SUPPLEMENTAL MATERIALS

Supplemental Figure 1. Schematic maps of DNA fragments used for co-transformation.
Probes used for Southern analysis are indicated as numbered boxes below the fragment map.
A, PHP19340A fragment (2924 bp), containing the 2084 bp KT\textit{i3} promoter, the 597 bp \textit{gm-fad2-1} fragment, and the 196 bp KT\textit{i3} terminator. Location of restriction enzyme sites for \textit{Bam}HI, \textit{Hind}III, \textit{Nco}I, and \textit{Spe}I are marked. \textit{Bgl}II, \textit{Eco}RI, and \textit{Eco}RV do not cut the fragment. Probes indicated are: 1, KT\textit{i3} promoter; 2, \textit{gm-fad2-1}; 3, KT\textit{i3} terminator. B, PHP17752A (4512 bp), containing the 1311 bp \textit{SAMS} promoter (including 5' UTR and intron), the 1971 bp \textit{gm-hra} gene, and the 652 bp \textit{als} terminator. Location of restriction enzyme sites for \textit{Bam}HI, \textit{Bgl}II, \textit{Eco}RI, \textit{Eco}RV, \textit{Hind}III, and \textit{Nco}I are marked. \textit{Spe}I does not cut the fragment. Probes indicated are: 4, \textit{SAMS}; 5, \textit{gm-hra}; 6, \textit{als} terminator.

Supplemental Figure 2. Southern Blot Analysis of 305423 Soybean. Genomic DNA isolated from leaf tissues of individual 305423 (lane 6-9) and control (lane 2-3, 5) soybean plants. About 3 \(\mu\text{g}\) of genomic DNA was digested and loaded per lane. Approximately one copy of plasmid containing the PHP19340A (lane 2) or PHP17752A fragment (lane 3) was spiked into 3 \(\mu\text{g}\) of control (Jack) soybean DNA. Lane 5 is control (Jack) soybean DNA alone. Lane 1 and 10 are molecular weight markers. Bands detected in 305423 soybean plants but not the control ones are indicated as b1-b8 (A) or b1-b2 (B). A. Genomic DNA were digested with \textit{Spe}I/\textit{Eco}RV and probed with PHP19340A probes (Figure 1A). B. Genomic DNA were digested with \textit{Nco}I and probed with PHP17752A probes (Figure 1B).

Supplemental Figure 3. Expression of \textit{KTi3} in 20 daf developing seeds of 305423 (lane 1-10) and control (lane 11-15) soybeans. Probes used are \textit{KTi3} (491 bp, coding region) and loading control \textit{Dap}A (538 bp, coding region).

Supplemental Figure 4. Deduced Amino Acid Sequence of the GM-HRA Protein Introduced into 305423 Soybean. Double underlining indicates the start of the mature protein at residue S53. The two boxed residues, A183 and L560, are differences from the
endogenous soybean acetolactate synthase protein. The first five amino acids in bold are derived from translation of the 15 nucleotides from the *als* 5′ UTR.
Supplemental Figure 1

A

B
Supplemental Figure 2

A

probe 1: KTi3 promoter
probe 2: gm-fad2-1
probe 3: KTi3 terminator

B

probe 4: SAMS
probe 5: gm-hra
probe 6: als terminator
Supplemental Figure 3
Supplemental Figure 4

1 MPHNT MAATA SRTTRFSSSS SHPTFPKRIT RSTLPLSHQT LTKPNHALKI
51 KCSISKPTA APFTKEAPTTP EPFVSRFASG EPRKGADILV EA LerQGT VTT
101 VFAYPPGASMAI EIQALTRSA AIRNVLP RHE QGGVFAAEGY ARSSGLPGVC
151 IATSGPGATN LVGLADALM DSVPVVAITG QVAR RMIGTD AFQETPIVEV
201 SRSITKHNYL IDVVDPVPRV VAEAFFVATS GRPGPV LIDI PKDVQQQLAV
251 PNWDEPVNLPGYLRALPRPP AEAQLEHIVR LIMEAQ KPVLYVVGGS LNSS
301 AELRRFVEILT GIPVASTLMG LGTFFPGDEY SLOMLGMH GTVYANYAVDNS
351 DLLLAFGVRF DDRVTGKLEA FASRA KIVHI DIDS AEGGK KQAHV SVCAD
401 LKLALKGINM ILEEPKVE G KFDLGGWRITE NVQHKFPLL G YKTFQDAISP
451 QHAIEVLDEL TNGDAIVSTG VGGHQMWAAQ FYKYKPRQW LTSGGLGAMG
501 FGLPAAILGA VANPAGVVVD IDGDGSFIMN V3LELATIRVE NLPVKILLLN
551 NHGLGMVQ L EDRF YKS NRA H T YL G DPSSE SEIF PNMLKF ADACG I PAAR
601 VTKKEELRAA IQRMLDT PQ YL LDIVPVHQ EH VLP MI PSN GSFKDVITEG
651 DGRRT
SUPPLEMENTAL METHODS

Molecular characterization of the inserted DNA in 305423 soybean
To identify and characterize the structure of the inserted DNA in 305423 soybean, two cosmid libraries were constructed from T4 generation genomic DNA that was partially digested with HindIII or MboI. Five positive clones were identified from the libraries using the KTi3 promoter fragment as hybridization probe (data not shown). Three of them were from the HindIII library (51-21, 51-9 and H3IIBB19) and two were from the MboI library (mbo30 and mbo22). These five clones were fully sequenced. The results showed that 51-21 and mbo30 were overlapping clones containing an identical insertion (Insertion 1, Figure 1A), 51-9 and mbo22 were overlapping clones containing another identical insertion (Insertion 2, Figure 1B), and H3IIBB19 was a unique clone containing Insertion 3 (Figure 1C). Primers were designed based on the cosmid clone sequences, and overlapping PCR products spanning the insertions, the insert/host genome junctions and the flanking genomic regions were obtained and sequenced.

However, when the total genomic DNA of 305423 soybean was digested with SpeI or BclI, and probed with gm-fad2-1 fragment and KTi3 promoter fragment for Southern analyses, additional signals were detected (data not shown), indicating the presence of additional DNA insertion(s). Since two signals of 2.8 kb and 5.1 kb were detected from the SpeI digestion, and two signals of 1.5 kb and 3.3 kb were detected from the BclI digestion, digested DNA fragments around these sizes were purified and cloned into plasmid libraries. These libraries were screened with the KTi3 promoter probe and positive clones were fully sequenced. A new DNA insertion was identified by this approach (Insertion 4, Figure 1D).

Detailed Southern analysis was performed using various restriction enzymes alone or in combinations for genomic DNA digestions. Specifically, genomic DNA from 305423 and control soybeans was digested with EcoRV and SpeI combined, as EcoRV does not cut the PHP19340A fragment and SpeI has a single recognition site at the end of the KTi3 terminator element (Supplemental Figure 1). The resulting digests were used for Southern blots and probed with DNA fragments derived from the KTi3 promoter, the gm-fad2-1 fragment, and the KTi3 terminator (Supplemental Figure 1, 2A). Single digestions using EcoRV or SpeI were used to support interpretation of the Southern double digestion (data not shown). The Southern results
revealed the existence of eight copies of the KTi3 promoter, seven copies of the gm-fad2-1 fragment, and five copies of the KTi3 terminator in the 305423 genome (Supplemental Figure 2A). Similarly, to characterize the PHP17752A insertion, genomic DNA was digested with NcoI, which cuts the PHP17752A fragment only once within the gm-hra region, and hybridized with probes derived from the SAMS region, the gm-hra gene, and the als terminator (Supplemental Figure 1, 2B). The Southern results showed one copy of the PHP17752A in 305423 soybean.

In addition, both the T4 and T5 generations of 305423 soybean were analyzed by Southern blot for the presence of backbone sequences derived from the source plasmids that were used to generate the transformation fragments PHP19340A and PHP17752A. When the hygromycin resistance gene or the plasmid origin region were used as probes, no hybridization signal was detected (data not shown), confirming the absence of all functional elements from the plasmid backbone in 305423 soybean. When a backbone probe (other than the hygromycin resistance gene region) was used, a hybridization signal was detected from the NcoI blot (data not shown). Detailed sequencing analysis showed that this hybridization signal came from a 495 bp fragment associated with Insert 3 (Figure 1C).

For inheritance analysis, since there is a single NcoI site at the KTi3 promoter region of the PHP19340A fragment (Supplemental Figure 1A), and a single NcoI site at the gm-hra coding region of the PHP17752A fragment (Supplemental Figure 1B), NcoI was chosen to digest the genomic DNA. Probes used were derived from the gm-fad2-1 fragment, the gm-hra gene, and the KTi3 promoter to detect all four insertions.

**Expression of FAD2-1 is suppressed in 305423 soybean seeds**

Gene expression was analyzed by northern analysis on leaves and developing seeds at 20 and 30 days after flowering (daf) from ten 305423 soybean plants of the T4 generation and five Jack control plants. Probes were derived from either the introduced gene fragment (gm-fad2-1) or the 3’ UTR (FAD2-2) and coding regions (FAD3, DapA) of the endogenous genes. A gene encoding for dihydrodipicolinate synthase (DapA), involved in amino acid biosynthesis, was used as a low-level expression control to validate northern blot sensitivity.

The expression patterns of the tested genes in developing seeds at 30 daf were very similar to those detected in 20 daf seeds, and therefore not shown here.