic modification. The transgene is not the cDNA but the genomic DNA of the target gene; therefore, the transgene contains its own promoter, exon, and intron. Once the transgene is transferred to the crop and the crop produces seeds and continues for next generations, the vector template, which is designed for deletion, can be selected out from the genome leaving only the whole gene in the transformed crop. Ordinary GM crops contain the cDNA of a target gene instead of the whole gene (Fig. 9.10).

When the genetic relationship between a donor and recipient is distant from each other, it usually takes a long breeding process (several years) using backcrossing to the recipient (recurrent) and self-crossing to fix the genomic background in the recipient crop. When cisgenesis is used, the recipient plant obtains the specific gene directly from the donor plant, therefore the time required to develop a new germplasm line can be reduced. For example, to construct a new tomato line, the average time would be 5–6 years using traditional methods, 7–8 years including the transformation, using ordinary GM methods, and 2–3 years using cisgenesis (Fig. 9.11).

For cisgenesis, the following conditions are required: (1) the whole target gene can be obtained; (2) a recombination-out vector can be constructed; and (3) all vector components, except the whole target gene, can be excluded from the host genome. Although this method uses genomic transformation, it leaves the intact whole gene in the genome and does not include any other vector components; therefore, technically, cisgenesis generates a non-GM plant.

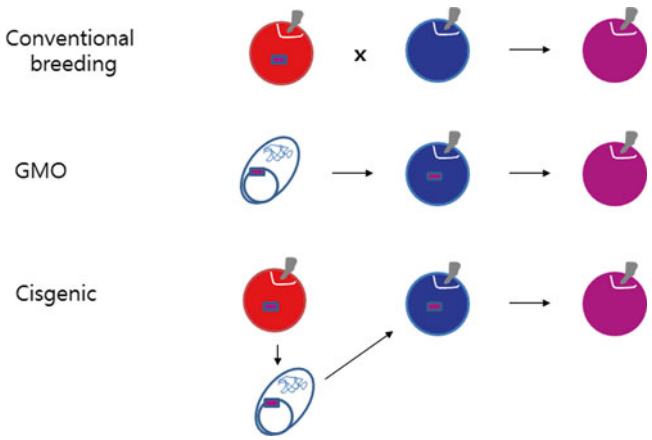

Fig. 9.10 Schematic representation of cisgenesis, genetic modification, and conventional breeding approaches. For conventional breeding, the *purple* gene in the red apple can be transferred to the *blue* apple by crossing; however, it would take a long time to conduct backcrosses and self-crosses to obtain the *purple* apple. Using genetic modification, the purple gene (cDNA) is transformed in a vector and transferred to the *blue* apple using a genetic transformation, which would generate *purple* apples containing the *purple* gene. Using cisgenesis, the whole *purple* gene (genomic DNA) in the *red* apple is transformed in a vector and transferred to the *blue* apple using a genetic transformation method. During the selection process, the vector components are deleted from the genome leaving the whole *purple* gene in the genome of the *blue* apple, generating a *purple* apple

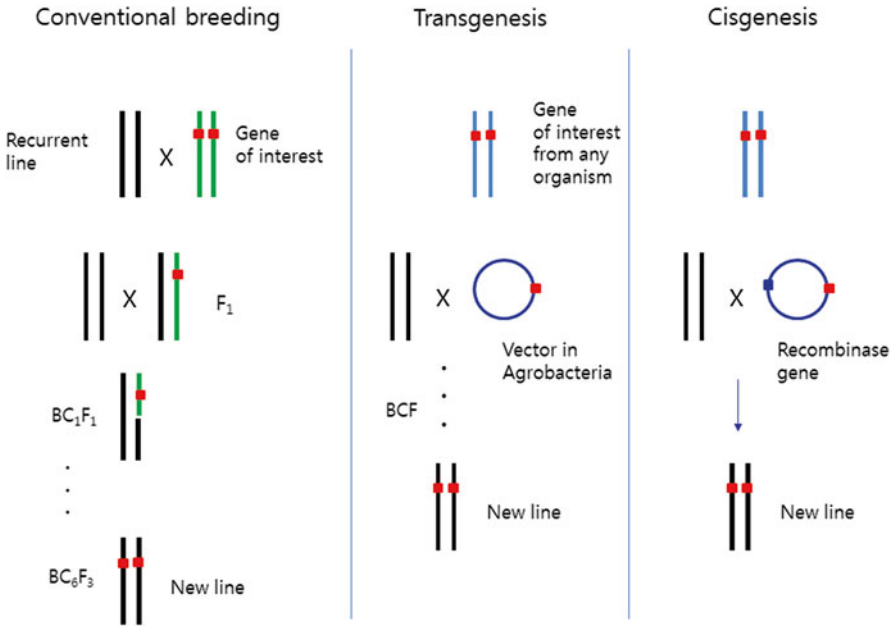

Fig. 9.11 Comparison of conventional breeding, transgenesis, and cisgenesis. Cisgenesis uses whole gene transfer in the same species, while transgenesis transfers a gene from any source such as animals, plants, humans and micro-organisms to another organism

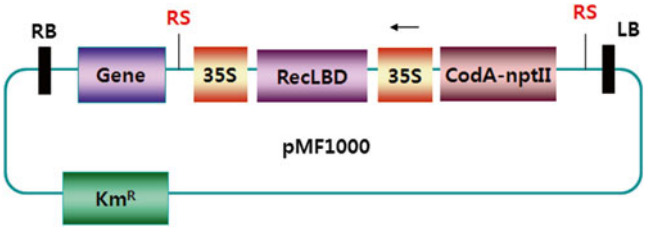

Fig. 9.12 A constructed vector for genetic transformation by cisgenesis. The vector contains there combinase gene (RecLBD) and a target gene. *35S* cucumber mosaic virus promoter, *Km*^{R} kanamycin resistant gene, *CodA* choline oxidase, *nptII* neomycin phosphotransferase type II, *LB* left border, *RB* right border, *RS* recombination site

9.4.1.2 Genetic Transformation for Cisgenesis

The constructed vector (Fig. 9.12) is transformed by *Agrobacterium* and the insert region between the right and left borders are located in the genome. The inserted region contains a gene encoding recombinase. It is advisable to choose an elite line

for genetic transformation; however, because the transformation efficiency depends on the genotype, the line that shows the highest regeneration rate should be used.

9.4.1.3 Recombination Mechanism

After the genetic transformation, explants are cultured on MS medium to which dexamethasone has been added to activate the recombinase (Fig. 9.13), which cuts the recombination site leaving only the target gene.

9.4.1.4 Successful Uses of Cisgenesis

A GM apple resistant to scab disease was constructed successfully with the scab resistance gene *HcrVf2* (Vanblaere et al. 2011) that has been used in the recombination vector (Fig. 9.14). The segment in the vector between the left and right borders is inserted into the host genome, and the segment between the recombination sites is removed by recombinase-mediated deletion (Fig. 9.14). This is a useful alternative approach that can help to avoid public controversy about the use of GM in food crops. In addition, Jo et al. (2014) developed a marker-free transformation pipeline to select potato plants functionally expressing a stack of late-blight-resistance genes using cisgenesis.

Therefore, cisgenesis is a promising breeding tool that introduces native genes from the same species to elite lines using GM technology, taking less breeding time and thereby retaining favorable characteristics of established varieties.

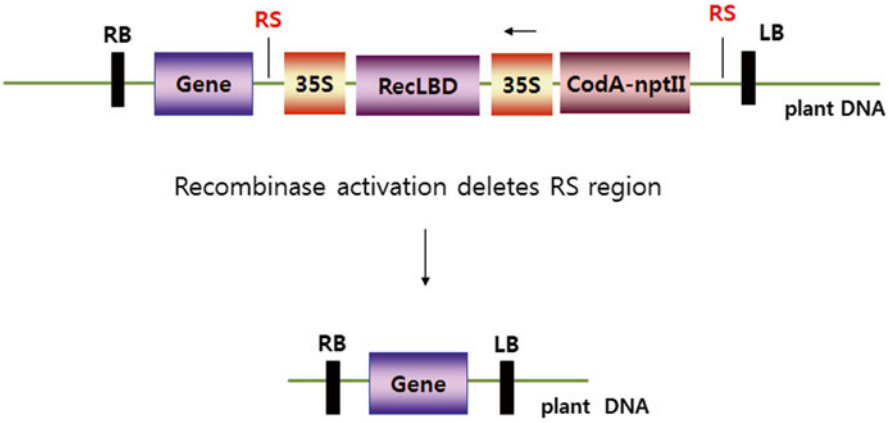

Fig. 9.13 Recombination mechanism is activated after genetic transformation using the constructed vector in Fig. 9.12. Recombinase is activated when dexamethasone is added, and the region between the recombination sites (RSs) with in the right and left boundaries (RB and LB) is deleted, leaving only the target gene in the host genome

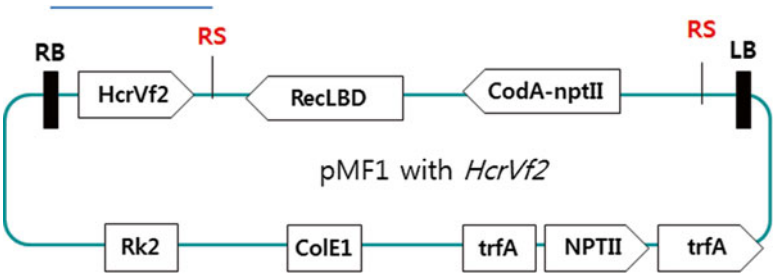

Fig. 9.14 Schematic representation of the pMF1 recombination vector containing the apple scab resistance gene (*HcrVf2*). The gene is controlled by its native regulatory sequences (Referred from Vanblaere et al. 2011). *HcrVf2* apple scab resistance gene from apple cv. Florina. Fusion marker gene *coda-nptII*, hybrid gene for positive (*nptII*) and negative (*codA*) selection. *RecLBD* translational fusion of recombinase R-LBD. Rk2 and ColE1, origins of replication. *trfA* replication gene. *nptIII* kanamycin resistance gene

9.4.1.5 Advantages and Disadvantages of Cisgenesis

Advantages

- Transferring a gene from wild-type in the same species
- Avoiding linkage drag from recessive alleles when backcrosses and self-crosses are conducted
- No need to fix the genome after cisgenesis; saving time for line construction
- Easier stacking of elite alleles.

Disadvantages

- Construction of the vector for recombination is difficult
- Low rate of transformation causes difficulty for cisgenesis
- It is not yet accepted that the modified plant is not a genetically modified organism.

9.4.1.6 Difference Between Cisgenesis and Intragenesis

Cisgenesis transfers a whole gene, including the native promoter, introns, exons, and terminator. Intragenesis (Schouten and Jacobsen 2008) uses the same method to transfer the gene but the components of the gene are not native; instead, they are constructed *in vitro* with other promoters and coding regions. In other words, cisgenesis uses an intact gene directly from the same species, while intragenesis uses a gene from the same species but a promoter and terminator from another gene.

9.4.2 Reverse Breeding

9.4.2.1 Introduction

To develop F_1 hybrids, breeders construct elite lines by crossing lines to develop and select the best horticultural characteristics. Parental lines often do not look particularly good, so it is interesting to consider how breeders select parental lines that contain the vigorous characteristics required in the hybrid. In ordinary breeding practice, the selection of elite offspring (F_1) is not easy and, as a result, reverse breeding was introduced. Reverse breeding is used to generate homozygote lines using F_1 and F_2 that have already been segregated, by blocking recombination during the cell division process (Fig. 9.15). In this way, the original parental lines can be obtained from F_1 and F_2 and new genetic sources with various genetic combinations can be obtained as homozygotes. Therefore, reverse breeding can provide new sources for breeding; therefore, this technology has had a significant impact in breeding programs.

When germ cells are fertilized, chromosome recombination occurs and the cells divide to become daughter cells. If no recombination occurs during the meiotic division, unaltered chromosomes will be transferred to the daughter cells. Pollen cells

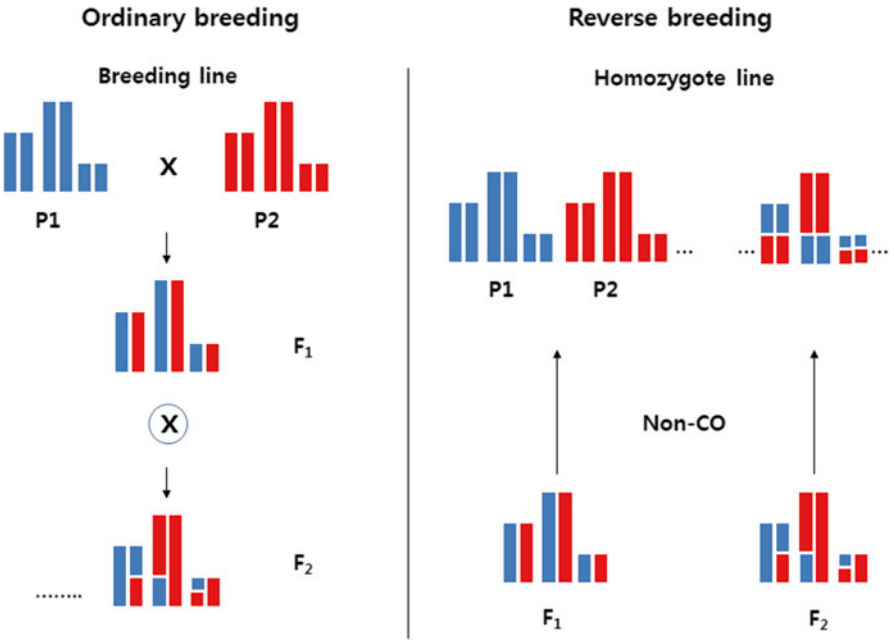

Fig. 9.15 Comparison of F_1 breeding and reverse breeding

can then be obtained and cultured to generate a doubled haploid (DH) line. To ensure that no recombination occurs during meiosis, the specific gene that controls recombination is blocked.

9.4.2.2 Preparation of Reverse Breeding

- Vector construction and genetic transformation: A small region of the disrupted meiotic cDNA1 gene (*DMC1*), including several hundred base pairs of the highly conserved region, are isolated and prepared as a transgene (Fig. 9.16) (Wijnker et al. 2012).
- An RNAi vector containing the transgene leads to gene silencing by knocking out *DMC1*.
- For genetic transformation, it is important to use genotypes that show a high regeneration rate.

9.4.2.3 Selection of Non-recombinant Plants

Normally, chromosome recombination occurs before meiotic division during germ cell development and chromosomes are separated equally to the two daughter cells. Sometimes, however, chromosomal non-disjunction occurs and the chromosomes are not equally distributed. When this occurs, normal germ cells will not develop. If the cross-over is blocked intentionally using genetic transformation, chromosome recombination is blocked and the non-recombinant chromosomes will either be balanced or non-balanced in the daughter cells (Figs. 9.17, 9.18, and 9.19). Non-balanced individuals do not survive and balanced individuals are obtained, but this depends on the numbers of chromosomes.

Theoretically, the probability of normal balancing is $(0.5)^x$ (x = number of chromosomes); therefore, the more chromosomes a plant contains, the lower is the probability of getting balanced non-recombinant individuals. For example, for *Arabidopsis* the probability of normal balancing is $(0.5)^5 = 0.0313$ (3 %), while for pepper, the probability is $(0.5)^{12} = 0.0002$ (0.02 %).

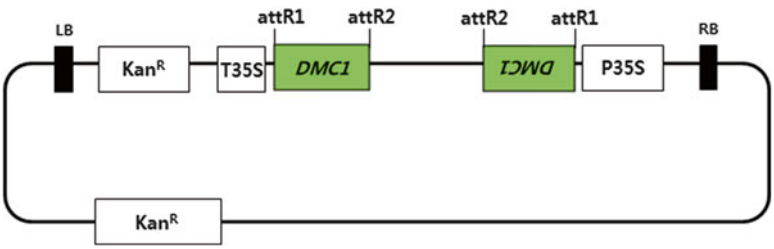

Fig. 9.16 RNAi vector with a part of the *DMC1* gene for vector construction

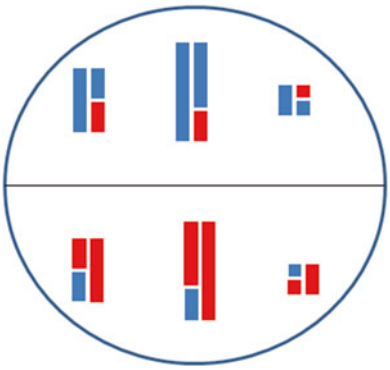

Fig. 9.17 Meiotic cell division with normal recombination of chromosomes

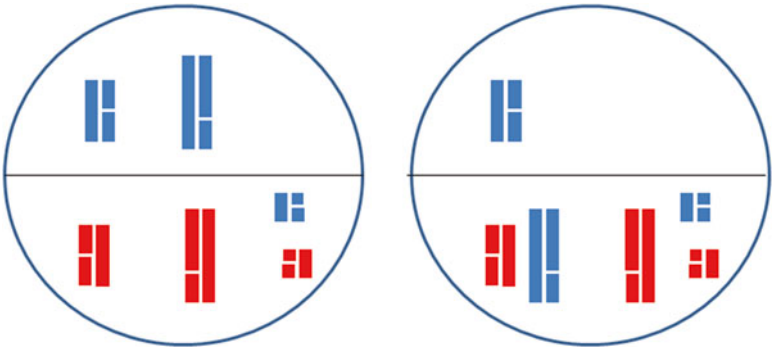

Fig. 9.18 Meiotic cell division with non-recombination and abnormal balancing of chromosomes

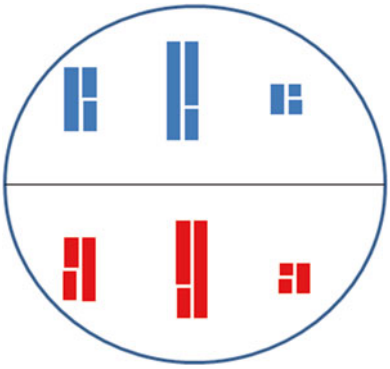

Fig. 9.19 Meiotic cell division with non-recombination and normal balancing of chromosomes

9.4.2.4 Procedure of Reverse Breeding

Transforming F_1 The F_1 hybrid is a product of crossing male and female parental lines and is genetically heterozygote. F_1 hybrids can be transformed with a vector that carries the *DMC1* gene sequence. The transformant selected as the one that blocks chromosome recombination would be a homozygote. The reverse breeding process is described in Fig. 9.20 (modified from Dirks et al. (2009)).

A F_1 heterozygote is the starting hybrid. F_1 seeds are planted to obtain the explants tissues. Explants are transformed with the transgene; the chromosomes of the transformants would not be recombined. After meiotic division, various combinations of chromosomes are generated. The pollen is cultured to obtain the DH line, which is a homozygote. The more DH lines obtained, the better the chance of obtaining chromosome substitution lines. By self-crossing the selected DH lines, new genetic sources can be maintained.

Parental lines for F_1 as well as various combination lines can be found among homozygotes and substitution lines, parental lines for F_1 can be found as well as various combination lines (Wijnker et al. 2012) and new lines can be obtained

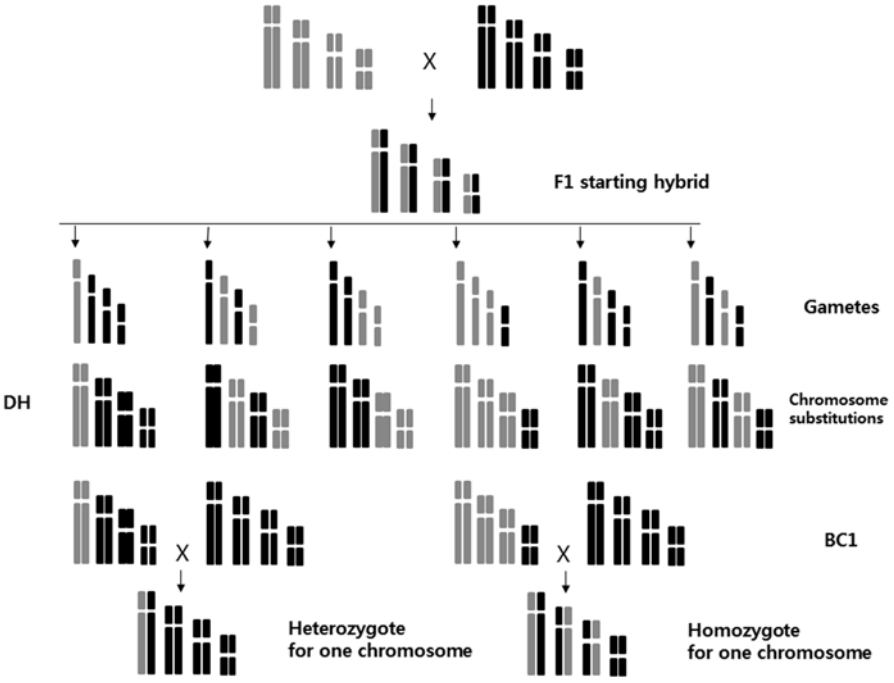

Fig. 9.20 Reverse breeding process using F_1 (Modified from Dirks et al. 2009). After meiotic division, various combinations of chromosomes are obtained: complete *black*, complete *gray*, or one *black* one *gray* as in the F_1 starting hybrid. The doubled haploid (DH) line is a homozygote. The more DH lines obtained the better the chance of generating chromosome substitution lines. By self-crossing the selected DH lines, new genetic sources are maintained

when these various combinations are crossed. When the markers for each chromosome are known, then the chromosomal composition of the new lines can be identified, indicating that the tailor-made breeding for each chromosome could be performed.

Transforming F_2 Chromosomal composition is more segregated in F_2. Each individual plant has its own chromosomal composition based on the recombination. Various compositions would be better as breeding sources (Fig. 9.21).

F_2 is the starting hybrid. The F_2 seeds are planted to obtain the cotyledon or hypocotyl tissues. The explants are transformed with the transgene; the chromosomes of the transformants would not be recombined. After meiotic division, various combinations of chromosomes are generated. The pollen is cultured to obtain the DH line, which is a homozygote. The more DH lines obtained, the better the chance of obtaining chromosome substitution lines. By self-crossing the selected DH lines, new genetic sources can be maintained. Compared with F_1 transformation, F_2 transformation generates more varied homozygotes because the chromosomal composition of the F_2 starting hybrids is more segregated.

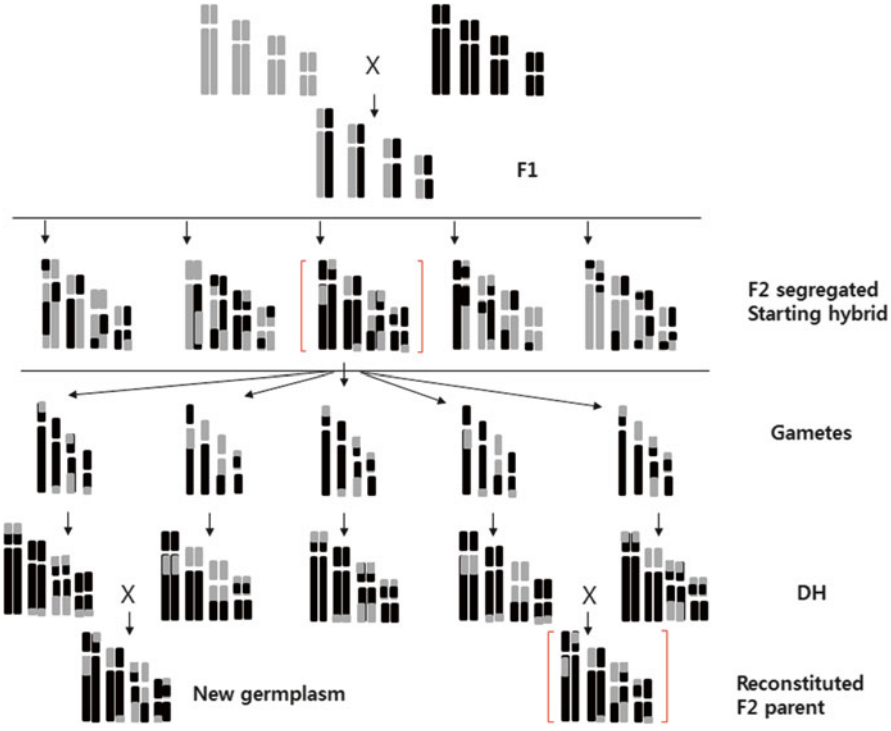

Fig. 9.21 Reverse breeding process using F_2 (Modified from Dirks et al. 2009). After meiotic division, various combinations of chromosomes are obtained. The doubled haploid (DH) line is a homozygote

Selection of Individuals After Reverse Breeding Because the transgene is inserted, it should be selected from the genome to obtain non-GM plants. The creation of non-GM hybrids is the final target for reverse breeding. Figure 9.22 shows that about 50 % of the germ cells in F_2 reverse breeding contain the transgene (red spot). Therefore, when the DH line is obtained, individuals that contain the transgene can be detected by PCR analysis and removed.

When homozygotes, which do not contain the transgene, are selected, the lines are maintained by self-crossing and the selected lines can be crossed to obtain new and various genetic germplasm. This germplasm will provide varied sources because of recombination. Therefore, reverse breeding is an important breeding technology to develop and create genetic sources.

9.4.2.5 Advantages and Disadvantages of Reverse Breeding

Advantages

- Various chromosome combinations can generate balanced parental lines as well as various homozygotes that can become new genetic sources.
- Tailor-made breeding using chromosome substitution lines is possible.
- GM technology can be used to generate new non-GM sources.

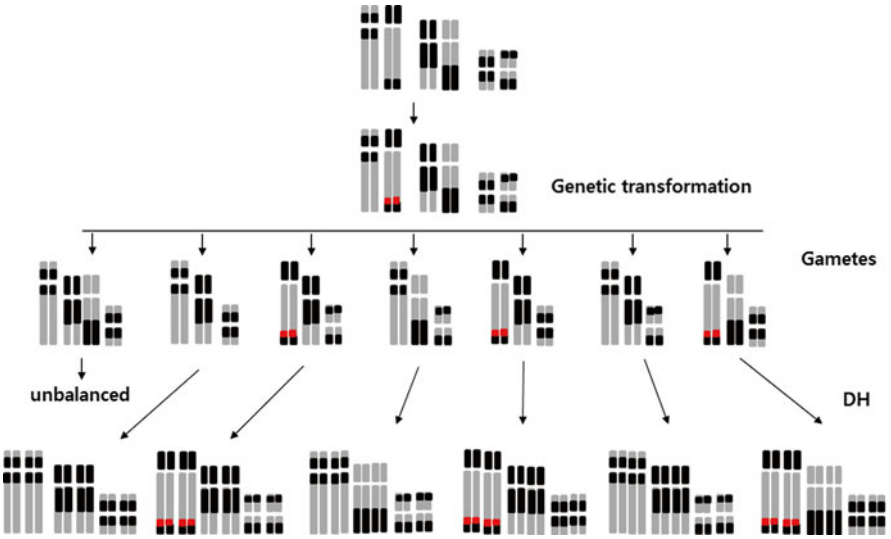

Fig. 9.22 Selection process of F_2 reverse breeding (modified from Wijnker and de Jong 2008). About 50 % of the germ cells in F_2 reverse breeding contain the transgene (*red spot*). DH, doubled haploid

Disadvantages

- When the number of chromosomes is more than 12, it is hard to obtain balanced germ cells.
- It would take a long time to confirm that the selected plant is non-GM.

References

Aswath CR, Mo SY, Kim DH et al (2007) Agrobacterium an biolistic transformation of onion using non-antibiotic selection marker phosphomannose isomerase. Plant Cell Rep 25:92–99

Carbone K (2013) Cultivars: chemical properties, antioxidant activities and health benefits. Nova Science Publishers, Inc. New York, pp 237–251

Cheng M, Fry JE, Pang S et al (1997) Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiol 115:971–980

Chilton MD, Drummond MH, Merlo DJ (1977) Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. Cell 11:263–271

Choi PS, Soh WY, Kim YS et al (1994) Genetic transformation and plant regeneration of watermelon using *Agrobacterium tumefaciens*. Plant Cell Rep 13:344–348

Dai S, Zheng P, Marmey P et al (2001) Comparative analysis of transgenic rice plants obtained by Agrobacterium-mediated transformation and particle bombardment. Mol Breed 7:25–33

Dale PJ, Hampson KK (1995) An assessment of morphogenic and transformation efficiency in a range of varieties of potato (*Solanum tuberosum* L.). Euphytica 85:101–108

Dan Y, Yan H, Munykwa T et al (2006) MicroTom-a high throughput model transformation system for functional genomics. Plant Cell Rep 25:432–441

Dirks R, Van Dun K, De Snoo CB et al (2009) Reverse breeding: a novel breeding approach based on engineered meiosis. Plant Biotechnol J 7:837–845

Eady CC (1995) Review towards the transformation of onions (*Allium cepa* L.). NZ J Crop Hortic Sci 23:239–250

Ellul P, Garcia-Sogo B, Pineda B et al (2003a) The ploidy level of transgenic plants in *Agrobacterium* mediated transformation of tomato cotyledons (*Lycopersicon esculentum* L. Mill.) is genotype and procedure dependent. Theor Appl Genet 106(2):231–238

Ellul P, Rios G, Atares A et al (2003b) The expression of the Saccharomyces cerevisiae HAL1 gene increase salt tolerance in transgenic watermelon (*Citrullus lanatus* Thunb.) Matsum. & Makai. Theor Appl Genet 107:462–469

Filichkin SA, Gelvin SB (1993) Formation of a putative relaxation intermediate during T-DNA processing directed by the *Agrobacterium tumefaciens* VirD1, D2 endonuclease. Mol Microbiol 8:915–926

Frame BR, Zhang H, Cocciolone S (2000) Production of transgenic maize from bombarded Type II callus: effect of gold particle size and callus morphology on transformation efficiency. In Vitro Cell Dev Biol Plant 36:21–29

Gaba V, Zelcer A, Gal-On A (2004) Cucurbit biotechnology-the importance of virus resistance. In Vitro Cell Dev Biol Plant 40:346–358

Gahakwa D, Maqbool SB, Fu X et al (2000) Transgenic rice as a system to study the stability of transgene expression: multiple heterologous transgenes show similar behavior in diverse genetic backgrounds. Theor Appl Genet 101:388–399

Gaspar T, Kevers C, Penel C et al (1996) Plant hormones and plant growth regulators in plant tissue culture. In Vitro Cell Dev Biol Plant 32:272–289

James C (2014) Global status of commercialized biotech/GM crops: 2014. *ISAAA* Brief No. 49. Ithaca, NY

Jo KR, Kim CJ, Kim SJ et al (2014) Development of late blight resistant potatoes by cisgene stacking. BMC Biotechnol 14:50–60
Lee YH, Kim HS, Kim JY et al (2004) A new selection method for pepper transformation: callus-mediated shoot formation. Plant Cell Rep 23:50–58
Lee YH, Jung M, Shin SH et al (2009) Transgenic peppers that are highly tolerant to a new CMV pathotype. Plant Cell Rep 28:223–232
Mysore KS, Nam J, Gelvin SB (2000) An Arabidopsis histone H2A mutant is deficient in *Agrobacterium* T-DNA integration. Proc Natl Acad Sci 97:948–953
Nandakumar R, Babu S, Wang ZY (2011) DNA delivery systems. In: DanY, Ow DW (eds), Historical technology developments in plant transformation technology revolution. Bentham Science Publishers, USA, pp 3–24
Nugent GD, ten Have M, van der Gulik A et al (2005) Plastid transformants of tomato selected using mutations affecting ribosome structure. Plant Cell Rep 24:341–349
Olhoft PM, Somers DA (2001) L-Cysteine increases Agrobacterium-mediated T-DNA delivery into soybean cotyledonary node cells. Plant Cell Rep 20:706–711
Olhoft PM, Flagel LE, Donovan CM et al (2003) Efficient soybean transformation using hygromycin B selection in the cotyledonary-node method. Planta 5:723–735
Olsen FL (1987) Induction of microspore embryogenesis in cultured anthers of *Hordeum vulgare*: the effects of ammonium nitrate, glutamic acid and asparagines as nitrogen sources. Carlsberg Res Commun 52:393–404
Ooms G, Burrell MM, Karp A et al (1987) Genetic transformation in two potato cultivars with T-DNA from disarmed *Agrobacterium*. Theor Appl Genet 73:744–750
Park SH, Morris JL, Park JE et al (2003) Efficient and genotype-independent Agrobacterium-mediated tomato transformation. J Plant Physiol 160:1253–1257
Paz MM, Martinez JC, Kalvig AB et al (2006) Improved cotyledonary node method using an alternative explants derived from mature seed for efficient Agrobacterium-mediated soybean transformation. Plant Cell Rep 25:206–213
Schouten HJ, Jacobsen E (2008) Cisgenesis and intragenesis, sisters in innovative plant breeding. Trends Plant Sci 13:260–261
Shrawat AK, Becker D, Lorz H (2007) *Agrobacterium* mediated genetic transformation of barley (*Hordeum vulgare* L.). Plant Sci 172:281–290
Sigareva M, Spivey R, Wilits MG et al (2004) An efficiency mannose selection protocol for tomato that has no adverse effect on the ploidy level of transgenic plants. Plant Cell Rep 23:234–245
Tingay S, McElroy D, Kalla R (1997) *Agrobacterium* tumefaciens-mediated barley transformation. Plant J 11:1369–1376
Tzfira T, Citovsky V (2000) From host recognition to T-DNA integration: the function of bacterial and plant genes in the *Agrobacterium*–plant cell interaction. Mol Plant Pathol 1:201–212
Vanblaere T, Szankowski I, Schaart J et al (2011) The development of a cisgenic apple plant. J Biotechnol 154:304–311
Vaucheret H, Beclin C, Elmayan T et al (1998) Transgene-induced gene silencing in plants. Plant J 16:651–659
Wan Y, Lemaux PG (1994) Generation of large numbers of independently transformed fertile barley plants. Plant Physiol 104:37–48
Wang G, Xu Y (2008) Hypocotyl-based Agrobacterium-mediated transformation of soybean (Glycine max) and application for RNA interference. Plant Cell Rep 27:1177–1184
Wijnker E, de Jong H (2008) Managing meiotic recombination in plant breeding. Trends Plant Sci 13:640–646
Wijnker E, van Dun K, de Snoo CB et al (2012) Reverse breeding in Arabidopsis thaliana generates homozygous parental lines from a heterozygous plant. Nat Genet 44:467–470
Zhang ZY, Xing AQ, Staswick P et al (1999) The use of glufosinate as a selective agent in Agrobacterium-mediated transformation of soybean. Plant Cell Tissue Organ Cult 56:37–46
Zhang W, Wang K, Lin ZS (2014) Production and identification of haploid dwarf male sterile wheat plants induced by corn inducer. Bot Stud 55:1–8

Zhao P, Wang K, Zhang W (2014) Review and inspiration of plant proteins involved in the transformation processing of T-DNA initiated by *Agrobacterium*. Sctientica Agric Sin 47(13):2504–2518

Zheng SJ, Khrustaleva L, Henken B et al (2001) *Agrobacterium tumefaciens*-mediated transformation of *Allium cepa*L.: the production of transgenic onion and shallots. Mol Breed 7:101–115

Zhou H, Arrowsmith J, Fromm M et al (1995) Glyphosate-tolerant CP4 and GOX genes as a selectable marker in wheat transformation. Plant Cell Rep 15:159–163

Zhu Y, Nam J, Carpita NC (2003) *Agrobacterium*-mediated root transformation is inhibited by mutation of an Arabidopsis cellulose synthase-like gene. Plant Physiol 133:1000–1010