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Cotransformation and differential expression of introduced genes into potato (*Solanum tuberosum* L.) cv Bintje

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Summary. The Dutch potato cultivar Bintje has been transformed by *Agrobacterium* strain LBA1060KG, which contains two plasmids carrying three different DNAs (TL- and TR-DNA on the *Agrobacterium rhizogenes* plasmid and TKG-DNA on the pBI121 plasmid). Several transformed root clones were obtained after transformation of leaf, stem, and tuber segments, and plants were then regenerated from these root clones. The expression of the various marker genes [*rol*, opine, β-glucuronidase (*GUS*), and neomycin phosphotransferase (*NPTII*)] was determined in several root clones and in regenerated plants. The selection of vigorously growing root clones was as efficient as selection for kanamycin resistance. In spite of the location of *NPTII* and *GUS* genes on the same T-DNA, 17% of the root clones did not show *GUS* activity. Nevertheless, Southern blot analysis showed that these root clones contained at least three copies of the *GUS* gene. Sixty-four per cent of the root clones contained opines. The expression of these genes, however, was negatively correlated with plant regeneration capacity and normal plant development. The differential expression of the marker genes in the transgenic potato tissues is discussed.

Key words: *Agrobacterium rhizogenes* – Binary vector – Cotransformation – *GUS* gene – Kanamycin resistance gene

Introduction

Many cultivated varieties require improvement in important characteristics such as disease resistance. In this regard, genetic engineering provides a promising tool. The capacity to introduce single genes into the plant genome has already resulted in some plant species with agronomically interesting properties (Comai et al. 1985; Shah et al. 1986; Anzai et al. 1989). This has led to an interest in the introduction and expression of more than one gene in plants. Two or more foreign genes can be introduced into the plant genome either successively or simultaneously. However, the mode of expression of foreign genes and their effect on the resident genes play a major role in the ultimate realization of varietal improvement by genetic manipulation. Recently, Matzke et al. (1989) reported the suppression of transgenic expression by a second gene introduced later into the plant genome.

The Dutch potato cultivar Bintje is the most important variety in The Netherlands. Improvement of this cultivar has not been possible thus far by classical breeding because of autotetraploidy, heterozygosity, and male sterility. Therefore, current cell and molecular biological methods are now being used in order to produce transgenic plants. We have recently established transformation protocols for Bintje leaf, stem, and tuber explants using *Agrobacterium rhizogenes* (Hänisch ten Cate et al. 1987, 1988; Ottaviani et al. 1990). This procedure enables genetically stable "hairy root clones" and plants to be produced at a high frequency. Simpson et al. (1986) showed that *A. rhizogenes*, carrying a wild type of the Ri-plasmid, was capable of transferring the T-DNA from a second plasmid. With this approach a number of plant species have now been transformed (Simpson et al. 1986; Sukhapinda et al. 1987; De Vries-Uijtewaal et al. 1989; Visser et al. 1989).

The present article reports results on the transformation of the cultivar Bintje, using a binary vector of *Agrobacterium* carrying three different T-DNAs (TL-, TR-DNA from *Agrobacterium rhizogenes*, and TKG-DNA from pBI121). Four marker genes [*rol*, opine synthesis, neomycin phosphotransferase conferring resis-

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tance to kanamycin (*NPTII*), and β-glucuronidase (*GUS*)] were present on these T-DNAs, which were introduced simultaneously into the potato genome. The effect of selection pressure on (co)-transfer and (co)-expression of these genes was analyzed in a large number of transformants. Moreover, the mode and stability of gene expression in transformed root clones and regenerated plants are presented.

Materials and methods

Plant material

Tubers of the potato cv Bintje (*Solanum tuberosum* L.) were obtained from the Center for Variety Research and Seed Technology (CRZ) at Wageningen, The Netherlands. The tubers were stored in the dark at 4 °C until use. Leaves and stems were obtained from 2- to 3-month-old plants grown in a greenhouse.

Bacterial strain

The *Agrobacterium* strain LBA1060KG is derived from the LBA1060 strain (kindly supplied by Dr. P. J. J. Hooykaas, State University, Leiden, The Netherlands). This strain contains the agropine pRi1855 plasmid with TL- and TR-DNA and the pBI121 plasmid with TKG-DNA. The TL-DNA, with four *rol* loci (*A*, *B*, *C*, and *D*), is responsible for the induction of transformed roots and for the hairy (Ri) phenotype of roots and plants (White et al. 1985). The TR-DNA, with genes for the synthesis of opines (Jouanin 1984) and auxin (Offringa et al. 1986), affects the hormonal balance and development of the transformed plants. The TKG-DNA contains the *NPTII* gene, which confers resistance to kanamycin (Haas and Dowding 1975), and the bacterial *GUS* gene which is responsible for β-glucuronidase activity (Jefferson et al. 1987). The *NPTII* gene is under the control of the nopaline synthase (NOS) promoter and the *GUS* gene is under the control of the cauliflower mosaic virus (CaMV35S) promoter (Jefferson et al. 1987). The lack of intrinsic *GUS* activity in vegetative parts of untransformed potato plants and its easy and sensitive detection make the *GUS* gene a suitable reporter gene (Jefferson et al. 1987; Stiekema 1988).

Induction and culture of transformed root clones

Transformed root clones were obtained upon bacterial infection of tuber disks, stem, and leaf segments as described previously (Hänisch ten Cate et al. 1987). To examine the effect of kanamycin on root formation, part of the explants were cultured in the presence of kanamycin (100 mg/l), which was added 2 week after infection. The other explants were cultured in the absence of kanamycin. Transformed root clones were obtained after excision of single roots and propagated monthly on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) in a growth chamber under controlled conditions (24 °C; 16 h light, 3,000 lx; 8 h dark). The roots of clones that grew to a length of about 2 cm or more per week were considered vigorously growing and were selected for further analysis. The poorly growing root clones were not analyzed.

Plant regeneration and shoot culture

Shoot regeneration from root segments was obtained using the procedure described previously (Ottaviani et al. 1990). Shoots were propagated on MS medium. For phenotypic characterization and tuber formation, at least three plants per shoot line were grown in a greenhouse.

Test for kanamycin resistance

Root clones were considered kanamycin resistant (Km^r) when they continued to grow even after 2 months on MS medium supplemented with kanamycin (50 mg/l). Axenic shoot lines and greenhouse-grown plants were considered kanamycin resistant when they developed roots on stem or leaf segments in the presence of kanamycin (50 mg/l).

Opine test

The presence of opines (specific amino acid analogues, i.e., agropine and mannopine) in transformed tissues was established by paper electrophoresis and silver staining, as described by Petit et al. (1983).

β-Glucuronidase assay and determination of protein content

The β-glucuronidase (*GUS*) activity in various tissues was assayed by a fluorometric test with 4-methyl umbelliferyl glucuronide (MUG) (Jefferson et al. 1987). Protein concentration of plant extract was determined by the method of Bradford (1976) with a reagent supplied by Bio-Rad Laboratories.

Southern blot analysis

The extraction of DNA from young leaf material was carried out by phenol/chloroform extraction and ethanol precipitation, according to the method of Dellaporta et al. (1983). The samples of DNA (10 µg) were digested with HindIII and EcoRI, electrophoresed on 0.7% agarose gels, and blotted onto Hybond-N filters. Labelling of DNA fragments was carried out by random primer incorporation of digoxigenin-labelled deoxyuridine triphosphate. The labelled DNA fragments were conjugated with antibodies and subsequently detected by an enzyme-catalyzed color reaction with 5-bromo-4-chloro-3-indolphosphate and nitroblue tetrazolium salt (Boehringer Mannheim, Germany).

Nomenclature

Root clones were numbered from 1 to 73. Calli induced from these root clones were indicated by capital letters from A to D. Sister shoot lines were obtained from the same root clone and numbered with roman numerals I to IV, when they were obtained from the same callus. Thus, shoot line 6AII is the second primary shoot originated from callus A induced from root clone 6.

Results

Induction, selection, and characterization of root clones

To study the effect of selection pressure on root formation, induction of roots was performed in the presence or absence of kanamycin. In total, 165 explants were cultured in the presence of kanamycin and 85 in the absence of kanamycin [Table 1 (a)]. In the presence of kanamycin (100 mg/l), added after 2 weeks of incubation, 114 explants formed roots while 68 explants formed roots when cultured without kanamycin [Table 1 (a)]. This indicates that kanamycin slightly inhibited the root formation ($P = 0.05$).

Table 1. Root formation on explants of the potato cv Bintje and characterization of root clones obtained with or without kanamycin selection

	Root clones				
	With kanamycin		Without kanamycin		Total no.
	No.	%	No.	%	
(a) Root formation[a]					
Explants analyzed	165		85		250
Explants showing roots	114	69	68	80	182[e]
(b) Expression of marker genes in isolated clones					
Clones tested	60		30		
Vigorous growth	55	92	18	60	73[e]
Km^r	55	92	18	60	73
GUS^+	49	82	23	77	72[b]
$Opine^+$	43	71	13	44	56[d]
(c) Characteristics of 73 vigorously growing root clones					
GUS^+, $opine^+$	34	62	8	44	42
GUS^+, $opine^-$	13	24	6	33	19
GUS^-, $opine^+$	5	9	0	0	5
GUS^-, $opine^-$	3	5	4	23	7

[a] Pooled data from explants of leaf, stem, and tubers
P values indicate the significance of difference between two groups (i.e., with and without kanamycin), determined by a Chi-square test for a 2*2 table with Yates's continuity correction:
[b] $P=0.4$
[c] $P=0.05$
[d] $P=0.01$
[e] $P=0.005$
Km^r: Root clones resistant to kanamycin
GUS^+, GUS^-: Root clones with or without β-glucuronidase activity
$Opine^+$, $opine^-$: Root clones with or without opine

Table 2. Growth rate and *GUS* activity measured during different weeks of subcultures 6 and 10 on MS medium in transformed root clones of the potato cv Bintje

Root clone number	Growth rate of root clones (cm/week)	*GUS* activity (nmol MU/min/mg protein)				
		Subculture 6 (week)				Subculture 10
		1	3	5	6	3
5	4	30.0	24.0	35.0	30.0	24.0
63	2	1.0	1.5	1.9	2.1	2.2
6	3	0.8	0.4	0.4	0.8	0.8
31	3	0	0	0	0	0

The expression of marker genes was studied on 60 root clones obtained with kanamycin and 30 root clones obtained without kanamycin [Table 1 (b)]. The results show that 55 of the former and 18 of the latter exhibited vigorous growth (2–3 cm/week) suggesting that kanamycin promoted the formation of vigorously growing root clones ($P<0.01$). Moreover, the number of opine-positive root clones was also increased by the presence of kanamycin ($P=0.01$). In contrast, the presence of *GUS* activity was not affected by the presence of kanamycin ($P=0.4$). Irrespective of the presence of kanamycin, all 73 vigorously growing root clones were kanamycin resistant [Table 1 (b)]. Of these 73 root clones, 61 showed *GUS* activity, 47 expressed the opine synthesis gene, and 42 expressed both marker genes [Table 1 (c)].

Plant regeneration

After culture on shoot induction medium, 16 of the 47 opine-positive and 19 of the 26 opine-negative root clones regenerated plants ($P<0.01$). Shoot induction and culture were carried out in the absence of kanamycin. Nevertheless, all shoot lines maintained the kanamycin resistance trait, since they grew well and rooted in the presence of kanamycin (50 mg/l).

Stability of gene expression

Stable expression of foreign genes is a prerequisite for succesful genetic manipulation of crop plants. Furthermore, early selection of transgenic tissue at the root level is highly desirable. Therefore, the expression of opine and *GUS* genes in five root clones (four $opine^+$ and GUS^+, and one $opine^+$ and GUS^-) was compared with that in the respective regenerated plants.

Opine expression. The root tissues of the root clones showed opine production when observed at four different periods spread over 1 year. Each time, four plants per root clone were regenerated. Initially, two of the opine-positive root clones each regenerated four plants which showed no opine synthesis. However, the 72 plants regenerated later were found to be opine positive. In total, 8 of the 80 regenerated plants showed no opine expression.

GUS expression. In contrast to the occasional loss of opines, *GUS* expression was consistent in all regenerants. All shoots regenerated from the four *GUS*-positive root clones were *GUS* positive (including the regenerants which had lost opine expression), whereas the shoots regenerated from the *GUS*-negative root clones lacked *GUS* activity.

Activity of GUS at the root and plant levels

Out of 73 kanamycin-resistant root clones, 61 root clones showed *GUS* activity which varied by 500-fold (from 0.1 to 50 nmol MU/min/mg protein). Of these root clones, 4 with approximately the same growth rate, but showing different *GUS* activities, were analyzed to investigate

Table 3. Activity of *GUS* in tissues of various parts of plants regenerated from four different potato cv Bintje transformed root clones. Per root clone, two to three sister shoot lines were regenerated. *GUS* activity was measured in one plant of each shoot line

Root clone and shoot line number	*GUS* activity (nmol MU/min/mg protein) in tissues of various plants					
	Root	First leaf (juvenile)	Third leaf	Eight leaf (mature)	Internode	Tuber
Root clone 5	24.0[a]					
Shoot line						
5AII	2.8	0.23	0.7	2.8	12.7	1.5
5BI	15.3	3.8	3.1	5.5	12.7	8.2
Root clone 6	0.8[a]					
Shoot line						
6AI	6.1	0.04	0.3	0.4	1.8	0.9
6AII	1.5	0.02	0.1	0.6	1.5	1.0
6BI	6.3	0.08	0.7	1.6	2.1	0.7
Root clone 8	0.9[a]					
Shoot line						
8AI	11.0	0.1	0.39	2.2	11.0	7.9
8BI	18.0	2.8	2.9	5.5	10.0	12.0
Root clone 63	2.2[a]					
Shoot line						
63AI	2.8	0.06	0.42	2.1	8.6	11.1
63BI	7.3	0.03	0.09	1.8	2.9	NT
Root clone 31	0[a]					
Shoot line						
31CI	0	0	0	0	0	0

[a] Tenth subculture of root clones on MS medium
NT = plant 63BI did not form tubers

GUS activity during various period of subculture. No significant variation in *GUS* activity during the subcultures was observed (Table 2). Furthermore, *GUS* activity was determined in tissues of various parts of plants regenerated from five root clones, including those mentioned above (Table 3). In all shoot lines, *GUS* activity in leaves increased with the age of the plants. The highest activity was observed in stem, roots, or tubers, irrespective of the original root clone. A considerable variation in *GUS* activity was observed in sister shoot lines (plants regenerated from the same root clone). Nevertheless, *GUS* activity was generally higher in plants regenerated from root clones with high *GUS* activity.

Presence of the GUS gene

To investigate whether the absence of *GUS* activity in some of the regenerants was due to the absence or inactivity of the gene, the length and copy number of the *GUS* gene in the genome of three *GUS*-negative (31AIII, 36BIII, 53BI) and four *GUS*-positive (5AII, 6AI, 8BI, 63BII) shoot lines were determined by Southern blot analysis. In all lines the predicted 3-kb EcoRI/HindIII fragment was observed. In three shoot lines (*GUS*$^-$ and *GUS*$^+$), addition hybridizing fragments of different lengths were found (Fig. 1). The copy number of the *GUS* gene in the plant genome was estimated by compar-

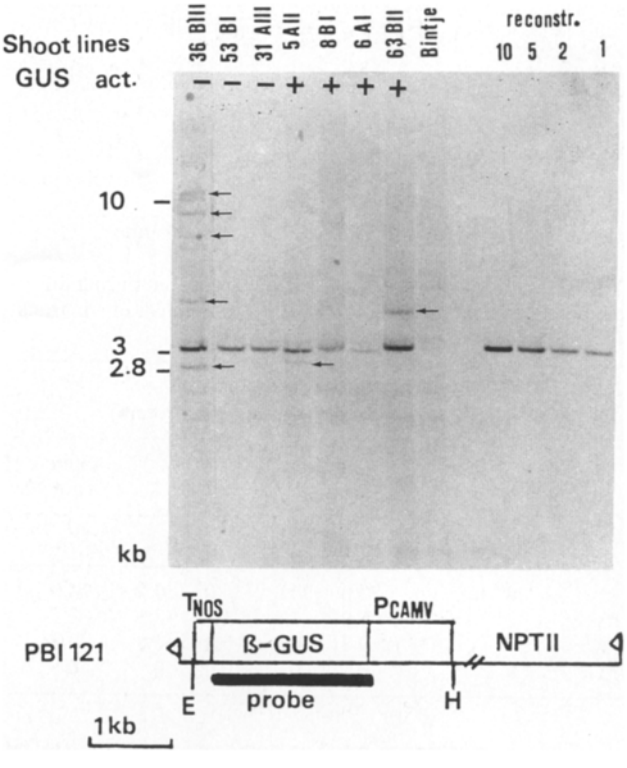

Fig. 1. Southern blot of DNA extracted from plants regenerated from various root clones and digested with EcoRI and HindIII. The filter was hybridized with the fragment containing the coding region of the *GUS* gene. The *arrows* indicate extra DNA fragments

Fig. 2. Phenotypic variation among mature plants and tubers of seven transformed shoot lines of the potato cv Bintje

Table 4. Root growth on MS medium in the presence (Km^+, 50 mg/l) or absence (Km^-) of kanamycin, *GUS* activity, and the estimated copy number of the *GUS* gene in transgenic shoot lines of the potato cv Bintje

Shoot line numbers	Growth of roots (cm/week)		*GUS* activity in the 3rd leaf (nmol/MU/min/mg protein)	Copy number of *GUS* gene
	Km^+	Km^-		
6AI	3	3	0.3	2
63BII	2	2	0.4	6
5AII	4	4	3.1	5
8BI	4	4	2.9	2
31AII	3	3	0	3
53BI	2	1	0	10
36BIII	3	2	0	10

ing the intensity of hybridizing bands with those of the *GUS* reconstruction. It varied from two to ten copies and was not correlated with the level of *GUS* activity (Fig. 1, Table 4). Moreover, no strict correlation was observed between the level of *GUS* activity and resistance to kanamycin (50 mg/l) in the shoot lines (Table 4).

Phenotypic characterization of transformed plants

Table 5 gives data on phenotypic variation and opine expression in transformed plants. Plants were analyzed that had been grown to maturity in a greenhouse. These came from seven shoot lines, selected on their distinct levels of *GUS* expression and the presence of opines.

Table 5. Phenotypic characterization and presence of opines of mature plants from control and seven regenerated shoot lines of the potato cv Bintje. Three plants per shoot line were scored and pooled data are presented

Shoot line number	Phenotypic characters				Opine
	Leaf form	Plant habit	Tuber form	Tuber weight (g/plant)	
5AII	N	N	Ri	210± 54	−
8BI	N	N	Ri	210± 32	−
36BIII	N	Ri	Ri	174± 41	−
31AIII	N	Ri	Ri	133± 20	−
53BI	D	Ri[a]	Ri	7± 2	+
6AI	Ri	Ri[b]	Ri	51± 3	+
63BII	D	Ri[c]	Ri	3± 1	+
Bintje	N	N	N	229±100	−

[a] Early flowering and senescence
[b] Additional axillary tuber formation
[c] Poorly growing plants with early tuber formation
N: Normal Bintje phenotype
Ri: Presence of Ri traits
D: Deviant from normal and Ri phenotype

Variation was observed in the expression of the Ri phenotype, i.e., diminished length and decreased apical dominance of plants, wrinkled leaves, oblong tubers with many deep-set eyes, and accelerated flowering and senescence. Differences were obvious between plants of different shoot lines, as well as between organs of the same

plant (Fig. 2). All shoot lines had developed typical Ri tubers, irrespective of the plant phenotype. In shoot lines that showed a pronounced Ri phenotype and contained opines, tuber yield was extremely low.

Discussion

The results on selection pressure showed that kanamycin supplemented 2 weeks after transformation with the binary vector of *Agrobacterium* tended to inhibit root formation on potato cv Bintje explants, confirming the results of Hamill et al. (1987). All root clones selected for vigorous growth were kanamycin resistant. This suggests that TL- and TKG-DNAs were integrated simultaneously into the plant genome. This conclusion is supported by the data for the large number of transformants analyzed, and confirms the results of Hamill et al. (1987), who showed complete cointegration of T-DNAs from two plasmids (pRi and pBin19 plasmids) into plant genome without selection pressure. In contrast to the simultaneous expression of genes carried by TL- and TKG-DNAs, the opine genes of TR-DNA were expressed in only 64% of the root clones. This might be due to the failure of TR-DNA integration or to the absence of expression of the TR-DNA genes (Taylor et al. 1985). The selection for vigorous growth is probably a negative selection on the expression of TR-DNA genes in the transformed potato. This might be the result of a negative effect of higher auxin concentration on root growth, due to the expression of the auxin synthesis genes of the TR-DNA. However, in tobacco, Vilaine and Casse-Delbart (1987) observed an increased root induction upon transformation with *A. rhizogenes* plasmids containing TL- and TR-DNA, compared to transformation with deleted plasmids containing TL- or TR-DNA only. In *Daucus*, Cardarelli et al. (1987) attributed the stimulation of TR-DNA on root formation to an auxin effect.

The expression of TR-DNA might also interfere with the regeneration capacity of opine-containing root clones, and with plant development and tuber formation of opine-positive shoot lines. Apparently, the phenotypic expression of the *rol* genes of TL-DNA (Spena et al. 1987; Schmulling et al. 1988) is enhanced by the expression of auxin synthesis genes of TR-DNA. This conclusion is supported by the fact that: (1) transformed root clones require less auxin than control roots for the formation of shoot-competent calli (Ottaviani et al. 1990), (2) transformed cells are more sensitive to auxin than untransformed cells (Shen et al. 1988), and (3) a high cytokinin/auxin ratio is required for shoot regeneration and plant development (Skoog and Miller 1957).

All shoot lines, including those that were phenotypically similar to normal cv Bintje plants, formed Ri tubers, suggesting a differentially organ-specific expression of the Ri phenotype. Previously in cv Bintje we have observed root clone-dependent variation in the Ri phenotype (Hänisch ten Cate et al. 1988). Reduced apical dominance, as well as internodal length, are correlated with the expression of the *rol C* locus (Spena et al. 1987; Oono et al. 1987). Moreover, a higher number of transcripts of *rol C* is found in tubers compared to that in stem or leaf tissue of potato (Ooms et al. 1986). Thus, it is likely that the phenotype of the oblong Ri tubers containing many deep-set eyes is due to the tuber-specific or enhanced expression of the *rol C* locus.

All root clones of cv Bintje obtained after transformation and all regenerated plants were kanamycin resistant, indicating that the *NPTII* gene is stably expressed in the transformed root clones. However, 17% of the root clones and their regenerated plants showed no expression of the *GUS* gene. This absence of activity was not due to the presence of an inhibitor, because addition of purified commercial enzyme to plant extracts resulted in the expected additional activity (data not shown). In spite of the absence of detectable *GUS* activity in three shoot lines, at least three copies of the *GUS* gene were present. The presence of additional hybridizing DNA fragments in three shoot lines indicates that deleted or rearranged fragments also occurred. These additional fragments were present in both *GUS*-negative and *GUS*-positive shoot lines, and therefore their occurrence was not correlated with the absence of *GUS* activity. Furthermore, there was no correlation between *GUS* activity and its copy number. It is generally expected that both the *GUS* and *NPTII* genes are located at the same sites in the plant genome, because in the *Agrobacterium* they were located on one plasmid between the same T-DNA borders. The observed differences in the expression of the two genes might be due to specific methylation of the chimeric (CaMV35S/*GUS* gene leading to gene inactivation (Hepburn et al. 1983; Saedler et al. 1989). However, Matzke et al. (1989) showed that the NOS promoter can also be methylated. The lack of correlation of expression of *NPTII* and *GUS* genes might be more likely ascribed to differential regulation of the NOS and CaMV35S promoters by enhancers located outside the T-DNA (Dynan and Tijan 1985).

The present data reveal a high frequency of transformation and gene expression, using *A. rhizogenes* plasmid as virulence vector, and confirm that the *vir* genes on Ri plasmid act efficiently in *trans* (Simpson et al. 1986; De Vries-Uijtewaal et al. 1989; Visser et al. 1989). Furthermore, this study shows that the expression of cointroduced genes can vary independently, irrespective of their original location on the same or separate T-DNAs in the *Agrobacterium* vector. It is not likely that the variation in expression is caused by a chimeric origin of the transformed tissues. This is supported by a unicellular origin of roots induced by *A. rhizogenes*, as described previously (Tepfer 1983; Hänisch ten Cate et al. 1990). Further

knowledge regarding the effect of gene insertion site, different promoters, as well as the border sequences is highly desirable for the successful realization of genetic manipulation as a technique in varietal improvement.

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