Corporation obtaining approval, the name of its representative, and the address of its main office

Name of Entity: Monsanto Company
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Approved Type 1 Use Regulation

Name of the type of Living Modified Organisms:	Low-lignin alfalfa (*CCOMT, Medicago sativa* L.) (KK179, OECD UI: MON-ØØ179-5)
Contents of Type 1 Use etc. of Living Modified Organisms	Use for provision as food or animal feed purposes, cultivation, processing, storage, transportation and disposal, and other acts attendant with these.
Methods of Type 1 Use etc. of Living Modified Organisms	—

Overview of the Biological Diversity Risk Evaluation Report

1st Information collected for biological diversity risk evaluation

1 Information regarding the preparation of living modified organisms

Monsanto Company and Forage Genetics International (FGI) developed "low-lignin alfalfa (*CCOMT*, *Medicago sativa* L.) (KK179, OECD UI: MON-ØØ179-5)(hereinafter called the "recombinant alfalfa")," which lowers lignin content in plant bodies by depressing expression of caffeoyl CoA 3-O-methyltransferase, which is a main enzyme in a lignin biosynthetic pathway, (hereinafter called "CCOMT protein").

The recombinant alfalfa is produced by introducing an inverted repeat, partial sequence of *CCOMT* gene, alfalfa endogenous gene (hereinafter called *"CCOMT"*"gene fragment").

This transcriptional product in an inverted repeat sequence forms double-stranded RNA (dsRNA) and depresses expression of alfalfa endogenous *CCOMT* gene by RNA i[1].

Lignin contents in plant bodies are reduced by depressing the *CCOMT* gene expression (Figure , p14).

Lignin exercises negative effects on ruminants' feed digestion rate(Chen et al., 2006). This is because the combination of lignin with carbohydrate, which forms cell walls, prevents microorganisms in the digestive tract of ruminants from proteolysis(Akin, 1988). A pastures of lower digestibility is regarded as a low quality and its values is affected. In addition, if a pasture is harvested later than the optimal time, lignin is accumulated in plant bodies, which lowers the quality of the pasture.

The total lignin content derived from the ground segment of the recombinant alfalfa is equivalent to the content of conventional varieties of feed harvested a few days earlier under the similar production condition. Therefore, in the growth period when the feed quality starts to be remarkably deteriorated, the feed quality of the recombinant alfalfa is not. Producers can

[1] RNAi is a mechanism that typically occurs to regulate gene expression in a eukaryote. RNAi is caused by cutting double-stranded RNA (dsRNA) with enzyme called Dicer and forming 21-25 base siRNA. After coupled to RNAi-induced silencing complex (RISC), siRNA is coupled to mRNA having a target complementary sequence(Siomi and Siomi, 2009). mRNA coupled to siRNA is degraded by RISC and protein production is inhibited. Having a high specificity and a high gene expression inhibiting effect, RNAi is used to give specific characters and analyze gene functions(Kusaba, 2004).

harvest the recombinant alfalfa a few days later in comparison with conventional varieties. Specifically, such expansion of the harvest period brings the following benefits to producers:

- The purpose is high-quality feed production: The recombinant alfalfa harvested in a general harvesting time has lower lignin contents in comparison with conventional varieties harvested in the same period. It is likely that the quality as feed is equal to or more than the quality standard target of producers. The yield is maintained comparable with those of conventional varieties.
- In case of the recombinant alfalfa aiming for high yield: Producers can ensure high yield without significantly impairing quality by delaying harvests for a few days. During alfalfas' reproducing/growing seasons, the dry matter weight of alfalfa can be increased by 225 kg per hectare a day(Undersander et al., 2009). Therefore, by slightly retarding the harvest timing, the yield greatly increases. if the recombinant alfalfa is harvested a few days later, the accumulation of lignin is smaller and the alfalfa has the same quality as conventional varieties harvested a few days earlier. If similarly, conventional varieties are harvested a few days later, the yield is equal to Line KK179, but lignin contents are larger, and the quality as feed is lower.
- Unexpected harvest delay reduces the deterioration of quality: Unseasonable weather events such as rain and failure of equipment may unexpectedly delay the harvest. In many cases, lignin accumulation during such delay rapidly lower the quality as feed and economic losses. In case of the recombinant alfalfa, less lignin is accumulated during the delay of harvest, which reduce quality losses as feed until harvest, and a short-time delay of harvest is endurable. Producers can improve flexibility of the cultivation management.

(1) Information concerning provided nucleic acid

a. Configuration and Origins of Components

The configuration and the origins of components of provided nucleic acid used to produce the recombinant alfalfa are shown in Fig. (p5) and Table (p6-8).

b. Component functions

(1) Objective gene, expression-regulating region, localization signal, selection marker, and each function of components of other provided nucleic acids

The functions of components of provided nucleic acid used to produce the recombinant alfalfa are shown in Table (p6-8).

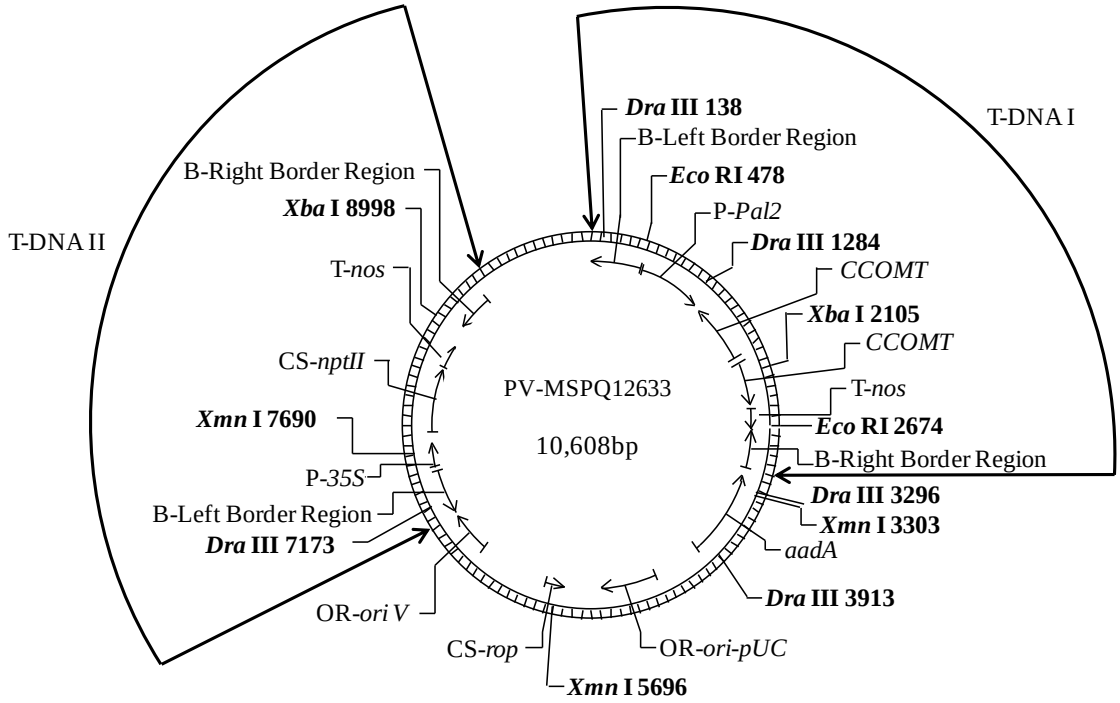


Fig. 2 The plasmid map of PV-MSPQ12633 used to produce the recombinant alfalfa[2]

The relative position of the cleavage site of a restriction enzyme is shown on the right of an enzyme name.

Selected individual pieces having T-DNAI region but not T-DNAII region in the drawing above in the process of producing the recombinant alfalfa.

[2]Rights and responsibilities of contents related to the information shown in this drawing belong to Monsanto Japan Ltd.

Table 2 Configuration of Provided Nucleic Acid and Origins and Function of Components[3]

Components	Location in a Plasmid	Origin and Function
T-DNA I region		
B[Note1]-Left Border Region	1-442	Includes the left border sequence used to transmit T-DNA in DNA region derived from *Agrobacterium tumefaciens* (Barker et al., 1983).
Intervening Sequence	443-490	Sequence used at the time of DNA cloning
P[Note2]-*Pal2*	491-1,567	Promoter of *Pal2* gene coding phenylalanine ammonia-lyase (PAL) derived from common bean (Phaseolus vulgaris) (Cramer et al., 1989). It responds to an endogenous signal, which promotes the formation of vascular bundles, and specially expresses in a lignin deposition site in a mature plant(Leyva et al., 1992; Guo et al., 2001).
Intervening Sequence	1,568-1,584	Sequence used at the time of DNA cloning
CCOMT[*]	1,585-2,103	A partial sequence of CCOMT gene coding CoA3-O-methyltransferase derived from alfalfa(*Medicago sativa*) (Inoue et al., 1998). It constitutes gene suppression cassettes
Intervening Sequence	2,104-2,110	Sequence used at the time of DNA cloning
*CCOMT**	2,111-2,410	A partial sequence of *CCOMT* gene coding caffeoyl CoA3-O-methyltransferase derived from alfalfa(*M. sativa*) (Inoue et al., 1998). It constitutes gene suppression cassettes
Intervening Sequence	2,411-2,418	Sequence used at the time of DNA cloning
T[Note3]-*nos*	2,419-2,671	It induces polyadenylation in the untranslated region of 3' terminal of the nopaline synthetase gene coding NOS derived from *A. tumefaciens* pTi (*nos*) (Bevan et al., 1983; Fraley et al., 1983b).
Intervening Sequence	2,672-2,727	Sequence used at the time of DNA cloning
B-Right Border Region	2,728-3,084	Includes the right border sequence used to transmit T-DNA in DNA region derived from A. tumefaciens.(Depicker et al., 1982; Zambryski et al., 1982)

[3]Rights and responsibilities of contents related to the information shown in this table belong to Monsanto Japan Ltd.

Table 2 (cont.) Configuration of Provided Nucleic Acid and Origins and Function of Components

Components	Location in a Plasmid	Origin and Function
Exoskeleton region (not exist in the recombinant alfalfa)		
Intervening Sequence	3,085-3,199	Sequence used at the time of DNA cloning
aadA	3,200-4,088	Germ promoter, coding sequence and untranslated region of 3' of 3" (9) -*O*-nucleotidyltransferase (aminoglycoside modified enzyme) of transposon *Tn7.* (Fling et al., 1985) Provide spectinomycin and streptomycin resistance.
Intervening Sequence	4,089-4,618	Sequence used at the time of DNA cloning
OR[Note 4]-*ori-pUC*	4,619-5,196	It is the Origin of Replication derived from pUC plasmid and the autonomous replication function is provided in *Escherichia coli*(Vieira and Messing, 1987).
Intervening Sequence	5,197-5,623	Sequence used at the time of DNA cloning
CS[Note5]-*rop*	5,624-5,815	The copy number of plasmid in coding sequence of repressor of primer protein deprived from ColE1 plasmid should be kept in *E. coli*(Giza and Huang, 1989).
Intervening Sequence	5,816-6,552	Sequence used at the time of DNA cloning
OR-*ori V*	6,553-6,949	It is the replication initiation region derived from wide host range of plasmid RK2 and the autonomous replication function is provided in *Agrobacterium*(Stalker et al., 1981).
Intervening Sequence	6,950-7,035	Sequence used at the time of DNA cloning
T-DNA II region (not exist in the recombinant alfalfa)		
B-Left Border Region	7,036-7,477	Includes the left border sequence used to transmit T-DNA in DNA region derived from *A. tumefaciens* (Barker et al., 1983).
Intervening Sequence	7,478-7,527	Sequence used at the time of DNA cloning
P-*35S*	7,528-7,851	cauliflower mosaic virus (CaMV) 35S promoter(Odell et al., 1985). Transfers are constantly induced in plant cells.

Table 2 (cont.) Configuration of Provided Nucleic Acid and Origins and Function of Components

Components	Location in a Plasmid	Origin and Function
T-DNA II region (not exist in the recombinant alfalfa)		
Intervening Sequence	7,852-7,884	Sequence used at the time of DNA cloning
CS-*nptII*	7,885-8,679	Gene derived from *E. coli* transposon Tn5 and coding neomycin phosphotransferase II (Beck et al., 1982). Provide neomycin and kanamycin resistance(Fraley et al., 1983a).
Intervening Sequence	8,680-8,710	Sequence used at the time of DNA cloning
T-*nos*	8,711-8,963	It induces polyadenylation in the untranslated region of 3' terminal of the nopaline synthetase (nos) gene coding NOS derived from *A. tumefaciens* pTi (Fraley et al., 1983a; Bevan, 1984).
Intervening Sequence	8,964-9,048	Sequence used at the time of DNA cloning
B-Right Border Region	9,049-9,405	Includes the right border sequence used to transmit T-DNA in DNA region derived from A. tumefaciens (Depicker et al., 1982; Zambryski et al., 1982).
Exoskeleton region (not exist in the recombinant alfalfa)		
Intervening Sequence	9,406-10,608	Sequence used at the time of DNA cloning

Note [1]B, Border (border sequence)

Note [2]P, Promoter (promoter)

Note [3]T, Transcription Termination Sequence

Note [4]OR, Origin of Replication

Note [5]CS, Coding Sequence

* T-DNAI region has 2 *CCOMT* gene fragments. 1,654th through 1,953th sequences (sense strand) in the first *CCOMT* are reverse complementary sequences of 2,111 through 2,410 in the second *CCOMT*. The sequences between a sense strand and a anti-sense strand become loop structures in forming dsRNA.

(2) If a function of a protein generated by the manifestation of an objective gene and a selection marker and the protein are allergic, and there is a homology to the protein, a statement to that effect

The *CCOMT* gene fragment introduced to the recombinant alfalfa is a part of *CCOMT* gene, an endogenous gene of alfalfa (Table , p6-8), dsRNA is produced from a transcriptional product from an inverted repeat sequence, and endogenous *CCOMT* gene expression is specifically inhibited by RNAi.

The nucleic acid like RNA universally exists in flora and fauna. It is reported that there are thousands of RNAs having high homology with genes of humans and livestock animals in rice grains. A part of 21bp short RNAs in rice grains have 100 % homology with important human gene sequences (Ivashuta et al., 2009). In addition, there are dsRNA, a precursor of siRNA inducing RNAi in many eukaryotes (Ivashuta et al., 2009; Parrott et al., 2010). Moreover, there are many endogenous dsRNA, miRNA, and siRNA in plant cells. At the same time, dsRNA derived from extraneous virus accumulated in cells(Gould and Francki, 1981; Fukuhara et al., 1993; Cock et al., 1997; Heisel et al., 2008; Ivashuta et al., 2009; Jensen et al., 2013; Petrick et al., 2013). These show vertebrates have a long history of safely eating various RNAs.

Furthermore, there is no report that RNA is allergic and toxic. Nucleic acid has a long history of safely being eaten and received a GRAS (generally recognized as safe) [4] from the U.S. Food and Drug Administration (FDA)(FAO-WHO, 1991; U.S. FDA, 1992).

(3) If nucleic acid changes host's metabolic system, the details

First of all, the formulation and the role of lignin in plant bodies, and alfalfa's lignin biosynthetic pathway are as follows.

There are two types of plat cells: the primary cell wall formed between cells in their growing processes; and the strong secondary cell wall formed inside the primary one after the growth stops. Lignin is one of main components of the secondary cell wall in conjunction with cellulose and hemicellulose. The function of lignin in plant bodies is the maintenance of cell wall architecture and related to the substance transport in plants and disease

[4] Foods generally recognized as safe by FDA

resistance(Vance et al., 1980).

In the lignin biosynthetic pathway, three kinds of lignin subunits are predominately synthesized, undergo polymerization, and produce lignin. There are three kinds of lignin subunits: guaiacyl lignin (hereinafter called “G lignin”), syringyl lignin (hereinafter called “S lignin”) , and *p*-hydroxyphenyl (H) lignin (hereinafter called H lignin) (Figure, p11)(Boerjan et al., 2003; Vanholme et al., 2010). The ratio of lignin subunits in lignin varies depending on the plant classification and the organization(Boerjan et al., 2003). In case of alfalfa, the ratio of G lignin and S lignin in lignin should comprise 95 % at most(Chen et al., 2006).

To produce G and S lignin in a lignin biosynthetic pathway, two *O*-methyltransferases are required: CCOMT protein and caffeine acid *O*-methyltransferase (hereinafter called “COMT protein”). *O*-methyltransferase is a group of enzymes that methylates oxygen atoms of secondary metabolite production such as phenylpropanoid, flavonoid, and alkaloid(Lam et al., 2007). CCOMT protein methylates caffeoyl CoA to produce feruloyl CoA in the lignin biosynthetic pathway, COMT protein methylates caffeoyl aldehyde to produces coniferyl aldehyde, and methalates 5-hydroxyphenyl aldehyde to produce sinapaldehyde respectively (Figure, p11). With regard to producing lignin in alfalfa, COMT protein is specifically involved in the formation of S lignin and COMT protein has been known to be also involved in the formation of G lignin(Figure, p11)(Guo et al., 2001; Zhou et al., 2010). It is reported that by suppressing CCOMT protein of these two enzymes, the production of G lignin is lowered (Guo et al., 2001; Chen et al., 2006).

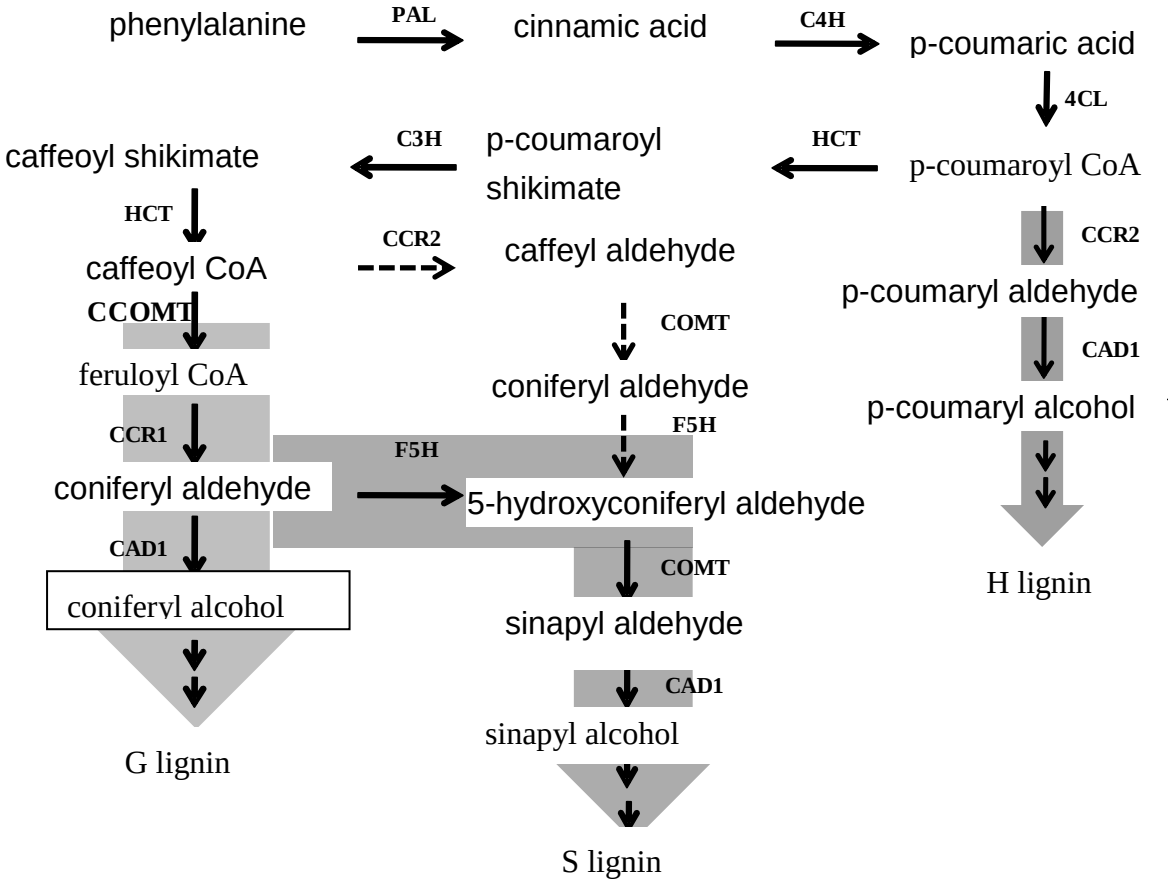


Figure 3 Lignin Biosynthetic Pathway in Alfalfa[5]

PAL: Phenylalanine ammonia-lyase

C4H: Cinnamate 4-hydroxylase

4CL: 4-coumarate: CoA ligase

HCT: Hydroxycinnamoyl - CoA: shikimate hydroxycinnamoyl transferase

C3H: *p*-coumarate -3-hydroxylase

CCOMT: Caffeoyl CoA 3-*O*-methyltransferase

COMT: Caffeine acid *O*-methyltransferase

CCR1, CCR2: Cinnamoyl CoA reductase

F5H: Ferulate 5-hydroxylase

CAD1: Cinnamyl alcohol dehydrogenase

If a route catalyzed from caffeoyl CoA to CCOMT and CCR1 is suppressed in a non-recombinant alfalfa, the route shown by a dashed line (caffeoyl CoA → caffeoyl aldehyde → coniferyl aldehyde) works(Zhou et al., 2010). Gray shading shows the biosynthesis phase of each lignin/subunit.

[5]Rights and responsibilities of contents related to the information shown in this drawing belong to Monsanto Japan Ltd.

Next, the activity of *CCOMT* gene fragment in the recombinant alfalfa is described.

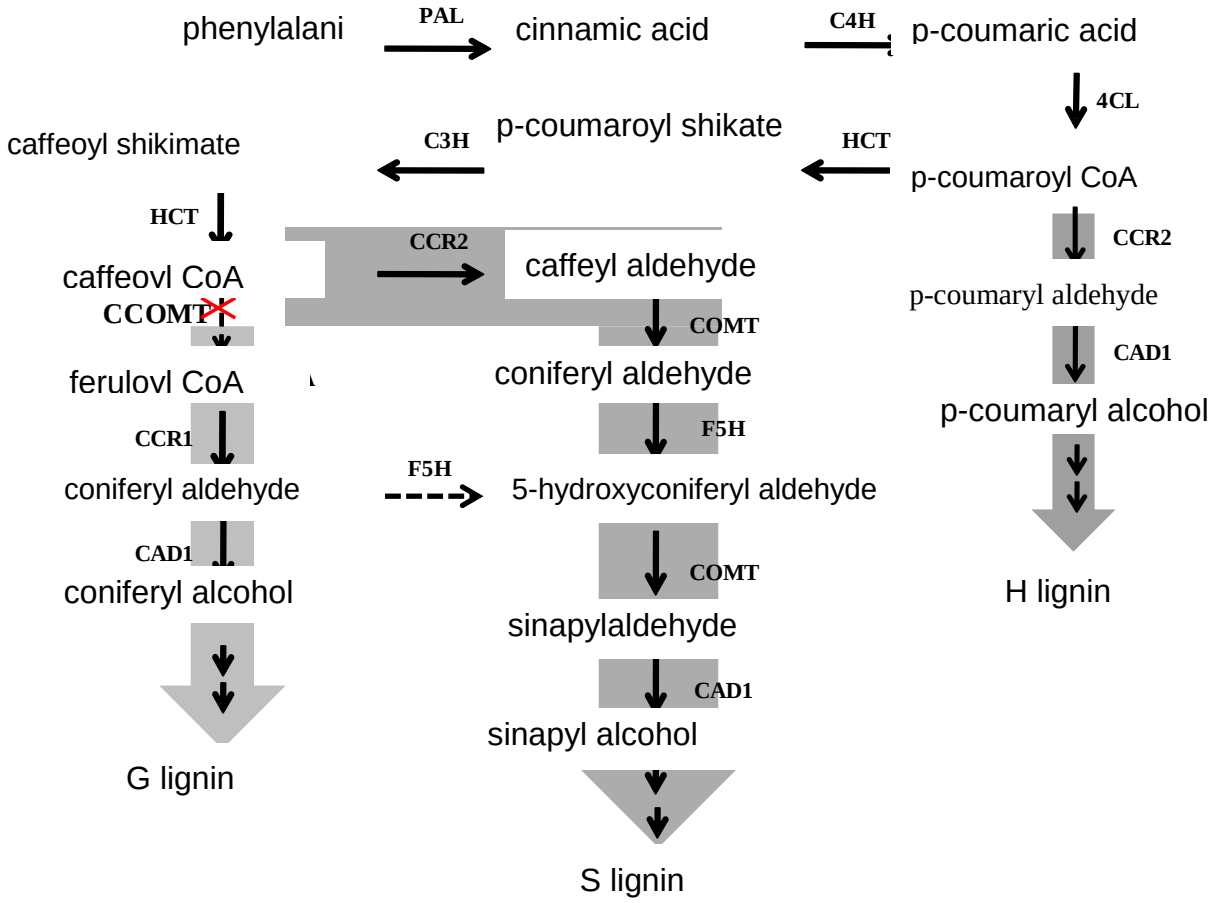
The gene expression control cassette in the recombinant alfalfa is designed so that the transcriptional product produced from the inverted repeat sequence of endogenous *CCOMT* gene fragments can be double-stranded RNAs (dsRNA). By degrading mRNA derived from alfalfa endogenous *CCOMT* gene, the target *CCOMT* gene expression is suppressed.

Furthermore, the introduced *CCOMT* gene expression control cassette is controlled by the *Pal2* promoter derived from phenylalanine ammonia-lyase (PAL) gene of common bean (*Phaseolus vulgaris*). *PAL* gene responds to an endogenous signal, which promotes the formation of vascular bundles, and specially expresses in a lignin deposition site in a mature plant(Leyva et al., 1992; Guo et al., 2001). Therefore, *CCOMT* gene fragments of the recombinant alfalfa develops in an organization where much lignin deposition is found (Leyva et al., 1992; Guo et al., 2001).

Next, a change in the metabolism system generated by the suppression of *CCOMT* gene fragment in the recombinant alfalfa is described.

It has been reported that even if CCOMT protein enzyme activity is reduced in *M. truncatula*, a congenic plant of *CCOMT* gene-deleted alfalfa, S lignin biosynthesis continues in another route in the lignin biosynthetic pathway. This biosynthesis is conducted through conversion from caffeoyl CoA to caffeoyl aldehyde by CCR2 (Figure , p14) (Zhou et al., 2010). In addition, it has been reported that the effect of suppressing expression of CCOMT protein is limited to the decline of G lignin production in the alfalfa where the expression of *CCOMT* genes are suppressed(Chen et al., 2006). The decline of this G lignin lowers the relative ratio of G lignin contained in the lignin. It has been confirmed that the decline of G lignin increases the relative ratio of S lignin but that the absolute amount of S lignin does not increase(Chen et al., 2006). As a result, the percentage of G lignin has decreased, and the rate of S lignin in all subunits has increased. Changes in the percentages of lignin subunits can be confirmed as the ratio of S- to G-lignin, namely, S/G-ratio lignin, and the increase of S/G lignin ratio is a characteristics shown when *CCOMT* genes in alfalfa are suppressed(Chen et al., 2006).

It has been confirmed that if actually, *CCOMT* gene expression control cassette specifically works on *CCOMT* genes, only G lignin decreases without changing S and H lignin (Table , p17 and Table 4, p18). In addition, it has been confirmed that the total lignin content is reduced with a reduced G lignin content (Table , p19). The following are details of each analysis results.



5 Figure 4 Lignin Biosynthetic Pathway in the Recombinant Alfalfa[6]

The official names of enzymes in a lignin biosynthetic pathway in the drawing are shown in Figure (p11).

✕ shows endogenous enzymes (CCOMT protein) gene expression is suppressed in the recombinant alfalfa.

A route shown by a dashed line weakens the action in the line where the expression of CCOMT protein is suppressed(Zhou et al., 2010).

[6]Rights and responsibilities of contents related to the information shown in this drawing belong to Monsanto Japan Ltd.

Changes in Lignin Subunits in the Ground segment of the Recombinant Alfalfa(Attached reference **1)**

Lignin subunits were analyzed to confirm G lignin decreases by suppressing the expression of *CCOMT* genes in the recombinant alfalfa. In 2011, samples were taken from first cut of recombinant alfalfa, reference non-recombinant alfalfa (C0-Syn1 generation[7]), and conventional commercial species, which were raised in six (6) fields in the U.S. (States of California, Iowa, Illinois, Kansas, Texas, and Wisconsin (Attached reference **1**, p12-14).

For the collected ground segment, H and G lignin, and S lignin caffeyl lignin (derived from caffeyl aldehyde, Figure , p14) and 5-hydroxyguaiacyl lignin (derived from 5-hydroxyconiferyl aldehyde, Figure , p14) were analyzed. Caffeyl lignin and 5-hydroxyguaiacyl lignin are not regarded as major lignin subunits in alfalfa but were analyzed because it has been reported that they are decreased when the expression of *CCOMT* genes in coniferous trees. Furthermore, two lignin subunits of caffeoyl lignin and 5-hydroxyguaiacyl lignin were excluded from a statistical analysis because all actual measured values were less than limits of quantification (LOQ). In addition, the S/G lignin ratio was calculated based on respective values of S lignin and G lignin shown by the µmol/g CWR unit. In addition, values of H, G, and S lignin were shown as percentages of respective lignin subunits in the total HGS lignin.

As a result of the analysis, a statistically significant difference was recognized between the recombinant and the reference non-recombinant alfalfas in G lignin content ($p<0.05$) (Table , p17). The mean value of G lignin of the recombinant alfalfa was 68.10 µmol/g CWR, and lower by 15.62 µmol/g CWR (18.66%), as compared with the mean value 83.72 µmol/g CWR of the reference non-recombinant alfalfa. Meanwhile, as a comparison between the contents of S and H lignin of the recombinant alfalfa and the reference non-recombinant alfalfa, no statistically significant difference was recognized (Table , p17).

In addition, the percentage of G lignin of the recombinant alfalfa in the total of H, G, and S lignin was 53.69%, which was lower by 8.00 %, in comparison to the reference non-recombinant alfalfa (61.69%) (Table , p18). Due to the reduction of this G lignin content, the S/G ratio increased from 0.58 of the reference non-recombinant alfalfa to 0.80 (Table , p17).

From the above, it was recognized that in the recombinant alfalfa, the production of G lignin decreased by depressing the expression of CCOMT genes, the percentage of G lignin in the total HGS lignin and the S/G ratio increased in comparison to the reference non-recombinant alfalfa.

[7]The C0-Syn1 generation was produced by crossing non-recombinant alfalfa Lines R2336 and Ms208 in the similar raising method as the Syn1 generation of the recombinant alfalfa (Drawing 1, p23).

Moreover, to look through the influence of the reduction in G lignin content over the total lignin content, the total lignin content (acidic detergent fiber: ADL[8]) was measured. In 2011, the ground segment of the first cut of the recombinant and the reference non-recombinant alfalfas (C0-Syn1 generation), which had been collected in six (6) fields in the U.S., were offered for an experiment. As a result of analysis of the ground segment of the recombinant alfalfa, a decrease in the total lignin (ADL) was confirmed. The mean values of the total lignin (ADL) in the recombinant alfalfa was 5.39% DW in dry weight and lower by 1.53 % ($p<0.05$) in comparison of the mean value 6.93 % DW of the reference non-recombinant alfalfa (Table , p19 and Attached reference **2** Table 1, p15). From the above, it was confirmed that the total lignin (ADL) of the recombinant alfalfa was significantly low in comparison to the reference non-recombinant alfalfa harvested in the same developing stage (Attached reference **2**).

[8]Lignin obtained by treating ghosting extracted from acid detergent solution with sulfuric acid (Association of Feed Analysis Methods (Written and ed.) 2004).

Table 3 Lignin Subunit Contents and S/G Ratio of the Recombinant Alfalfa and Reference Non-Recombinant Alfalfa in Contrast[9]

Analysis component (unit)[1, 2]	The recombinant alfalfa[3] mean values (S.E.)[4] (range)	Reference variety[5] mean value (S.E.) (range)	Significant difference (p-value)	Commercial species 99%T.I.[6] (range)
Lignin subunit (µmol/g CWR)				
G lignin	68.10 (9.48) (21.17-134.96)	83.72 (9.40) (33.11-131.40)	0.027*	8.83, 176.39 (25.34-153.11)
S lignin	55.96 (8.83) (9.82-87.67)	50.41 (8.78) (12.20-91.89)	0.302	0, 120.96 (5.64-110.93)
H lignin	5.05 (0.45) (2.20-10.84)	3.88 (0.43) (0.58-5.49)	0.077	1.59, 6.91 (0.29-8.26)
Comparisons between S and G lignin				
S/G ratio	0.80 (0.061) (0.43-1.16)	0.58 (0.060) (0.35-0.70)	<0.001*	0.21, 0.96 (0.22-0.92)

[1]In 2011, samples were taken from first cut of recombinant alfalfa, reference non-recombinant alfalfa (c0-Syn1 generation), and conventional commercial species, which were raised in six (6) fields in the U.S. (States of California, Iowa, Illinois, Kansas, Texas, and Wisconsin).

[2] CWR: Cell Wall Residue; S/G ratio: S lignin content divided by G lignin content.

[3]The recombinant alfalfa harvested at 1-10% flowering time, a normal harvest time (same condition as reference and commercial species).

[4]Mean value (S.E.) = least mean square (standard error)

[5]As the reference non-recombinant alfalfa, C0-Syn1 produced from Line R2336, a line of non-recombinant alfalfa, was used.

[6] The range fixed to include 99 % of commercial species in the 95% level of confidence. The minimum value was set to 0.

* A significant difference is found ($p<0.05$)

[9]Rights and responsibilities of contents related to the information shown in this table belong to Monsanto Japan Ltd.

Table 4 Lignin Subunit Contents the Recombinant Alfalfa and Reference Non-Recombinant Alfalfa (the Percentage in the Total HGS Lignin) [10]

Analysis component (unit)[1, 2]	The recombinant alfalfa[3] mean values (S.E.)[4] (range)	Reference variety[5] mean value (S.E.) (range)	Significant difference (p-value)	Commercial species 99%T.I.[6] (range)
Lignin subunits (% total HGS lignin)				
G lignin	53.69 (1.87) (44.92-63.78)	61.69 (1.87) (56.88-70.56)	<0.001*	46.69, 76.44 (50.02-76.69)
S lignin	42.09 (2.35) (26.98-52.01)	35.24 (2.35) (24.60-40.26)	<0.001*	17.39, 53.32 (17.07-46.14)
H lignin	4.22 (0.54) (2.04-9.78)	3.07 (0.54) (0.34-5.18)	0.001*	0, 6.74 (0.18-6.23)

[1]In 2011, samples were taken from first cut of recombinant alfalfa, reference non-recombinant alfalfa (c0-Syn1 generation), and conventional commercial species, which were raised in six (6) fields in the U.S. (States of California, Iowa, Illinois, Kansas, Texas, and Wisconsin.
[2]The total HGS lignin is the total of H, G, and S lignin (% total HGS lignin**)**.
[3]The recombinant alfalfa harvested at 1-10% flowering time, a normal harvest time (same condition as reference and commercial species).
[4]Mean value (S.E.) = least mean square (standard error)
[5]As the reference non-recombinant alfalfa, C0-Syn1 produced from Line R2336, a line of non-recombinant alfalfa, was used.
[6] The range fixed to include 99 % of commercial species in the 95% level of confidence. The minimum value was set to 0.
* A significant difference is found ($p<0.05$)

[10]Rights and responsibilities of contents related to the information shown in this table belong to Monsanto Japan Ltd.

Table 5 Total Lignin Contents of the Recombinant Alfalfa and the Reference Non-Recombinant Alfalfa[11]

Analysis component (unit)[1, 2]	The recombinant alfalfa[3] mean values (S.E.)[4] (range)	Reference variety[5] mean value (S.E.) (range)	Significant difference (p-value)	Commercial species 99%T.I.[6] (range)
Total lignin[7] (% DW)	5.39 (0.64) (2.73-7.60)	6.93 (0.64) (2.23-10.10)	0.004*	1.39, 12.54 (1.70-10.03)

[1]In 2011, samples were taken from first cut of recombinant alfalfa, reference non-recombinant alfalfa (c0-Syn1 generation), and conventional commercial species, which were raised in six (6) fields in the U.S. (States of California, Iowa, Illinois, Kansas, Texas, and Wisconsin.

[2] DW = dry weight

[3]The recombinant alfalfa harvested at 1-10% flowering time, a normal harvest time.

[4]Mean value (S.E.) = least mean square (standard error)

[5]As the reference non-recombinant alfalfa, C0-Syn1 produced from Line R2336, a line of non-recombinant alfalfa, was used.

[6] The range fixed to include 99 % of commercial species in the 95% level of confidence. The minimum value was set to 0.

[7] An analysis was conducted using the semi-automation ANKOM method (Weston et al., 2006).

* A significant difference is found ($p<0.05$)

[11]Rights and responsibilities of contents related to the information shown in this table belong to Monsanto Japan Ltd.

(2) Information concerning vector

a. The name and origin

PVMSPQ12633 used to produce the recombinant alfalfa is constructed based on plasmid pBR322 derived from *E. coli*. Details are shown in Table (p6).

b. Characteristics

(1) The number of vector bases and base sequences

The total number of bases of PV-MSPQ12633 used to produce the recombinant alfalfa is 10,608bp. The base sequence of PV- MSPQ12633 is described in Attached reference **3**.

(2) Functions of base sequences having specific functions if any

As a selection marker gene of a construct vector in *E. coli*, *aadA* genes derived from transposon *Tn7* imparting resistance to spectinomycin and streptomycin exist outside of the T-DNA region.

(3) The presence or absence of vector infections and the information concerning the host range if it is infectious.

The vector does not include a sequence with any known infection.

(3) Method for the preparation of living modified organism

a. Overall structure of nucleic acid transformed into the host

The components of the plasmid vector transformed into the host are shown in Table (p6-8). The location of the components of provided nucleic acid in a vector and the cleavage site of the restriction enzyme are shown in Fig. (p5).

b. A method to transform nucleic acid into the host

PV-MSPQ12633 was introduced to Line R2336 by the Agrobacterium method.

c. The development progress of living modified organism

(1) The method of selecting cells where nucleic acid is transformed

After co-location cultivation of Line R2336 leaf tissue and *A. tumefaciens* ABI including PV-MSPQ12633, the cell transformed by a tissue culture medium, where kanamycin and ticarcillin- clavulanate were added, was selected.

(2) If the method for introducing nucleic acid is the Agrobacterium method, the existence or absence of remaining agrobacterium fungi

Agrobacterium fungi were removed with the transformation from the tissue culture medium, where ticarcillin- clavulanate were added Furthermore, a PCR analysis was conducted with a target of the exoskeleton region of PV-MSPQ12633, which was used for transformation in the Syn1 generation of the recombinant alfalfa[12] found out that PV-MSPQ12633 exoskeleton region did not exist (Attached reference **4**). This confirmed that no agrobacterium fungi used for transformation survived in the recombinant alfalfa (Table 1, p10 in Attached reference **4**).

(3) The breeding process from nucleic acid cells to the line where it has been confirmed that a copy of the transferred nucleic acid exists, the line provided to the Isolated Field Test, and the line used to collect information necessary to other biological diversity risk assessments

Transformed redifferentiated plants (T0) crossbreed with the conventional variety Line Ms208 to produce the P0 generation. In the P0 generation, plants with the T-DAI region and without T-DNAII region with PCR and Southern blot analysis. By using excellent phenotype, transgenes, and others as indications, the recombinant alfalfa was ultimately selected as the commercialization line.

Alfalfa is tetraploid and has four sets of eight chromosomes (2n=4x=32). Predominately, alfalfa is self - incompatibility and shows inbreeding depression and heterosis (Cooper and Brink, 1940; Wilsie, 1958; Hill, 1983).

[12]Harvested seeds were bulked and about 200 grains were randomly picked up to extract DNA for PCR analysis.

Seeds of commercial species are created through natural mating between multiple parental lines with characteristics requiring bees as pollinating insects. A species produced in this method is called synthetic variety. A synthetic variety is produced through multiple hybridization mating of genetic superior lines. A plant in a synthetic variety has different genotypes and shows multiple phenotypes. In general, cultivated species are genetically fixed(Rumbaught et al., 1988). As for the recombinant alfalfa, the Syn1 generation is produced through voluntary hybridization in the MBC2 generation and Syn1 Adv generation through voluntary hybridization in the Syn1 generation.

The raising drawing of the recombinant alfalfa is depicted in Drawing 1 (p23). Furthermore, objects of the application are P0 generation and all posterity families of hybridization mating derived from P0 generation.

[Nondisclosed due to confidentiality]

Drawing 1 The raising drawing of the recombinant alfalfa

(4) The state of existence of nucleic acid transferred into cells and the stability of phenotypic expression by the nucleic acid

(1) Space where duplicates of transferred nucleic acid exist

Transformed redifferentiated plants (T0) crossbreed with the conventional variety Line Ms208 to produce the P0 generation. In the P0 generation, plants with the T-DAI region and without T-DNAII region with PCR analysis. MBC1 was produced through mating between the obtained P0 generation with FD4 conventional variety population. MBC2 was produced through mating pollen obtained from 20 individual plants of MBC1, where transgenes are confirmed, with FD4 conventional variety population. Similarly, MBC3 was produced through mating pollen obtained from 24 individual plants of MBC2, where transgenes are confirmed, with FD4 conventional variety population.

Furthermore, Syn1 was produced through mating pollen obtained from 80 individual plants of MBC2, where transgenes are confirmed, with FD4 conventional variety population. The existence or absence of transgenes in MBC2, MBC3, and Syn1 individual plants was examined through End point TaqMan PCR.

In an examination of segregation rate, consideration was given to a fact that alfalfa is autotetraploid. In autotetraploid, if genes are linked with centromere, chromosome segregation is known to occur according to Mendel's law of segregation. Whereas, genes are known to be separated depending on the formation of duplicate reduction gametes and the presence or absence of chiasmata according to the maximum equational segregation and the chromatid segregation(Muramatsu, 1987). Therefore, as for transgenes of the recombinant alfalfa, segregation ratios were examined in case of three gene segregations: 1) chromatid segregation, 2) maximum equational segregation, and 3) chromatid segregation.

First of all, combination of gametes generated from the MBC2 generation of the recombinant alfalfa was examined (Table, p25). In the MBC2 generation, only individual plants including transgenes in 1/4 of chromosomes (Aaaa) were selected to use for progeny raising.

Next, the segregation ratio of genotypes in the syn1 generation comprising gametes Table (p25) were examined (Table , p25).

In addition, expected values of the phenotype segregation ratio in the Syn1 generation were examined and compared with the actual results of examining the Syn1 generation of the recombinant alfalfa (Table, p25). As a result, it was confirmed that observation values of the phenotype segregation ratio in the Syn1 generation of the recombinant alfalfa (2.93:1) were different from expected values of the segregation ratio (2.41:1 and 2.48:1) based on the maximum equational segregation and the chromatid segregation.

Whereas, no statistically significant difference was found between observation values of the phenotype segregation ratio in the Syn1 generation (378:126) and expected values of the chromosome segregation (378:126) in the chi-square test (Table , p26; Table1, p7 of Attached reference5). Therefore, transgenes of the recombinant alfalfa are considered to exist on chromosomes.

Table 6 Expected values of the gamete in MBC2 generation (genotype Aaaa) of the recombinant alfalfa [13]

	AA	Aa	aa
Chromosome segregation	0	1	1
Maximum equational segregation	1	10	13
Chromatid segregation	1	12	15

*A shows a transgene locus with transgenes and a shows a transgene locus without any transgenes.

Table 7 Expected and observed values of the genetic type in Syn1 generation of the recombinant alfalfa [14]

	AAAA	AAAa	AAaa	Aaaa	aaaa
Chromosome segregation	0	0	9	18	9
Maximum equational segregation	1	20	126	260	169
Chromatid segregation	1	24	174	360	225

Table 8 Phenotype expected and observed values in Syn1 generation of recombinant alfalfa [15]

	Positive population	Negative population	Negative is 1 Segregation ratio
Chromosome segregation (expected value)	378	126	3 : 1
Maximum equational segregation (expected value)	356	148	2.41 : 1
Chromatid segregation (expected value)	359	145	2.48 : 1
Observation value in Generation Syn1	376	128	2.93 : 1

[13]Rights and responsibilities of contents related to the information shown in this table belong to Monsanto Japan Ltd.
[14]Rights and responsibilities of contents related to the information shown in this table belong to Monsanto Japan Ltd.
[15]Rights and responsibilities of contents related to the information shown in this table belong to Monsanto Japan Ltd.

Table 9 Separation Form of *CCOMT* Gene Fragment in the Cultivation Process of the Recombinant Alfalfa[16]

Generation	No. of Experiments[1]	Observation Value[2]		Expected Value of Segregation Ratio Set at 1:1		χ^2	P-value[3]
		Positive population	Negative population	Positive population	Negative population		
MBC2	261	119	142	130.5	130.5	2.03	0.154
MBC3	263	132	131	131.5	131.5	<0.01	0.951

Generation	No. of Experiments[1]	Observation Value[2]		**Expected Value of Segregation Ratio Set at** 3:1		χ^2	P-value[3]
		Positive population	Negative population	Positive population	Negative population		
Syn1	504	376	128	378	126	0.04	0.837

[1] 261 individual plants in the MBC2 generation, 263 individual plants in the MBC3 generation, and 504 individual plants in the Syn1 generation were cultivated by bulking all seeds of cultivated parent generations and using randomly selected seeds from them.
[2] The presence of absence of *CCOMT* gene was examined with TaqMan PCR.
[3]The segregation ratio obtained from the above 3 generations was analyzed by a chi-square test ($p<0.05$).

[16]Rights and responsibilities of contents related to the information shown in this table belong to Monsanto Japan Ltd.

(2) The copy number of replication of transferred nucleic acid and the transmission stability over multigenerations of nucleic acid replication

As a result of transgene analysis in the Southern blot analysis, it has been confirmed that one copy of T-DMAI region is integrated with one area of nuclear genome of the recombinant alfalfa (Figure4-7, p39-42 of Attached reference 6) and this transmission is stable over multi-generations (P0、MBC1、MBC2, and Syn1) (Figure4, p19 of Attached reference **7**). In addition, it has been confirmed by the Southern blot analysis that T-DNAII and the exoskeleton regions have not been introduced (Figure8~10, p43-45 of Attached reference 6). The raising drawing of the recombinant alfalfa is depicted in Fig. (p28).

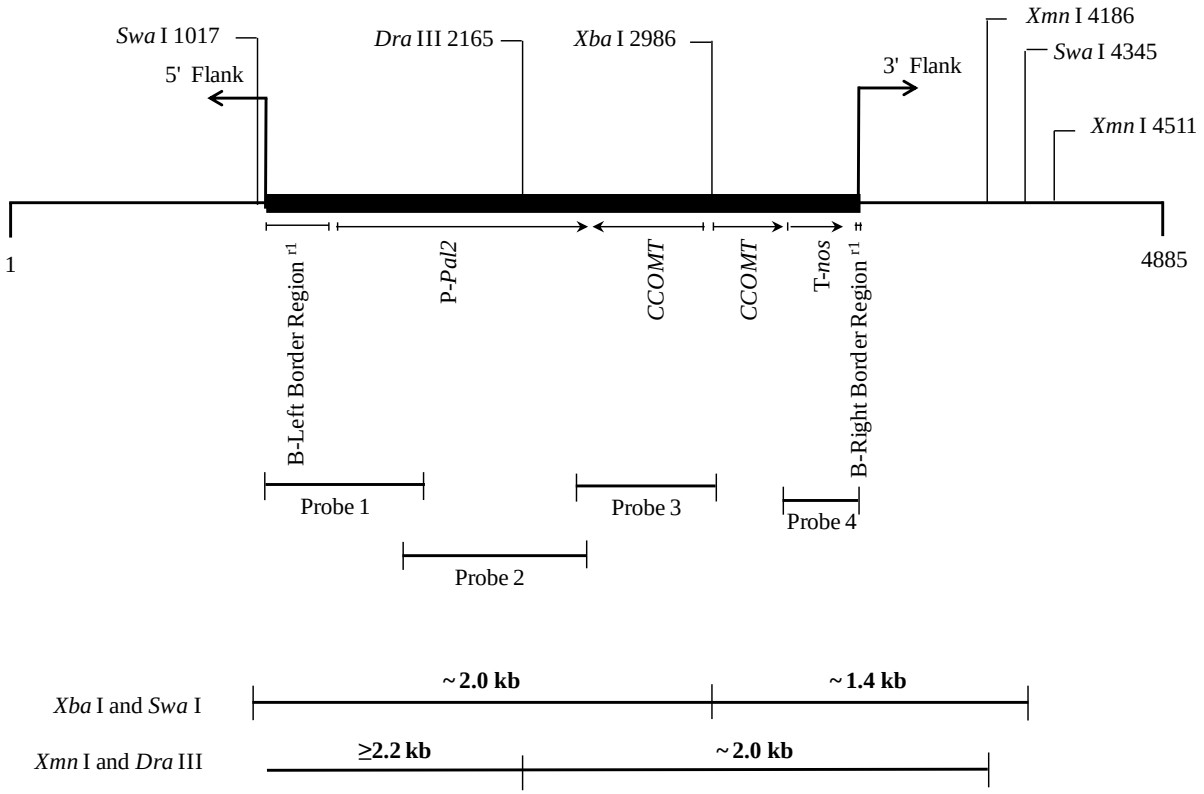


Fig. 6 Transgene Map of the Recombinant Alfalfa and Pattern Diagrams of Inserted Genome Neighbor Sequence and Restriction Enzyme Cleavage Site[17]

The upper part of the figure is an alfalfa transgene pattern diagram and components in the transgenes and the restriction enzyme cleavage site used for the Southern blot analysis are shown. Arrows in the upper part of the figure show start positions of genome DNA sequences neighboring 5' and 3' flanks of the transgene.

The middle part of the figure shows relative sizes and positions of T-DNAI probe. The lower part of the figure shows sizes of DNA fragment expected after cut with restriction enzyme. Furthermore, components, restriction enzyme cleavage sites, and each probe roughly shows the positions. rls show B-Right Border Region and B-Left Border Region are shorter than PV-MSPQ12633 in the recombinant alfalfa.

[17]Rights and responsibilities of contents related to the information shown in this drawing belong to Monsanto Japan Ltd.

(3) If more than one copy is on a chromosome, whether they are adjacent or separated

N/A because the number of copies is one (Figure4-7, p39-42 of Attached reference 6).

(4) As for features explained in details in (6)(1) the stability of expression between individuals and generations under natural conditions

In 2010, ADL of multi-generation (MBC1, Syn1, and Syn1-Adv1) of the recombinant alfalfa and the reference non-recombinant alfalfa were analyzed as the total lignin of the ground segment at fields in two U.S. places (States of Iowa and Wisconsin).

As a result, it has been confirmed that the lignin contents have decreased in the recombinant alfalfa (Table, p30; Table 1, p4 of Attached reference **8**). The lignin contents of generations of the recombinant alfalfa (MBC1, Syn1, and Syn1-Adv1) are decreased by 17.71%, 13.27%, and 15.34% respectively in comparison to the reference non-recombinant alfalfa (Table, p30; Attached reference **8** of Table 1, p4).

In addition, in 2013, the transfer quantity of dsRNA and siRNA derived from the *CCOMT* gene control cassette over multi-generation (MBC2, Syn1, and Syn1-Adv1) of the recombinant alfalfa and the reference non-recombinant alfalfa grown at the greenhouse in the U.S. Forage Genetic International (FGI) was analyzed by the northern blot analysis.

As a result, it has been confirmed that the RNA transfer quantity of endogenous CCOMT protein over generations of the recombinant alfalfa has decreased and that siRNA derived from *CCOMT* has been transferred (Figures 3 and 4, pp16-17 of Attached reference **9**).

Table 10 Total Lignin Contents in the Recombinant Alfalfa and Reference variety in Contrast[18]

Under test generation[2]	Mean value (S.E.)[1] (Range) Recombinant Alfalfa	Reference variety [3]	P-value[4]
MBC1	4.37 (0.36) 3.55 (5.63)	5.31 (0.36) 4.42 (7.11)	0.002
Syn1	4.02 (0.36) 3.30 (4.31)	4.64 (0.36) 4.15 (5.39)	0.034
Syn1 Adv1	3.91 (0.36) 3.61 (4.42)	4.62 (0.36) 3.72 (4.89)	0.016

*Analyzed acid detergent lignin as the total lignin content.

[1]Mean value (S.E.) = least mean square (standard error)

[2]Values are obtained from dry weight %.

[3] Used C0- MBC1, C0-Syn1 and C0-Syn1-Adv1 generations produced from Line R2336 as the reference non-recombinant alfalfas.

[4] Statistical processing was conducted by the analyses of variance (significant in 6 repeats in one sample/repeat $p<0.05$).

[18]Rights and responsibilities of contents related to the information shown in this table belong to Monsanto Japan Ltd.

(5) If nucleic acid transferred through virus infection and another route may be propagated to wild fauna and flora, the existence or absence and the degree of the transmissibility

As the transferred nucleic acid sequence has no function to realize the transmission, no nucleic acid may be transferred to wild fauna and flora through virus infection and another route.

(5) Detection of living modified organisms (LMOs), identifying methods, their sensitivity and reliability

The recombinant alfalfa can be detected and identified by using a primer set that are capable of binding to specific DNA sequence present at the recombinant alfalfa in the End-Point TaqMan PCR method(Attached reference **10**). The recommended DNA concentration for the test is 5-10ng per one reaction. The test can be done by using a part of leaf (leaf disk).

To check the repeatable precision of the method, 46 samples of the recombinant alfalfa and 134 samples of non-recombinant alfalfa were used for a confirmation test (p.6 of Attached reference **10**).

(6) Difference from a host or its species

(1) Physiological or ecological characteristics imparted by the expression of reproductions of transferred nucleic acid

CCOMT gene fragments introduced to the recombinant alfalfa depress the expression of *CCOMT* genes coding for main enzymes in the lignin biosynthetic pathway. By depressing the expression of *CCOMT* genes, G lignin is reduced out of G, S, and H lignin that forms three-dimensional network structures of lignin. The total lignin content of alfalfa is reduced by polymerizing S and H lignin and reducing G lignin that forms three-dimensional network structures of lignin. Actually, as a result of analysis of the ground segment of the recombinant alfalfa, it was confirmed that the total lignin (ADL) was decreased in comparison with the reference non-recombinant alfalfa by 1.53% ($p<0.05$) (Table , p19; Attached reference **2**).

(2) The presence or absence of any difference of the following physiological or ecological characteristics between genetically modified crops and host's species and the degree if there is any difference[19]

Isolated field tests for the recombinant alfalfa were carried out at isolated fields set in of the Institute of Livestock and Grassland Science, the National Agriculture and Food Research Organization (NARO) in 2012 through 2013 (hereinafter called the Isolated Field Test). For the test, the Syn1 generation of the recombinant alfalfa was offered for an experiment (Drawing 1, p23). As for the reference non-recombinant alfalfa, Line R2336 was used as a mother plant, and the C0-Syn1 generation produced in the similar raising method as the Syn1 generation of the recombinant alfalfa.

Furthermore, low or high temperature resistance tests at the early growth stage (Item b, p33) were conducted at a U.S. growth chamber. Alfalfa is an allogamous plant that shows the self - incompatibility and mainly uses bees, leaf-cutting bees, honeybees, etc. as pollinating insects for forming seeds through pollination by insect(Lesins and Lesins, 1979; Quiros and Bauchan, 1988; Barnes and Sheaffer, 1995). However, an area to survey morphologies and growth characteristics is covered with insect screens at the Isolated Field Tests for the recombinant alfalfa as hybridization prevention measures, out of tests to evaluate the productivity of seeds, the threshing property, dormancy property and the germination percentage (Items e and p34), investigations of the productivity of seeds and the threshing property were conducted at the U.S. fields (Idaho) and seeds' germination percentages were investigated at the U.S. greenhouses.

a Characteristics of Morphologies and Growth

To evaluate characteristics of morphologies and growth, seven items were evaluated: start (date), the number of plants in springtime, the longest stem length at the second cut (cm), lodging at the second cut, flower color, the number of flowers around inflorescence axes.

Statistical processing were conducted for the number of plants in springtime, the longest stem length (at the second cut) (cm), the fresh weight of the ground segment (at the second cut) (g), and the number of flowers around inflorescence axes but not for the germination start (date) and the flower color. In addition, as for lodging (at the second cut), as all of the recombinant alfalfa and the reference non-recombinant alfalfa fell, the statistical processing was not conducted.

As a result, no difference was recognized between the recombinant alfalfa and the reference non-recombinant alfalfa (Table 2, p.11 of Attached reference

[19]Rights and responsibilities of contents related to the following information shown in a-h in this paragraph belong to Monsanto Japan Ltd.

11).

b Low or high temperature resistance at the early growth stage

As alfalfa is a perennial plant, low and high temperature resistance was investigated at the early growth stage. Furthermore, low or high temperature resistance tests at the early growth stage were conducted at a growth chamber in the U.S. Monsanto Company in 2011.

To investigate the low temperature resistance at the early growth stage, seedling of the recombinant and the reference non-recombinant alfalfa on the 26th day after seeding and 4 lines of conventional commercial species were transferred into three stages of temperature conditions (day/night) (suitable temperature: 28/23 deg C, slightly low temperature: 15/8 deg C, low temperature: 7/5 deg C), grown for 21 days, and investigated. Developing stages, plant's vigor, and the longest stem length were investigated on the 7th, 14th, and 21st days before/after low-temperature processing.

Furthermore, in this test, among cultivated individual plants, only those confirmed to have transgenes were investigated.

As a result of the investigation, no statistically significant difference in the longest stem length that was statistically processed was recognized between the recombinant and the reference non-recombinant alfalfa while no difference between them in the developing stage when no strategic processing was conducted and plant's vigor was recognized (Table 1-3, pp.5-7 of Attached reference **12**).

To investigate the high temperature resistance at the early growth stage, seedling of the recombinant and the reference non-recombinant alfalfa on the 26th day after seeding and 4 lines of conventional commercial species were transferred into three stages of temperature conditions (day/night) (suitable temperature: 28/23 deg C, slightly high temperature: 35/33 deg C, high temperature: 40/38 deg C), grown for 21 days, and investigated. Developing stages, plant's vigor, and the longest stem length were investigated on the 7th, 14th, and 21st days before/after high-temperature processing.

Furthermore, in this test, among cultivated individual plants, only those confirmed to have transgenes were investigated.

As a result of the investigation, no statistically significant difference in the longest stem length that was statistically processed was recognized between the recombinant and the reference non-recombinant alfalfa while no difference between them in the developing stage when no strategic processing was conducted and plant's vigour was recognized (Table 1-3, pp.5-7 of Attached reference **13**).

c Overwintering of Adults

To evaluate the overwintering of adults, the numbers of the recombinant and the reference non-recombinant alfalfas at the germination start and in springtime were compared in an area to survey morphologies and growth characteristics. As a result, no statistically significant difference was recognized in any item between the recombinant and the reference non-recombinant alfalfas (Table 2, p.11 of Attached reference 11).

d Fertility and Size of Pollen

Pollen collected from the recombinant and the reference non-recombinant alfalfas was dyed with Alexander solution and its fertility (circularity) and size were measured. As results of statistic processing of these items, no statistically significant difference was recognized in either pollen fertility (circularity) or sizes between the recombinant and the reference non-recombinant alfalfas (Tables 7 and 3, p.12 of Attached reference 11).

e The production volume, shattering, dormancy property, and germination percentage

Alfalfa is an allogamous plant that shows the self incompatibility and mainly uses bees, leaf-cutting bees, honeybees, etc. as pollinating insects for forming seeds through pollination by insect(Lesins and Lesins, 1979; Quiros and Bauchan, 1988; Barnes and Sheaffer, 1995). However, an area to survey morphologies and growth was covered with insect screens as the hybridization mating prevention measures during blossoming at the Isolated Field Tests, the productivity of seeds, shattering, and germination percentage were investigated at the U.S. fields and greenhouses.

To investigate the production volume and shattering, 7 items (the number of establishment, plant's vigor, lodging, seed weight per 50 grains, the number of seeds per pod, the number of shuttering, and seed yield) of the recombinant alfalfa [20] , the reference non-recombinant alfalfas, and 7 conventional commercial species were investigated in one U.S. field (Idaho) in 2010.

As a result of the investigation, a statistically significant difference was not recognized between the recombinant and the reference non-recombinant alfalfas in all items (Table 2, p.6 of Attached reference14).

[20]In the Syn1 generation of the recombinant alfalfa used for this test, individual plants without transgenes account for 25 % in a theoretical value.

In addition, the germination percentage of seeds harvested from the recombinant alfalfa, the reference non-recombinant alfalfa, and 4 lines of conventional commercial species cultivated as greenhouses was investigated. The possibility to increase hard seeds has been reported if the seeds are obtained at a greenhouse (Bass et al., 1988; Copeland and McDonald, 2001). Germination percentages were investigated for harvested seeds excellent in water resistance with forming a flaw on the seed coat to improve water absorption as well as harvested seeds without forming a flaw on the seed coat to remove the influence. Germination tests are conducted with four repetitions per 100 grains at 20 deg C under the dark condition. Germinating seeds were measured after classification into normal and abnormal germination and non-germinating seeds were measured after classification into dead, water-absorbing swollen, and hard seeds before measurement(AOSA, 2010; AOSA/SCST, 2010). In the Syn1 generation of the recombinant alfalfa used for this test, individual plants without transgenes account for 25 % in a theoretical value.

As a result of investigation, in seeds without forming a flaw on a seed coat (without treatment), a statistically significant difference was recognized in normal germination percentages: in case of the recombinant alfalfa, 86.0%; and in case of the reference non-recombinant alfalfa, 72.5%. In addition, a statistically significant difference was recognized in hard seed percentages: in case of the recombinant alfalfa, 13.0%; and in case of the reference non-recombinant alfalfa, 25.3% (Table 1, p.7 of Attached reference **15**).

As for seeds with a flaw on a seed coat, a statistically significant difference was recognized in normal germination percentages: in case of the recombinant alfalfa, 93.5%; and in case of the reference non-recombinant alfalfa, 89.8% (Table 1, p.7 of Attached reference **15**).

f Hybridization Rate

Related species, which are considered to be able to be hybridized with alfalfa, are 3 species: *M. prostrata, M. cancellata,* and *M. saxatilis* of the *genus Medicago*.(Lesins, 1961; Lesins, 1962; Lesins, 1970; Quiros and Bauchan, 1988) These do not exist in Japan (Ohashi, 1999; Ohashi, 2003). Therefore, no hybridization rate is tested.

g Productivity of toxic substances

To confirm neither soil microorganism nor material influencing other plant is produced from the recombinant alfalfa, soil microorganism phase tests, plow-in tests, and subsequent crop tests were conducted. As a result, no statistically significant difference was recognized in either item (Tables 4-6, p.14 of Attached reference 11).

h Resistance against Insect Pest

CCOMT gene fragments introduced to the recombinant alfalfa depress the expression of *CCOMT* genes coding for main enzymes in the lignin biosynthetic pathway. As a result, the lignin contents have decreased in the recombinant alfalfa. By reducing lignin contents in plant bodies, plant body structures are get weaker, and weak against the physical stress from the environment. In addition, it is contemplated that the resistibility against disease damages and pests deteriorate, too. If the sensitivity against diseases, etc. increases due to the reduction in lignin contents in the recombinant alfalfa plant bodies, it is likely that diseases, etc. proliferate in the recombinant alfalfa and that diseases, etc. of other wild plants increase.

Therefore, investigations were conducted on the degrees of damages of plant bodies of the recombinant alfalfa and the reference non-recombinant alfalfa by *hypera postica* and *aphididae*, principal pests in the alfalfa cultivation in Japan, and *eptosphaerulina briosiana*, a principle disease. As a result, no difference was recognized between the recombinant alfalfa and the reference non-recombinant alfalfa (Table 7, p.16 of Attached reference 11).

Second Results of Examinations at the Biological Diversity Risk Evaluation Review Meeting

A review was made by persons with specialized knowledge and experience concerning Adverse Effect on Biological Diversity (called Experts) for possible Adverse Effect on Biological Diversity caused by the use in accordance with the Type 1 Use Regulation for Living Modified Organism based on the Law concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms. Results of the review are listed below.

1 Results of Biological Diversity Risk Evaluation

The recombinant alfalfa introduces and produces T-DNA I area of plasmid PV-MSPQ12633 constructed based on plasmid pBR322 deprived from fecal bacteria (Escherichia coli) in the Agrobacterium method.

The recombinant alfalfa is designed to produce double-stranded RNAs (dsRNAs) causing RNA interference by incorporating DNA fragment combining CCOMT gene partial sequence in the shape of the inverted repeat sequence, which codes caffeoyl CoA3-O-methyltransferase derived from alfalfa (hereinafter called “CCOM gene fragment”). It has been confirmed by the separation form of the gene and the Southern blot analysis that one copy of T-DNA region including CCOMT gene fragment is integrated on a chromosome and stably transmitted over multi-generations.

In addition, it has been confirmed by the northern blot that the intended gene fragment stably expresses over multi-generations.

(1) Preponderance in Competition

Alfalfa was introduced to Japan in the first year of Meiji era (1868) and it has been said that wild alfalfas are growing by the roadside and in a meadow all over Japan. Still, it is believed that such habitats are scattered and do not form a big plant colony. In addition, because young alfalfa plants are less competitive than weeds, low in moisture resistance, and do not like acid soil, the plants are not suitable for Japan's humid climate and acid soil. Therefore, it is not likely that the alfalfa itself can be one of invasive foreign species that detrimentally affect the biodiversity by driving out Japanese native species.

Comparison tests between the recombinant alfalfa and the reference non-recombinant alfalfa were conducted on characteristics of morphologies and growth, overwintering of plant bodies, pollen fertility and sizes at Japanese isolated fields, but no statistically significant difference was recognized.

In addition, in the U.S., as a result of investigations on the seed productivity,

shattering, germination percentage, and others, no statistically significant difference was recognized.

CCOMT gene fragments introduced to the recombinant alfalfa reduce lignin contents in plant bodies through the suppression of the expression of CCINT genes participating in the lignin biosynthetic pathway. In general, the reduction of lignin contents weakens plant body structures and become weak to the physical stress from the environment. In addition, there is a possibility that the resistibility against disease damages and pests deteriorate, too. Therefore, it is hard to think the competitive advantage can be raised only with the statistically significant difference in the germination percentage of the recombinant alfalfa.

From the above, wild fauna and flora that may be under influence were not identified and we determined the conclusion of the applicant was proper: Adverse Effect on Biological Diversity could not arise.

(2) Productivity of toxic substances

It has been reported that an alfalfa, a host species, shows allelopathy to cucumbers, lettuce, sorghum, and barley. However, it is considered that CCOMT gene fragments introduced to the recombinant alfalfa reduce lignin contents in plant bodies through the suppression of the expression of CCINT genes participating in the lignin biosynthetic pathway and are not likely to affect the other metabolic pathways.

In addition, comparison tests between the recombinant alfalfa and the reference non-recombinant alfalfa were conducted on harmful substance productivity through subsequent crop tests, plow-in tests, and soil microorganism phase tests but no statistically significant difference was recognized.

From the above, we have determined the conclusion of the applicant was proper: the recombinant alfalfa do not affect biodiversity resulting from the productivity of harmful substance other than the allelopathy held originally by the recombinant alfalfa.

(3) Hybridization

Related species, which are considered to be able to be naturally hybridized with alfalfa, are 3 species: M. prostrata, M. cancellata, and M. saxatilis of the genus Medicago. These do not exist in Japan. In addition, though black medic, a perennial plant of genus Medicago grow naturally in Japan, hybridization mating is not likely to occur.

Therefore, no wild fauna and flora, which may be influenced by the biodiversity resulting from the hybridization, was identified.

From the above, we have determined the conclusion of the applicant was proper: Adverse Effect on Biological Diversity could not arise resulting from hybridization.

2. A Conclusion Based on the Biological Diversity Risk Evaluation

Thus, a conclusion of the Biological Diversity Risk Evaluation is valid: if the recombinant alfalfa is used according to the Type 1 Use Regulation, the biological diversity may not be adversely affected in Japan.

Documents for Reference

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Attachment List

[Nondisclosed due to Confidentiality]

Attached reference 1 Amended Report for MSL0023982: Composition of Lignin of Forage from KK179 Alfalfa Grown in the United States during the 2011 Growing Season (MSL0024403) (Confidential)

Attached reference 2 Analyses of Lignin in Forage from KK179 Alfalfa Grown in the United States during the 2011 Growing Season (MSL0024120) (Confidential)

Attached reference 3 Sequence of Genetic Elements in PV-MSPQ12633 (Confidential)

Attached reference 4 Summary of PCR analysis to confirm the absence of *Agrobacterium tumefaciens* used to produce KK179 (MSL0025515) (Confidential)

Attached reference5 Heritability of the KK179 insert in the MBC2, MBC3, and Syn1 Populations (RPN-2010-0705)(Confidential)

Attached reference 6 Molecular Characterization of Reduced Lignin Alfalfa KK179 (MSL0023299) (Confidential)

Attached reference 7 Stability of the DNA Insert in KK179 Across Multiple Generations (REG-2011-0081)(Confidential)

Attached reference 8 Lignin Analysis of Forage from Multiple Generations of KK179 Alfalfa (RAR-2011-0129) (Confidential)

Attached reference 9 Demonstration of the Presence or Absence of *CCOMT* Transcripts in Alfalfa Forage Samples across Multiple Generations of KK179 (MSL0024762) (Confidential)

Attached reference 10 Alfalfa KK179-2 EndPoint TaqMan PCR with *PUB*

Internal Control (BQ-QC-10768-01) (Confidential)

Attached reference 11 Report of Biological Diversity Risk Assessment in Isolated Fields of Low-Lignin Alfalfa (*CCOMT*, *Medicago sativa* L.) (KK179, OECD UI: MON-ØØ179-5)

Attached reference 12 An Assessment of the Effect of Cold Stress on Biotechnology Derived Alfalfa KK179 under Growth Chamber Conditions (PLC-2011-0007) (Confidential)

Attached reference 13 An Assessment of the Effect of Heat Stress on Biotechnology Derived Alfalfa KK179 under Growth Chamber Conditions (PLC-2011-0007)(Confidential)

Attached reference14 Phenotypic Evaluation During Seed Production of Biotechnology Derived Alfalfa KK179 in a U.S. Field Trial During 2010 (FGI-10-001) (Confidential)

Attached reference 15 Dormancy and Germination Evaluation of Biotechnology Derived Alfalfa KK179 Using Seed Produced in a Greenhouse (PLC-10-212)(Confidential)

Attached Reference 16 Monitoring Results Report (Confidential)