(54) Title: EFFICIENT METHOD FOR THE DEVELOPMENT OF TRANSGENIC PLANTS BY GENE MANIPULATION

pGB(11.4kb)

(57) Abstract: The present invention relates to a method for transforming plants by using a gene manipulation technique, and more particularly, to the method for transforming plants with Agrobacterium containing bar gene having resistance to a herbicide by using an effective wounding method to an appropriate target tissue. A more improved breed than the conventional breed of plant can be produced by the transforming method according to the present invention.
EFFICIENT METHOD FOR THE DEVELOPMENT OF TRANSGENIC
PLANTS BY GENE MANIPULATION

Technical Field

The present invention relates to a method for transforming plants using a gene manipulation technique, and more particularly, to an improved transformation method of plants, comprising insertion of a useful target gene for plants, in particular soybean plant, into an appropriate target plant tissue using an effective wounding method, and a transformant produced using the method.

Background Art

Soybean is one of the most important crops in the world. It is widely cultivated on more than 50 million hectares of farmland, and soybean of more than 100 million-ton is produced every year. New breeds improved in terms of disease resistance, herbicide resistance and nutrition will provide substantial economic advantages. Up until now, conventional breeding techniques such as mutation, hybridization and natural screening have been employed to improve a breed of soybean, but have failed to provide satisfactory results. Therefore, development of new strategies for breeding of soybeans is needed. Methods that functional genes are transformed into plants and expressed using a molecular breeding method, thereby new plant breeds expressing desired characteristics being obtained, have been
developed. The molecular breeding method is different from the conventional hybridization breeding method, in that genetic characteristics of good breeds are kept intact during transplantation of new functional genes, and efficient breeding is accomplished within a short time (Altenbach et al., *Plant Mol. Biol.*, 13: 513-522, 1989).

Success of the molecular breeding method depends on selection of a suitable target plant cell, efficient transformation of the target cell with a target gene, and regeneration, by which the transformed cell is grown into a whole plant. Methods for efficient transformation of plant cells include the method for delivering foreign DNA into plant cell using a biological vector such as *Agrobacterium spp.*, and methods for delivering directly foreign DNA into plant cell such as microprojectile bombardment, electroporation, ultrasonic treatment and microinjection (Hansen and Wright, *Trends Plant Sci.*, 4:226-231, 1999). Though foreign genes have been transformed into main crops such as corn, rice, barley, wheat and soybean using microprojectile bombardment, there are problems in that the foreign genes must be injected into regenerative cells, and transplantation or injection pattern into plant cell of the foreign genes varies (De block M., *Euphytica.*, 71:1-14, 1993).

Currently, the most widely used method for transferring genes into plants is *Agrobacterium tumefaciens*-mediated transformation. *Agrobacterium* has the ability to transfer its T-DNA into a plant’s genome. The T-DNA is present in the tumor-inducing (Ti) or root-inducing (Ri) plasmid of the bacteria. Therefore, if a target gene is inserted into the T-DNA of *Agrobacterium* and the target gene-inserted *Agrobacterium* and the plant cell or tissue are co-cultivated, the plant cell or tissue can
be transformed with the target gene (Binns et al., Ann Rev Microbiol., 42:575-606, 1988). As detailed in the below, as for soybean transformation developed up until now, there are problems in that efficiencies of transformation and regeneration are very low, chimeric plants are produced, and reproducibility is low. Thus, in order to increase efficiency of transformation with Agrobacterium, there have been used methods such as cutting of the desired plant tissue with scalpel, microparticle bombardment (U.S. Patent No. 5,792,935), or ultrasonic treatment (U.S. Patent No. 5,693,512), but gene delivery efficiency is insignificant. Based on the fact that the regeneration of shoots can be induced at cotyledonary nodes (Barwale et al., Planta, 167:473-481, 1986), a plant transformation technique using the cotyledonary node as a target tissue has been reported (U.S. Patent No. 5,824,877). However, in this case, the cotyledonary node germinated for 4-5 days was used and the efficiencies of transformation and regeneration were very low. Accordingly, development of new soybean transformation techniques with higher efficiency and stability is needed.

According to those needs, the present inventors studied efficient techniques for developing new breeds of soybean plants. As a result, the inventors developed the following procedure and completed the present invention: the inventors wounded the cotyledonary node of cultivar soybean Jungery, which had been germinated for 1 day, as a target plant tissue, and inserted a target gene thereinto, thereby gene delivery efficiency being increased.

Disclosure of the Invention
Therefore, the present invention has been made in view of the above problems, and it is an object of the present invention to provide a method for transforming plants with *Agrobacterium tumefaciens* vector containing a target gene.

It is another object of the present invention to provide a transformed plant produced using the transformation method.

In accordance with one aspect of the present invention, the above and other objects can be accomplished by the provision of a method for transforming plants, comprising the steps of:

1) selecting the half a seed, which is germinated for one day and vigorously divided, as a target plant tissue;

2) wounding the target plant tissue using a bundle of needles;

3) inserting a target gene into the target plant tissue using *Agrobacterium tumefaciens* vector containing the target gene;

4) regenerating plants from the germinated cotyledonary node segments; and

5) selecting transformed plants.

In accordance with another aspect of the present invention, there is provided a transformed plant produced using the transformation method.

As used herein, the term “chimera” means a hybrid plant consisting of genetically different cells, i.e., a cell with a target gene and a cell with no target gene.

As used herein, the term “a target gene” means a gene encoding a useful protein, and which is introduced into a plant by using *Agrobacterium*.

As used herein, the term “transformant” or “transformed plant” means a plant into which *Agrobacterium* containing the target gene is introduced.
Brief Description of the Drawings

The above and other objects, features and other advantages of the present invention will be more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which:

Fig. 1 is a schematic view showing a recombinant plasmid pGB, constructed by introducing the bar gene and the gfp gene into the T-DNA of Agrobacterium;

Fig. 2 is an electrophoresis result for confirming that plasmid pGB for transformation is constructed;

Fig. 3 is a photograph showing half a seed germinated for one day, including cotyledonary node, as a target plant tissue;

Fig. 4 is a photograph showing multiple shoots regenerated from cotyledonary node segments;

Fig. 5 is a photograph showing the transformant selected in herbicide PPT (phosphinothricin)-containing medium, compared with a non-transformant;

Fig. 6 is a Southern blot showing presence of the bar gene in the transformant genome following PCR amplification of the bar gene, to confirm that a target gene (bar gene) is introduced into the plant;

Fig. 7 is a Southern blot showing presence of the bar gene in the transformant genome;

Fig. 8 is a Northern blot showing expression of the bar gene in the transformant;

Fig. 9 is a photograph of the transformant growing in soil;
Fig. 10 is a photograph showing resistance to herbicide of the transformant, compared with a non-transformant;

Fig. 11 is a photograph showing a second-generation progeny of the transformant, growing in soil;

Fig. 12 is a Southern blot showing presence of the bar gene and the gfp gene in the second generation transormant genome, following PCR amplification of the bar and gfp genes; and

Fig. 13 is a confocal fluorescence microphotograph showing expression of the gfp (green fluorescent protein) gene transformed into the transformant.

Best Mode for Carrying Out the Invention

Hereinafter, the present invention will be described in detail, using soybean as an example.

The present invention provides a method for producing a new breed of soybean by introducing a target gene into a soybean plant. The target gene is derived from animals, plants or microorganisms. Examples of the target gene which may be used in soybean transformation include a gene for an enzyme conferring resistance to damages by harmful insects and herbicide, a gene for an enzyme enhancing crop yield, or improving qualities of protein and fatty acid of soybean seed, and the like. The target gene may be any gene derived from microorganisms such as bacteria, fungi, yeasts, viruses, as well as plants and animals.

A target gene cassette according to the present invention is a useful gene
construct capable of imparting desired genetic characteristics to plants. Therefore, a characteristic of the transformed plant depends on the target gene, which is inserted into the plant. The DNA vector used for constructing the target gene cassette is containing an expression cassette consisted of a regulatory sequence such as a promoter and terminator, a transcription initiation region, a translation initiation codon, an amino acid coding region and a translation stop codon, and containing at least one origin of replication.

The gene construct used for transformation further contains a selectable gene, a marker gene, and a target gene, and the like. A gene conferring resistance to herbicide can be used as a target gene, at the same time as a selectable gene. Examples of genes, which may be used as a selectable gene, include genes conferring resistance to, NPTII (neomycin phosphotransferase), HPT (hygromycin phosphotransferase), CAT (chloramphenicol acetyltransferase), nitrilase and gentamicin. The genes provide resistance to biocides such as antibiotics, toxins and heavy metals.

Examples of genes which may be used as the marker genes include GUS (β-glucuronidase) emitting indigo, CAT, luciferase emitting visible light, and gfp (green fluorescent protein) gene producing green fluorescent material, and the like.

A target plant tissue for transformation is generally selected from the group consisting of meristematic tissue such as a germ, a stem, a shoot tip and a cotyledonary node, and induced meristematic tissue such as callus. In accordance with one embodiment of the present invention, the cotyledonary node of half a seed is used as a target plant tissue. As for the cotyledonary node of half a seed, which is germinated for one day, because it’s cells are vigorously dividing and DNA synthesis is high, the
possibility for transplantation of a target gene into the plant cells increases, upon inoculation of *Agrobacterium* (Binns et al., *Ann Microbiol.*, 42: 575-606, 1988). Moreover, cells of the cotyledonary node of half a seed germinated for one day, divide vigorously, but are not divided enough. Therefore, possibility for transformation increases in a single cell, capable of forming primordia, thereby reducing formation of chimera. However, it is known that if seedlings and their segments on or after 6 days of germination are used as a target plant tissue, primordia are already formed and thus the possibility for formation of chimera becomes high (Wright et al., *Plant Cell Reports.*, 5: 150-154, 1986). Specifically, the present inventors investigated transformation efficiencies using cotyledonary nodes, which had been germinated for 1, 2, 4 and 14 days, respectively. As shown in Table 4 below, the transformation efficiency of the cotyledonary node of half a seed germinated for 1 day as a target plant tissue was highest. In contrast, in case of using the cotyledonary nodes germinated for 2, 4 and 14 days, respectively, as a target plant tissue, their transformation efficiencies were remarkably reduced.

In accordance with another embodiment of the present invention, a recombinant vector is constructed to carry out transformation using the half a seed as a target plant tissue. In order to construct the recombinant vector, the *bar* gene conferring resistance to herbicide is used as a selectable gene, and the *gfp* gene, which causes green fluorescence, is used as a marker gene to select transformed plant in the beginning. As shown in Fig. 1, the *bar* and *gfp* genes are flanked by right and left borders of the T-DNA of *Agrobacterium*, respectively. The *bar* gene expression cassette is composed of the NOS promoter, the *bar* coding region, and the NOS terminator, while the *gfp*
cassette is composed of the 35S promoter, the gfp coding region, and the NOS terminator.

In accordance with still another embodiment of the present invention, cultivar soybean is transformed with a herbicide-resistance gene. A sterilized seed is germinated for 16 to 24 hours and then its half a seed is used as a target plant tissue (see Fig. 3). The half a seed is co-cultivated with Agrobacterium tumefaciens containing the herbicide-resistance gene to transform the seed. Then, shoots regenerated from the seed are selected in herbicide containing media (see Fig. 4). Finally, plants are re-selected by growing in media containing 1 ppm of PPT for 2 weeks (see Fig. 5). As a result, transformants with 2.5% transformation efficiency are obtained, as shown in Table 1 below.

In accordance with still another embodiment of the present invention, to increase transformation efficiency of plant tissue, a target plant tissue is wounded with a bundle of needles, preferably a bundle of 30 needles. In the course of regeneration of shoots from primary node or cotyledonary node of soybean, the shoots are generally induced in cells of a target plant tissue without inducing callus. Therefore, the transformation with Agrobacterium can be easily carried out only in the case where Agrobacterium directly accesses to the cells of a target plant tissue. To do this, it is very important to wound the cells of the target plant tissue, in which shoots are induced.

In the case where a target plant tissue is wounded to 1-2 mm depth using a bundle of 30 needles and then Agrobacterium is inoculated thereinto, the transformation efficiency is enhanced more than 8 times versus transformation efficiency without wounding (see Table 2). Furthermore, the transformation efficiency with a bundle of 30 needles is
remarkably higher than that of multiple wounding with one needle (see Table 3). The efficiencies for transformation also vary according to breed of plant. As shown in Table 5 below, when the herbicide-resistance gene was introduced into cultivar Jungery, Danyup and Paldal, respectively, with Agrobacterium, the transformation efficiency of cultivar Jungery was 15 times and 7 times higher than Paldal and Danyup, respectively. Accordingly, it can be seen that cultivar Jungery can be more effectively used in transformation of plant.

In accordance with yet another embodiment of the present invention, to confirm that the above transformed plant contains a target gene, the present inventors performed PCR amplification, Southern blotting and Northern blotting. As a result, the inventors confirmed that bar gene, the target gene, is stably expressed in the transformant (see Fig. 6, Fig. 7, Fig. 8). Further, to confirm that the transformant so produced has resistance to herbicide, the inventors applied 100 ppm of herbicide on leaves of the transformant and then compared with a non-transformant. As shown in Fig.10, as for the non-transformant, its leaves gradually wilted and died, while the transformant grew vigorously. Further, as shown in Fig. 11, second-generation plants can be obtained by germinating seeds obtained from the transformant. The bar gene of the second-generation plant was amplified by PCR, and then Southern blotting was carried out to detect the presence of the herbicide-resistance gene. As a result, it can be seen that the bar gene was stably introduced into the second-generation transformant (see Fig.12).

As can be seen from the above, a useful target gene-inserted transformant can be produced using the transformation method of the present invention. Examples of
plants which may be used in the transformation, include cultivar soybean; food crops such as rice, wheat, barley, corn, soybean, potato, red bean, oats and sorghum; vegetables such as Arabidopsis, Chinese cabbage, radish, red pepper, strawberry, tomato, watermelon, cucumber, cabbage, melon, pumpkin, green onion, onion and carrot; special crops such as ginseng, tobacco, cotton, sesame, sugar cane, sugar beet, perilla, peanut and rape; fruits trees such as apple, pear, jujube, peach, kiwi fruit (Actinidia arguta), grape, tangerine, persimmon, plum, apricot, and banana; ornamental plants such as rose, gladiolus, gerbera, carnation, chrysanthemum, lily and tulip; and forage crops such as ryegrass, red clover, orchard grass, alfalfa, tall fescue and perennial ryegrass and the like.

Examples

Hereinafter, the present invention will be illustrated by way of examples.

It is, however, to be borne in mind that the present invention is by no means limited to or by them.

Example 1: Construction of herbicide-resistance bar gene-inserted recombinant plasmid for transformation

Recombinant plasmid pGB was constructed to use in transformation of soybean (see Fig. 1). The recombinant plasmid pGB was constructed by inserting herbicide-resistance bar gene as a selectable gene and green fluorescent protein gfp gene as a marker gene into T-DNA plasmid pGA643 for plant transformation. The expression cassette of the bar gene for conferring resistance to herbicide PPT is
composed of the NOS (nopaline synthase) promoter, the *bar* coding region, and the NOS terminator. The expression cassette of the *gfp* gene is composed of the 35S promoter, the *gfp* coding region and the NOS terminator. The recombinant plasmid pGB was digested with restriction enzymes, *ClaI* and *EcoRI*, or *KpnI*, and then the digested fragments were separated by agarose gel electrophoresis (see Fig. 2). Finally, the sequence data of the recombinant plasmid pGB was obtained by sequencing.

**Example 2: Selection of target plant tissue for transformation and transformation using the same**

Target plant tissue was selected to introduce recombinant plasmid thereinto and then transformed with *Agrobacterium*.

2-1) Selection of target plant tissue

Cultivar Jungery, which is transformed at high efficiency, was used. Mature soybean seeds were immersed in 70% ethanol for 1 minute, and then in 20% Clorox solution (5.25% sodium hypochlorate) for 12 minutes to disinfect their surfaces and then washed four times with sterilized distilled water. The mature seeds so treated were immersed in sterilized distilled water for 16 to 24 hours so as to easily wound them, and germinated at room temperature. Because a whole seed is not easy to manipulate or wound, half a seed was used. A whole seed was divided into two equal parts in direction of cotyledonary node using a knife. As a result, the half a seed with bud was used as the target plant tissue of the present invention (see Fig. 3).
2-2) Transformation of target plant tissue with recombinant plasmid

The half a seed obtained from the example 2-1) and Agrobacterium containing recombinant plasmid pGB were co-cultivated in germination medium (MS salt solution (1/2 Murashige and Skoog), vitamin solution (Gamborg B5), benzyladenine 1 ppm) for 3 days, thus the half a seed is transformed with the recombinant plasmid pGB. 100 μM of acetosyringone was added to the medium to increase efficiency of transformation.

After co-cultivation, the half a seed was further germinated in the germination medium for 10 days. Then, the cotyledonary nodes of the seed were separated and cultured in 1 ppm of PPT-containing germination medium for 3 weeks to selectively induce multiple shoots (see Fig. 4). To select independent transformants, individual shoots were cut and the cuts were cultured and re-selected in 2 ppm of PPT-containing root inducing medium (MS salt solution (1/2 Murashige and Skoog), vitamin solution (Gamborg B5)) for 2 weeks. Non-transformed shoots wilted and died, while transformed shoots rooted (see Fig. 5). Shoots with roots were transferred to soil after acclimation in the manner of gradually opening the lid of their container. As shown in Table 1, transformation efficiency of Agrobacterium containing recombinant plasmid was 2.5%.

<table>
<thead>
<tr>
<th>Section</th>
<th>Number of cotyledonary nodes</th>
<th>Number of induced shoots</th>
<th>Number of PPT-resistant shoots</th>
<th>Number of PPT-resistant seedlings</th>
<th>Transformation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Agrobacterium</td>
<td>71</td>
<td>60</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+ Agrobacterium</td>
<td>120</td>
<td>100</td>
<td>5</td>
<td>3</td>
<td>2.5</td>
</tr>
</tbody>
</table>
Example 3: Wounding of target plant tissue to enhance transformation efficiency

Cotyledonary nodes of half a seed, inoculation sites for Agrobacterium, were wounded to enhance transformation efficiency.

3-1) Elevation of transformation efficiency by inoculations of Agrobacterium with a bundle of needles

Cotyledonary nodes of half a seed were wounded to 1-2 mm depth using a bundle of 30 needles in order to wound effectively and then Agrobacterium were inoculated thereinto. As shown in Table 2 below, the transformation efficiency was 21% and was enhanced more than 8 times versus transformation efficiency without wounding.

Table 2

<table>
<thead>
<tr>
<th>Number of inductions of shoots (number of cotyledonary nodes: 186)</th>
<th>Number of induced shoots</th>
<th>Number of PPT-resistant shoots</th>
<th>Number of PPT-resistant seedlings</th>
<th>Transformation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>76</td>
<td>10</td>
<td>10</td>
<td>5.4</td>
</tr>
<tr>
<td>Second</td>
<td>51</td>
<td>19</td>
<td>17</td>
<td>9.1</td>
</tr>
<tr>
<td>Third</td>
<td>48</td>
<td>22</td>
<td>12</td>
<td>6.5</td>
</tr>
<tr>
<td>Total</td>
<td>175</td>
<td>51</td>
<td>39</td>
<td>21.0</td>
</tr>
</tbody>
</table>

As for inoculation of Agrobacterium with no wounding, transformed shoots
were obtained once, while as for inoculation of *Agrobacterium* with a bundle of needles, transformed shoots continued to be regenerated at least three times from the inoculated cotyledonary nodes. Accordingly, inoculation of *Agrobacterium* with a bundle of needles makes it possible to wound target cells of the inoculated cotyledonary nodes. As a result, efficiency for transformation of soybean increases and thus stably transformed soybean plants are regenerated in large quantities.

3-2) Comparison of regeneration efficiency after uses of a single needle and a bundle of needles, respectively

To investigate transformation efficiency with a bundle of needles, the inventors compared regeneration efficiencies between the two conditions, i.e. when multiply wounding with a single needle and when once wounding with a bundle of needles. As for no wounding, regeneration efficiency of shoots was 188%, while as for once wounding with a bundle of needles, efficiency for regeneration of shoots was 191%. Judging from the fact that the two cases have little difference in regeneration of shoots, it can be seen that although cotyledonary nodes are wounded with a bundle of needles, differentiation of shoots from cotyledonary nodes is not affected. However, when cotyledonary nodes were wounded 20 times with a single needle, efficiency for regeneration of shoots was remarkably reduced to 38%. Based on these facts, it can be seen that cotyledonary nodes were severely damaged by the repeated wounding and regeneration was not easily induced. The results are presented in Table 3 below.
Table 3

<table>
<thead>
<tr>
<th>Treatment condition</th>
<th>Number of segments</th>
<th>Number of shoots</th>
<th>Regeneration efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>32</td>
<td>60</td>
<td>188</td>
</tr>
<tr>
<td>Single needle</td>
<td>60</td>
<td>23</td>
<td>38</td>
</tr>
<tr>
<td>Bundle of needles</td>
<td>70</td>
<td>134</td>
<td>191</td>
</tr>
</tbody>
</table>

As shown in the Table 3, it can be seen that wounding once with a bundle of 30 needles is more efficient than multiply wounding with a single needle, in terms of transformation efficiency.

Example 4: Comparison of transformation efficiency according to degree of growth of target plant tissue

Regeneration by culturing target plant tissue is necessary in transformation. Therefore, the inventors compared transformation efficiencies according to degree of growth of target plant tissue. The cotyledonary nodes of half a seed, of which degrees of growth are different, were transformed. As a result, the cotyledonary nodes of half a seed, which were germinated for one day, although their size was small, had the highest transformation efficiency. While, when the cotyledonary nodes of seedlings germinated for 2, 4 and 14 days, were used as target plant tissues, transformation efficiencies were remarkably reduced (see Table 4).
Table 4

<table>
<thead>
<tr>
<th>Target tissue</th>
<th>Number of segments</th>
<th>Number of PPT-resistant shoots</th>
<th>Transformation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cotyledonary node</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germinated for 1 day*</td>
<td>112</td>
<td>13</td>
<td>11.6</td>
</tr>
<tr>
<td>cotyledonary node</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germinated for 2 days*</td>
<td>115</td>
<td>6</td>
<td>5.2</td>
</tr>
<tr>
<td>cotyledonary node</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germinated for 4 days**</td>
<td>120</td>
<td>3</td>
<td>2.5</td>
</tr>
<tr>
<td>cotyledonary node</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Germinated for 14 days**</td>
<td>104</td>
<td>2</td>
<td>1.9</td>
</tr>
</tbody>
</table>

*: inoculation with a bundle of needles

**: wounding with knife

As shown in the Table 4, it can be seen that the cotyledonary nodes of seedlings are fast growing in the course of germination and thus inoculations and transformations with *Agrobacterium* are not easy. Actually, the cotyledonary nodes of half a seed germinated for 1 day or 2 days, were readily wounded with a bundle of needles. However, as for the cotyledonary nodes of half seeds germinated for more than 3 days, their tissues become hard, and wounding with needles was impossible. Instead of wounding with needles, the hardened tissues were wounded with a knife and then inoculated with *Agrobacterium*. Consequently, it can be seen that where transformation is performed using the cotyledonary nodes of germinating seedlings, young tissues germinated for less than 1 day are most efficient.
Example 5: Comparison of transformation efficiencies according to soybean breed

Transformation efficiencies were compared among the three soybean breeds: cultivars Jungery, Paldal and Danyup. After the three breeds were each transformed with *Agrobacterium*, efficiency for the transformation was calculated from the number of induced shoots. The results are presented in Table 5 below.

Table 5

<table>
<thead>
<tr>
<th>Breed</th>
<th>Number of segments</th>
<th>Number of induced shoots</th>
<th>Number of PPT-resistant shoots</th>
<th>Number of PPT-resistant seedlings</th>
<th>Transformation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paldal</td>
<td>120</td>
<td>48</td>
<td>2</td>
<td>1</td>
<td>0.8</td>
</tr>
<tr>
<td>Danyup</td>
<td>114</td>
<td>86</td>
<td>4</td>
<td>2</td>
<td>1.8</td>
</tr>
<tr>
<td>Jungery</td>
<td>130</td>
<td>122</td>
<td>20</td>
<td>16</td>
<td>12.3</td>
</tr>
</tbody>
</table>

Transformation efficiency of cultivar Jungery was 15 and 7 times higher than Paldal and Danyup, respectively. Accordingly, it can be seen that transformation with cultivar Jungery is most efficient.

Example 6: Transformant analysis

To confirm that selected transformants contain a target gene, the inventors isolated genomic DNAs of transformed plants and then PCR and Southern blotting were performed. A target gene was amplified by PCR using NOS promoter- and NOS terminator-specific primers having SEQ ID NO. 1 and SEQ ID NO. 2, respectively, and
then agarose gel electrophoresis was conducted. When Southern blotting was conducted using a bar gene-specific probe having SEQ ID NO. 3, no signal appeared in the non-transformant. While, as for the transformant, a 1.2 kb band was visualized. Based on the fact, it can be seen that the bar gene was transformed into a plant tissue (see Fig. 6). Furthermore, to confirm that a target gene is stably inserted into genomic DNA of the transformant, about 5 μg of genomic DNA was digested with SacII restriction enzyme, separated by 0.8% agarose gel electrophoresis, and transferred to a nylon membrane. As a result of a Southern blotting using bar gene-specific probe, a 0.6 kb fragment derived from a portion of the bar coding region, and 7 kb and 23 kb fragments derived from the expression cassette of the bar gene were visualized (see Fig. 7). Accordingly, it can be seen that a target gene was inserted into the genome of the transformed soybean plant. Furthermore, whole RNA was isolated from the transformed soybean plant, and a Northern blotting was performed. As a result, it could be seen that the inserted foreign gene was stably expressed in the transformant. As a result of the Northern blotting using bar gene-specific probe, no signal appeared in the non-transformant, while bar genes were stably expressed in the transformant (see Fig. 8).

Example 7: Test for acclimation and herbicide-resistance of transformant

Seedlings selected in media were transplanted into soil containers. When the seedlings reached the lids of the containers, they were acclimated by opening the lids. The acclimated seedlings were transplanted to soil and thus transformed soybean plants were obtained (see Fig. 9). To confirm that the transformant so produced had resistance
to herbicide, the inventors applied 100 ppm of herbicide to leaves of the transformant and the non-transformant. At 1 week after the application, as for the non-transformants, their leaves gradually wilted and died, while the transformants grew vigorously (see Fig. 10).

Example 8: Identification of presence of transplanted genes in second-generation transformants

Seeds obtained from soybean transformants were germinated in soil to obtain second-generation (T1) plants (see Fig. 11). To confirm that the second-generation plants had inserted foreign gene, genomic DNA was isolated and then PCR amplification and Southern blotting were performed. Specifically, a foreign gene was amplified by PCR using NOS promoter- and NOS terminator-specific primers, and then Southern blotting was carried out using a *bar* gene-specific probe. As a result, no signal appeared in the non-transformant, while strong signals appeared in transformant 2. In addition, in Southern blotting of *gfp* gene, strong signals appeared in all the transformants (see Fig. 12). When second-generation (T1) leaves of soybean transformant growing vigorously were observed under confocal fluorescent microscope, the inventors confirmed that *gfp* gene was strongly expressed in the leaves and roots of transformants (see Fig. 13). Accordingly, it can be seen that inserted foreign gene is expressed in progeny plants of transformants.
Industrial Applicability

As apparent from the above description, the present invention provides a useful method for transforming plants, in particular soybeans, by introduction of a target gene into the plants with *Agrobacterium*. Specifically, half a seed germinated for one day as a target tissue is suitably wounded and a herbicide-resistance gene is introduced thereinto. Then, transformed soybean plants are selected in herbicide-containing media. Accordingly, using the transformation method of the present invention, a new breed of transformed soybean, which is commercially valuable, can be developed and productivity of soybean can be remarkably enhanced.

Although the preferred embodiments of the present invention have been disclosed for illustrative purposes, those skilled in the art will appreciate that various modifications, additions and substitutions are possible, without departing from the scope and spirit of the invention as disclosed in the accompanying claims.
What is claimed is:

1. A method for transforming plants, comprising the steps of:
   1) selecting half a seed, which is germinated for one day as a target plant tissue;
   2) wounding the target plant tissue using a bundle of needles;
   3) inserting a target gene into the target plant tissue with *Agrobacterium tumefaciens* vector containing the target gene; and
   4) regenerating a whole plant from the target plant tissue.

2. The method according to claim 1, wherein the plant for transformation is selected from the group consisting of food crops, vegetables, special crops, fruit trees, ornamental plants and forage crops.

3. The method according to claim 2, wherein the food crops are selected from the group consisting of rice, wheat, barley, corn, soybean, potato, red bean, oats and sorghum; the vegetables are selected from the group consisting of *Arabidopsis*, Chinese cabbage, radish, red pepper, strawberry, tomato, watermelon, cucumber, cabbage, melon, pumpkin, green onion, onion and carrot; the special crops are selected from the group consisting of ginseng, tobacco, cotton, sesame, sugar cane, sugar beet, perilla, peanut and rape; the fruits trees are selected from the group consisting of apple, pear, jujube, peach, kiwi fruit (*Actinidia arguta*), grape, tangerine, persimmon, plum, apricot, and banana; the ornamental plants are selected from the group consisting of rose,
gladiolus, gerbera, carnation, chrysanthemum, lily and tulip; and the forage crops are selected from the group consisting of ryegrass, red clover, orchard grass, alfalfa, tall fescue and perennial ryegrass.

4. The method according to claim 1, wherein the plant for transformation is soybean.

5. The method according to claim 1, wherein the target plant tissue is half a seed, which is germinated for 16 to 24 hours.

6. The method according to claim 1, wherein the bundle of needles is composed of more than 2 needles.

7. The method according to claim 1, wherein herbicide-resistance bar gene is used as the target gene.

8. The method according to claim 1, wherein the wounding depth of the target plant tissue is 1-2 mm.

9. The method according to claim 1 or claim 5, wherein the target plant tissue is the cotyledonary node of half a seed.
pGB(11.4kb)

FIG. 1
FIG. 4
FIG. 5

non-transformant

transformant
non-transformant

transformant
12/13
FIG. 12

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