APPENDIX 1 :

Mode of Action and Evidence of Suppression of FATB and FAD2 Genes in MON 87705

Suppression of FATB and FAD2 RNAs in soybean decreases saturated fatty acids (16:0 palmitic acid and 18:0 stearic acid), increases 18:1 oleic acid, and decreases 18:2 linoleic acid.

The improved fatty acid profile in MON 87705 soybean oil is achieved through the use of endogenous soybean (*Glycine max* L.) gene segments configured to suppress *FATB* and *FAD2* genes. MON 87705 contains *FATB1-A* and *FAD2-1A* gene segments under the control of a seed promoter, limiting oil composition modification to the seed tissue. The assembled gene transcript has an inverted repeat that produces double stranded RNA (dsRNA) that, via RNA-based suppression (Siomi and Siomi, 2009), suppresses endogenous *FATB* and *FAD2* genes, thereby producing the desired fatty acid phenotype of decreased saturate, increased oleic and decreased linoleic fatty acid composition in the oil.

Acyl-acyl carrier protein (acyl-ACP) thioesterases (referred to herein as FATB enzymes) are localized in plastids and hydrolyze saturated fatty acids from the ACP-fatty acid moiety (Voelker and Kinney, 2001). The suppression of *FATB* RNAs ultimately results in a decrease in the transport of the saturated fats out of the plastid, thus retaining their availability for desaturation to 18:1 oleic acid (Figure A-1). Therefore, suppression of *FATB* RNAs decreases saturated fat content in the oil as well as increases 18:1 oleic acid (Kinney, 1996). Subsequently, this increased amount of 18:1 oleic acid is either delivered to the oil body or to the endoplasmic reticulum for further desaturation (Kinney, 1996). Delta-12 desaturases (referred to as FAD2 enzymes) desaturate 18:1 oleic acid to 18:2 linoleic acid. The suppression of *FAD2* RNAs in soybean seed ultimately causes reduced desaturation of 18:1 oleic to 18:2 linoleic acid, thus contributing further to the increase in 18:1 oleic acid while reducing 18:2 linoleic acid content in the oil (Dyer and Mullen, 2005). Therefore, the overall result of the MON 87705 RNA-based suppression cassette is a reduction in saturated 16:0 palmitic and 18:0 stearic acids, an increase in monounsaturated 18:1 oleic acid, and lower levels of polyunsaturated 18:2 linoleic acid, relative to commodity soybean.

To examine suppression of the RNA levels of the endogenous *FAD2-1A* and *FATB1-A* genes, RNA isolated from immature seed[1] of MON 87705 plants at the R5/6 growth stage were subjected to northern blot analyses and compared to a conventional control[2] (A3525) that has a genetic background similar to MON 87705.

To confirm the outcome of gene suppression on the seed fatty acid profile of MON 87705, fatty acid analysis was performed on MON 87705 compared to a conventional control. This analysis indicates the impact of the suppression of *FAD2* and *FATB* genes on the seed fatty acid profile. The details of the full compositional analyses of MON 87705 are in section 2.7.

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[1] For studies presented throughout this document referring to the use of MON 87705, conventional control, or reference material seed, the term "seed" refers to the harvested seed.

[2] For studies presented throughout this document comparing MON 87705 to the conventional control, the conventional control was a nontransgenic soybean variety with genetics similar to MON 87705, but lacked the introduced traits.

Northern blot analysis of FAD2-1A RNA level in MON 87705 confirms suppression.

PolyA enriched RNA (PolyA+ RNA) isolated from approximately 50 µg total RNA from immature seed was resolved on a formaldehyde/agarose gel, blotted, and hybridized to the FAD2-1A probe (Figure A-2A). Approximately 200 pg of the FAD2-1A probe template was loaded on the gel to serve as a positive control, and the probe template produced a hybridization signal at approximately 0.4 kb (Figure A-2A, lane 9).

Approximately 50 pg of actin probe template was loaded on the gel to serve as a negative control and, as expected, there is no hybridization signal produced (Figure A-2A, lane 10). The detection of the probe template hybridization control demonstrates that the probe is hybridizing only to the target DNA sequence.

PolyA+ RNA from each of four replicates of the conventional soybean control immature seeds (Figure A-2A, lanes 1, 3, 5, and 7) produced a strong hybridization signal at approximately 1.5 kb which is the expected size of the *FAD2-1A* transcript, whereas polyA+ RNA isolated from each of the four replicates of MON 87705 immature seeds (Figure A-2A, lanes 2, 4, 6, and 8) produced a very faint hybridization signal of approximately 1.5 kb at a greatly reduced level relative to the conventional control RNA. There were faint, nonspecific hybridization signals observed at approximately 1.7 kb, 3.2 kb, and 3.6 kb, which were detected in both the conventional soybean control and MON 87705. These data show a reduction in the levels of detectable *FAD2-1A* RNA in MON 87705 compared to conventional soybean.

In order to confirm the reduced *FAD2-1A* RNA levels in MON 87705 were not due to a reduced RNA loading on the gel or poor RNA quality, the FAD2-1A hybridization signal was removed and the blot was re-hybridized with the actin probe (Figure A-2B). The approximately 50 pg of actin probe template that was loaded on the gel as a positive hybridization control showed a band at approximately 0.9 kb (Figure A-2B, lane 10). There is a faint band at approximately 1.8 kb, which most likely resulted from dimerization of the actin probe template. In lane 9, there is a band at approximately 0.4 kb, which likely results from the incomplete removal of the FAD2-1A probe template. There is no hybridization signal with the actin probe at approximately 0.9 kb, as expected from the negative control (Figure A-2B, lane 9). The detection of the actin probe template hybridization control demonstrates that the probe is hybridizing only to the target DNA sequence.

PolyA+ RNA from conventional (Figure A-2B, lanes 1, 3, 5, and 7) and MON 87705 immature seeds (Figure A-2B, lanes 2, 4, 6, and 8) showed a strong hybridization signal at approximately 1.6 kb, which is expected for the actin transcript. The hybridization signals from conventional soybean and MON 87705 immature seeds have similar intensities, indicating that the RNA loading, RNA quality, and hybridization between conventional and MON 87705 are similar. Therefore, the difference in the *FAD2-1A* hybridization signals between conventional and MON 87705 reflects a large decrease in endogenous *FAD2-1A* RNA levels.

Northern blot analysis of FATB1-A RNA level in MON 87705 confirms suppression.

PolyA+ RNA isolated from approximately 100 µg total RNA from immature seed was resolved on a formaldehyde/agarose gel, blotted, and hybridized to the FATB1-A probe (Figure A-3A). Approximately 10 pg of the FATB1-A probe template was loaded on the gel to serve as a positive control. As expected, the probe template produced a hybridization signal at approximately 0.4 kb (Figure A-3A, lane 9). Approximately 50 pg of the actin probe template was loaded on the gel to serve as a negative control and, as expected, there is no signal produced (Figure A-3A, lane10). The detection of the FATB1-A probe template hybridization control demonstrates that the probe is hybridizing only to the target DNA sequence.

PolyA+ RNA from four replicates of the immature seeds from conventional soybean (Figure A-3A, lanes 1, 3, 5, and 7) produced a strong hybridization signal at approximately 1.8 kb, which is expected for the *FATB1-A* transcript, whereas polyA+ RNA isolated from four replicates of MON 87705 immature seeds (Figure A-3A, lanes 2, 4, 6, and 8) also produced a hybridization signal of approximately 1.8 kb, but at a much reduced level. In addition, there was a hybridization signal at approximately 1.5 kb, which was not present in the conventional soybean control. The approximately 1.5 kb signal is likely a degraded *FATB1-A* transcript. These data indicate a reduction in the levels of detectable *FATB1-A* RNA in MON 87705 compared to conventional soybean.

In order to confirm that the difference in the hybridization of the FATB1-A probe in MON 87705 is not due to a reduced RNA loading on the gel or poor RNA quality, the FATB1-A hybridization signal was removed and the blot re-hybridized with the actin probe after stripping (Figure A-3B). The approximately 50 pg of actin probe template that was loaded on the gel as a positive control showed a band at approximately 0.9 kb (Figure A-3B, lane 10). The FATB1-A probe template did not produce any signal, as expected from the negative control (Figure A-3B, lane 9). The detection of the actin probe template control demonstrates that the probe is hybridizing only to the target DNA sequence.

PolyA+ RNA from the conventional soybean control (Figure A-3B, lanes 1, 3, 5, and 7) and MON 87705 immature seed (Figure A-3B, lanes 2, 4, 6, and 8) showed a strong hybridization band at approximately 1.6 kb, which is expected for the actin transcript and the intensity of the bands are similar between the conventional control and MON 87705. These data indicate that the RNA loading, RNA quality, and hybridization between conventional soybean and MON 87705 are similar. Therefore, the difference in the FATB1-A hybridization signals between conventional and MON 87705 reflects the difference in endogenous *FATB1-A* RNA levels.

Fatty acid composition of MON 87705 shows intended phenotype.

An assessment of the fatty acid profile of seed collected from five field sites in Chile during 2007/2008 demonstrates MON 87705 has the intended changes in the levels of fatty acids. MON 87705 had a decrease in saturated fatty acids (from 15.33% to 5.67% of total FA: 16:0 palmitic acid plus 18:0 stearic acid), an increase in 18:1 oleic acid (from 22.81% to 76.47% of total FA), and a decrease in 18:2 linoleic acid (from 52.86% to

10.10% of total FA) compared to conventional soybean. Statistically significant differences reflecting intended changes in seed fatty acid levels were observed in the combined-site analysis (Table A-1) and at each individual site. Section 2.7 contains additional detailed results of the fatty acid composition of MON 87705.

Conclusion

The suppression of *FATB* RNAs in soybean seed decreases the major saturated fatty acids (16:0 palmitic acid and 18:0 stearic acid) and contributes to an increase in 18:1 oleic acid; suppression of *FAD2* RNAs in soybean seeds increases 18:1 oleic acid and subsequently decreases 18:2 linoleic acid in soybean seed (Dyer and Mullen, 2005). MON 87705 northern blot data confirm the suppression of endogenous *FAD2-1A* and *FATB1-A* RNAs. MON 87705 seed composition data demonstrate that suppression of the endogenous *FAD2-1A* and *FATB1-A* RNAs produces the intended alteration in seed fatty acid levels, which is a lower level of saturated fatty acids (16:0 palmitic acid and 18:0 stearic acid), a higher level of 18:1 oleic acid, and an associated lower level of 18:2 linoleic acid.

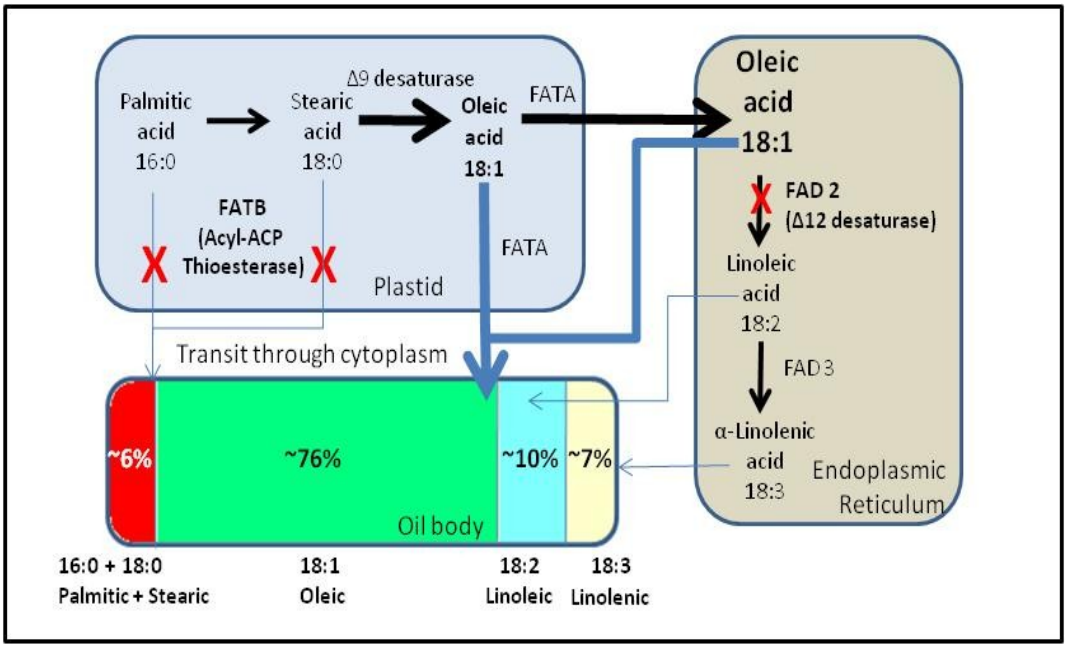

Figure A-1. Schematic of the Soybean Fatty Acid Biosynthetic Pathway
✕ indicates suppression of endogenous *FATB* and *FAD2* RNAs in MON 87705 seed

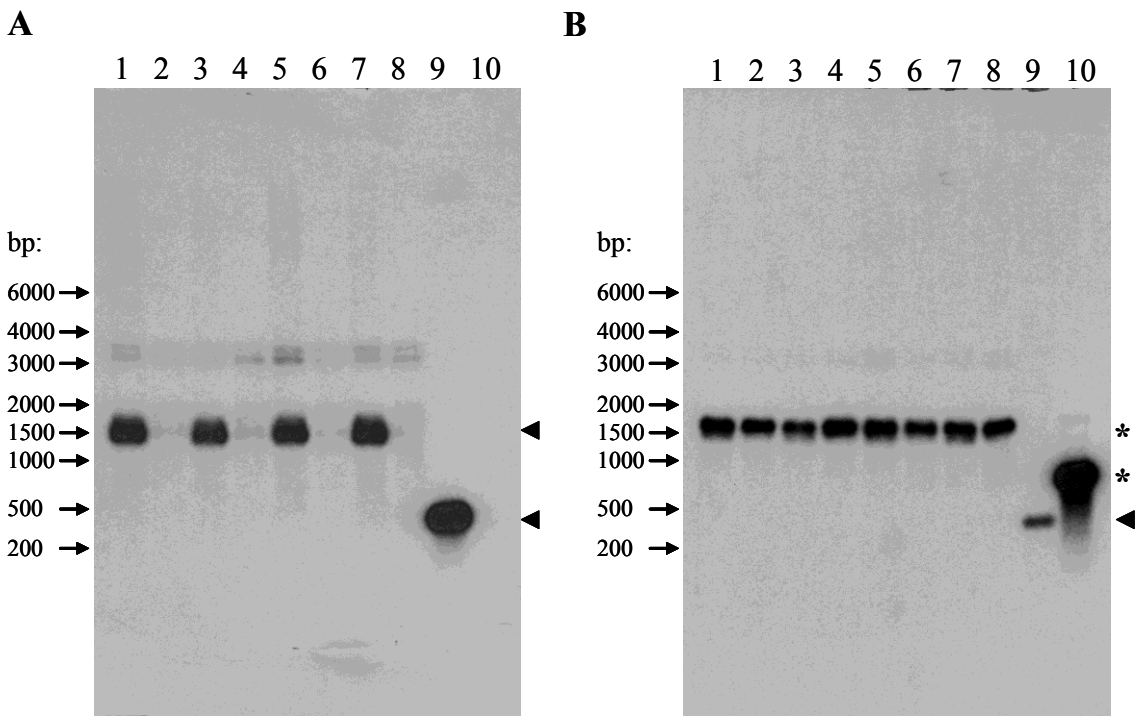

Figure A-2. Northern Blot Analysis of *FAD2-1A* RNA Level in MON 87705
Panel A and Panel B are the same northern blot containing polyA+ RNA isolated from stage R5/6 immature seed tissue. Panel A is hybridized with the FAD2-1A probe. Panel B is hybridized with the actin probe after removal of the FAD2-1A signal. Arrow heads indicate *FAD2-1A* hybridization signals and stars indicate the actin hybridization signal. Lane designations are as follows:

Lane 1: Conventional control (PolyA+ RNA replicate 1)
2: MON 87705 (PolyA+ RNA replicate 1)
3: Conventional control (PolyA+ RNA replicate 2)
4: MON 87705 (PolyA+ RNA replicate 2)
5: Conventional control (PolyA+ RNA replicate 3)
6: MON 87705 (PolyA+ RNA replicate 3)
7: Conventional control (PolyA+ RNA replicate 4)
8: MON 87705 (PolyA+ RNA replicate 4)
9: FAD2-1A probe template (200 pg)
10: Actin probe template (50 pg)

→ Symbol denotes size of RNA, in base pairs, obtained from MW markers on ethidium stained gel.

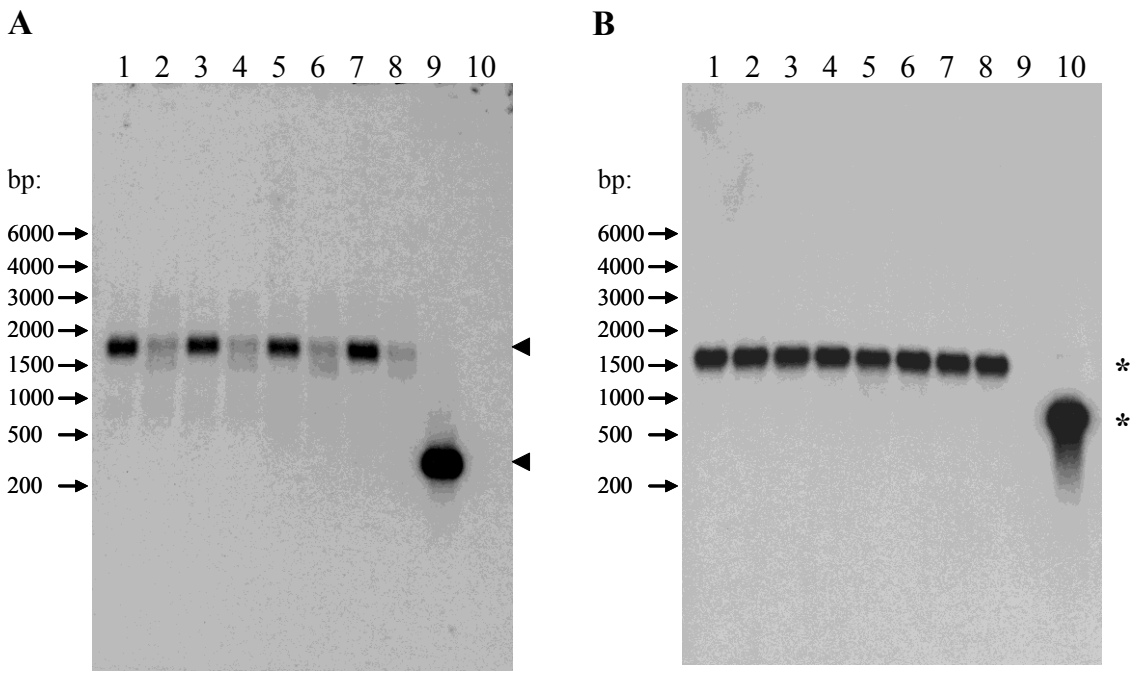

Figure A-3. Northern Blot Analysis of *FATB1-A* RNA Level in MON 87705
Panel A and Panel B is the same northern blot containing polyA+ RNA isolated from stage R5/6 immature seed tissue. Panel A is hybridized with the FATB1-A probe. Panel B is hybridized with the actin probe after removal of the FATB1-A signal. Arrow heads indicate *FATB1-A* hybridization signals and stars indicate the actin hybridization signal. Lane designations are as follows:

Lane 1: Conventional control (PolyA+ RNA replicate 1)
2: MON 87705 (PolyA+ RNA replicate 1)
3: Conventional control (PolyA+ RNA replicate 2)
4: MON 87705 (PolyA+ RNA replicate 2)
5: Conventional control (PolyA+ RNA replicate 3)
6: MON 87705 (PolyA+ RNA replicate 3)
7: Conventional control (PolyA+ RNA replicate 4)
8: MON 87705 (PolyA+ RNA replicate 4)
9: FATB1-A probe template (10 pg)
10: Actin probe template (50 pg)

→ Symbol denotes size of RNA, in base pairs, obtained from MW markers on ethidium stained gel.

Table A-1. Summary of Product Concept Fatty Acid Levels for MON 87705 vs. the Conventional Control (A3525) and Commercial Tolerance Interval

Component (Units)	MON 87705 Mean [Range]	Control Mean [Range]	p-Value	Commercial Tolerance Interval[1]
Statistical Differences Observed in Combined-Site Analysis Seed Fatty Acid (% Total FA)				
16:0 Palmitic	2.36 [2.25 - 2.44]	10.83 [10.51 – 11.08]	<0.001	[7.62, 12.55]
18:0 Stearic	3.31 [3.07 - 3.82]	4.50 [4.24 – 4.85]	<0.001	[2.87, 7.15]
18:1 Oleic	76.47 [73.13 - 79.17]	22.81 [21.41 – 25.08]	<0.001	[18.40, 30.22]
18:2 Linoleic	10.10 [7.85 - 12.42]	52.86 [51.68 – 53.89]	<0.001	[47.75, 56.46]

[1]With 95% confidence, interval contains 99% of the values expressed in the population of commercial soybean varieties. Negative limits were set to zero.

References

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