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(54) **Title**: DOMINANT EARLINESS MUTATION AND GENE IN SUNFLOWER (HELIANTHUS ANNUUS)

(57) **Abstract**: The subject invention relates in part to the discovery of a spontaneous sunflower mutation. The subject invention involves an 'early' mutation and related inbred/hybrid development. The subject invention further provides a single dominant gene that confers earliness in sunflower inbred isolines and near isogenic hybrids. There is no known prior teaching or suggestion of this gene's utility for hybrid development in the industry. The subject indention also provides a new and distinctive sunflower inbred line designated H 120R. The invention includes seeds that possess this mutated gene, plants produced by growing these seeds, and progeny thereof that possess this mutated gene and the associated earliness trait. The subject invention also includes methods for producing such sunflower seeds and plants, including inbreds and hybrids. Such plants can be produced by, for example, crossing such an inbred line with itself or with another sunflower line.

DOMINANT EARLINESS MUTATION AND GENE IN SUNFLOWER (*HELIANTHUS ANNUUS*)

BACKGROUND OF THE INVENTION

Sunflowers are an important and valuable field crop to supply food for both animals and humans. A continuing goal of plant breeders is to develop stable, high yielding sunflower hybrids that are agronomically sound so that the amount of seed produced on the land used is maximized. To accomplish this goal, the sunflower breeder must select and develop sunflower plants that have the traits that result in superior parental lines for producing hybrids.

Sunflower (*Helianthus annuus L.*) can be bred by both self-pollination and cross-pollination techniques. The sunflower head (inflorescence) usually is composed of about 1,000 to 2,000 individual disk flowers joined to a common base (receptacle). The flowers around the circumference are ligulate ray flowers with neither stamens nor pistil. The remaining flowers are hermaphroditic and protandrous disk flowers.

Natural pollination of sunflower occurs when flowering starts with the appearance of a tube partly exerted from the sympetalous corolla. The tube is formed by the five syngenesious anthers, and pollen is released on the inner surface of the tube. The style lengthens rapidly and forces the stigma through the tube. The two lobes of the stigma open outward and are receptive to pollen but out of reach of their own pollen initially. Although this largely prevents self-pollination of individual flowers, flowers are exposed to pollen from other flowers on the same head by insects, wind and gravity.

Promising advanced breeding lines are thoroughly tested and compared to appropriate standards in environments representative of the commercial target area(s) for three years at least. The best lines are candidates for new commercial cultivars; those still deficient in a few traits are used as parents to produce new populations for further selection.

These processes, which lead to the final step of marketing and distribution, usually take from eight to 12 generations from the time the first cross is made. Therefore, development of new cultivars is a time-consuming process that requires precise forward planning, efficient use of resources, and a minimum of changes in direction.

A most difficult task is the identification of individuals that are genetically superior, because for most traits the true genotypic value is masked by other confounding plant traits or environmental factors. One method of identifying a superior plant is to observe its performance relative to other experimental plants and to a widely grown standard cultivar. If a single observation is inconclusive, replicated observations provide a better estimate of its genetic worth.

Each year, the plant breeder selects the germplasm to advance to the next generation. This germplasm is grown under unique and different geographical, climatic and soil conditions, and further selections are then made, during and at the end of the growing season. The inbred lines that are developed are unpredictable. This unpredictability arises because the breeder's selection occurs in unique environments, with no control at the DNA level (using conventional breeding procedures), and with millions of different possible genetic combinations being generated. A breeder of ordinary skill in the art cannot predict the final resulting lines that will ultimately be developed, except possibly in a very gross and general fashion. The same breeder cannot produce the same line twice by using the exact same original parents and the same selection techniques. This unpredictability results in the expenditure of large research monies to develop a superior new sunflower inbred line.

Descriptions of breeding methods that are commonly used for different traits and crops can be found in one of several reference books (*e.g.*, Allard, 1960; Simmonds, 1979; Sneep *et al.*, 1979; Fehr, 1987).

A mutant sunflower was reported by Heaton *et al.*, but the locus of that mutation is unknown. T.C. Heaton, *et al.*, 1981, "Rapid Conversion of Maintainer Lines to Cytoplasmic Sterility," *Proceedings Sunflower Forum and Research Workshop,* p.23.

BRIEF SUMMARY OF THE INVENTION

The subject invention relates in part to the discovery of a spontaneous sunflower mutation. The subject invention involves an "early" mutation and related inbred/hybrid development. The subject invention further provides a single dominant gene that confers earliness in sunflower inbred isolines and near isogenic hybrids. There is no known prior teaching or suggestion of this gene's utility for hybrid development in the industry. The subject invention also provides a new and distinctive sunflower inbred line designated H120R.

The invention includes seeds that possess this mutated gene, plants produced by growing these seeds, and progeny thereof that possess this mutated gene and the associated earliness trait. The subject invention also includes methods for producing such sunflower seeds and plants, including inbreds and hybrids. Such plants can be produced by, for example, crossing such an inbred line with itself or with another sunflower line. The invention further relates to such plants and methods for producing such sunflower plants further containing in their genetic material one or more transgenes. Parts of a sunflower plant of the present invention are also provided, such as *e.g.*, pollen obtained from an inbred plant and an ovule of the inbred plant, wherein such parts comprise an early maturity gene of the subject invention.

The subject invention can significantly reduce the phenophase emergence flowering without affecting the filling period. This invention can also significantly increase the IC. This invention can also be used to convert very late, elite inbreds in earlier iso-lines for other geographies that require shorter maturity. This invention can also be used to increase density tolerance and for intercropping.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a photo of the H120R isoline showing flower development comparisons between the late Argentine line H120R and its early mutated version.

Figure 2 shows relationships between (A) leaf area index and (B) the proportion of the incident radiation which is intercepted by the crop (Qd) and the time from first anthesis for genotypes X223 (MG2em) and MG2. Vertical bars indicate standard deviation, when larger than the symbol.

Figure 3 shows bi-lineal relationship between seed weight and time from first anthesis for genotypes X223 (MG2em) and MG2. Vertical bars indicate standard deviations, when larger than the symbol.

Figure 4 shows bi-lineal relationship between harvest index (corrected for synthesis costs) and time from first anthesis for genotypes X223 (MG2em) and MG2. Vertical bars indicate standard deviations, when larger than the symbol.

Figure 5 shows a genetic map of a major locus for the early flowering (EF) gene. See Example 8.

Figure 6 illustrates a strategy for marker development.

Figure 7 shows markers flanking the early flowering gene of the subject invention.

Figure 8 illustrates an accelerated introgression strategy.

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NOs:1-60 are forward and reverse primers as discussed in Example 8.

SEQ ID NO:61 is the HA1805 forward primer.

SEQ ID NO:62 is the HA1805 reverse primer.

SEQ ID NO:63 is a genomic sequence comprising two single nucleotide polymorphism (SNP) loci as discussed in Example 9; SEQ ID NO:82 shows the SNPs as found in the early flowering / early maturing gene / line.

SEQ ID NO:64 is a forward primer for amplifying the "R" SNP locus.

SEQ ID NO:65 is a reverse primer for amplifying the "R" SNP locus.

SEQ ID NO:66 is a probe comprising the early-maturing nucleotide / polymorphism at the R locus.

SEQ ID NO:67 is a probe comprising the wild-type nucleotide at the R locus.

SEQ ID NOs:68-81 are marker sequences discussed in Example 9.

SEQ ID NO:82 is a genomic sequence comprising two single nucleotide polymorphisms (SNPs) as discussed in Example 9; SNPs as found in the early flowering / early maturing gene / line occur at residues 65 (the "Y" locus) and 125 (the "R" locus).

DETAILED DESCRIPTION OF THE INVENTION

The subject invention relates in part to the discovery of a spontaneous sunflower mutation. The subject invention involves an “early” mutation and related inbred/hybrid development. The subject invention further provides a single dominant gene that confers earliness in sunflower inbreds and hybrids, including inbred isolines and near isogenic hybrids. There is no known prior teaching or suggestion of this gene’s utility for hybrid development in the industry. The subject invention also provides a new and distinctive sunflower inbred line designated H120R. The mutation was discovered in nursery row 2290141 of a H792A inbred increase block. **Figure 1** is a photo of the

H120R isoline showing flower development comparisons between the late Argentine line H120R and its early mutated version.

This gene was originated by natural mutation in a sunflower breeding population. It was initially used to create hyper-early versions of early inbreds pursuing adaptation to short maturity regions. Later on its potential use to normalize hyper-late inbreds was understood and applied.

Inheritance of the subject traits conferred by the subject gene appears to be qualitative (single and incomplete dominance). The effect is seen as clearly dominant, but there are some indications of "gene dosage" effects.

Insertion of this gene (by backcrossing, for example) will allow the direct use of converted late sunflower inbreds in earlier environments. It can also be used for transgenic research and development in other crops.

The gene could allow late genotypes with desirable traits, quantitative and qualitative, to be moved into earlier (shorter season) environments. The same concept could be applied for the transgenic development of other crops. That is, this trait can also be bred or otherwise introduced into other, non-sunflower crops. For example, with successful applications, tropical corn germplasm could be made available for use in the central U.S. corn belt, for example. In addition, central corn belt germplasm could be moved north.

The early gene may also have utility as an aid in backcrossing traits, some examples of which include cytoplasmic male sterility or imidazilinone (IMI) resistance. If the heterozygote early flowering backcross F1 progeny are selected with the desired donor trait, the conversion cycle could be shortened. (Selfing would occur at the final stages of conversion when the desired maturity is selected.)

This gene can be transferred to other sunflower inbreds by the backcross method of breeding. Only one converted inbred is required to develop a hybrid conferring earlier maturity.

The early mutation gene appears to confer relatively proportionate decreases in days to flower, and thus maturity, for a wide range of conventional recurrent parent maturities. Proportionate flowering/maturity modifications are desirable, as it is undesirable for all inbreds, and thus hybrids, to mature in the same number of days for a restricted marketing area.

The invention includes seeds that possess this mutated gene, plants produced by growing these seeds, and progeny thereof that possess this mutated gene and the associated earliness trait. The subject invention also includes methods for producing such sunflower seeds and plants, including inbreds and hybrids. Such plants can be produced by, for example, crossing such an inbred line with itself or with another sunflower line. The invention further relates to such plants and methods for producing such sunflower plants further containing in their genetic material one or more transgenes. Parts of a sunflower plant of the present invention are also provided, such as *e.g.*, pollen obtained from an inbred plant and an ovule of the inbred plant, wherein such parts comprise an early maturity gene of the subject invention.

"Early Maturity" means a mean time to physiological maturity (where physiological maturity is defined as the time sunflower plant seed fill is complete), which ranges from between about 60 days to about 90 days. In some embodiments, this can be from about 60 days to about 70 days.

"Early Flowering" means a mean time to flowering for a sunflower plant which ranges from between about 48 days to about 66 days. In some embodiments, this can be from about 48 days to about 55 days.

By routine screening, it is expected that EM plants may vary in Early Maturity and Early Flowering by approximately 10%.

Head size (head periphery), dry seed weight and/or yield is statistically the same for EM and for wild-type.

CNE840B is the early mutant conversion of H840B. That is they are genetically the same except CNE840B has the mutation, and H840B does not. CNE840B is a backcross 5 derivation of H840B (as the recurrent parent) x an early mutant donor parent.

As part of this disclosure, at least 2500 seeds of early maturing sunflower line CNE840B, comprising the early maturity gene, have been deposited in accordance with the Budapest Treaty on October 17, 2007, and made available to the public without restriction (but subject to patent rights), with the American Type Culture Collection (ATCC) Manassas, VA 20110-2209. The deposit has been designated as ATCC Deposit No. PTA-8715. The deposit will be maintained without restriction at the ATCC depository, which is a public depository, for a period of 30 years, or five

years after the most recent request, or for the effective life of the patent, whichever is longer, and will be replaced if it becomes nonviable during that period.

The deposited seeds are part of the subject invention. Clearly, plants can be grown from these seeds, and such plants are part of the subject invention. The subject invention also relates to DNA sequences contained in these plants. Related early maturing progeny thereof, including the use of the parent plants and such progeny plants in crosses, are part of the subject invention. Detection methods and kits, of the subject invention, can be directed to identifying any of the deposited and/or progeny lines thereof.

In other aspects, the present invention provides regenerable cells, comprising such genes, for use in tissue cultures, for example. The tissue culture will preferably be capable of regenerating plants having the physiological and morphological characteristics of the foregoing sunflower plant, and of regenerating plants having substantially the same genotype as the foregoing inbred sunflower plant. Preferably, the regenerable cells in such tissue cultures will be embryos, pollen, ovules, leaves, stems, cortex, pith, involucral bracts, ray flowers, disk flowers, pappi, achenes, nectaries, interfloral bracts, receptacle, trichomes stigma, anther, style, filament, calyx, pericarp, seed coat, endosperm, embryo, roots, root tips, seeds and the like. Still further, the present invention provides early maturing sunflower plants regenerated from the tissue cultures of the invention.

Days to flower in the early isolines of H418R and H120R were 62 and 66 days, respectively, compared to 68 and 75 days for the recurrent parents. For comparisons involving normal early line conversions to early mutant, at one location, flowering occurred in as few as 35-37 days after planting in Group 1 F3 early mutant derivations (with the gene in Very Early segregating F3 derivations), versus 48 days for the normal (Group 1 derivations) Very Early (Group 1) inbred. At another location, days to flower for the early mutant isolines and its late maturing recurrent parent H840B (Argentine inbred) were 64 vs 80, respectively. Maturity classification changed from the recurrent parent's group 7 (very late) to the early mutant conversion of group 3 (moderately early).

The subject gene can also be stacked with other traits. This can be accomplished in a variety of ways. Cross-breeding with other lines (having other traits) is known in the art. *See e.g.* CLEARFIELD™ Sunflower (Helianthus annuus) Line X81359. Also, the subject trait and/or other traits can be genetically engineered to obtain a plant comprising the desired combination of traits.

For example, ornamental and confection (for human consumption) lines and varieties can be introgressed with the subject earliness gene. *See e.g.*:

Yue et al. (2007) "Experimenting with marker-assisted selection in confection sunflower germplasm enhancement." 29th Sunflower Research Workshop, January 10-11, 2007, Fargo, ND (available at website sunflowernsa.com/research/research-workshop/documents/Yue_Experiment_Marker_07.pdf).

Miller et al. (2006) "Registration of three low cadmium (HA 448, HA 449, and RHA 450) confection sunflower genetic stocks." *Crop Science*. 46:489-490 (January 1, 2006).

"Interspecific hybridisation and cytogenetic studies in ornamental sunflower breeding,"*J. Atlagic* et al., *Australian Journal of Experimental Agriculture* 45(1) 93–97, published 21 February 2005.

"Genes for pollen fertility restoration in sunflowers." *Euphytica*, Volume 27, Number 2 / June, 1978, pp. 617-627.

Some other examples of some traits and lines are in the following patent references:

U.S. Patent or U.S. Application Serial Number	**Title or subject matter**	**Application Filing Date** *(if applicable)*
61/015,591	Low Saturated-Fat Sunflower and Associated Methods	20 December 2007
USSN 11/245,991 (Published as 2006/0112450A1)	Sunflower Seed with High Delta-Tocopherol Content	7 October 2005
60/721,181	High Oleic Imidazolinone Resistant Sunflower	28 September 2005
USPNs 4,627,192 and 4,743,402	High Oleic Sunflower	
USPN 5,276,264	Sunflower Products having Lower Levels of Saturated Fatty Acids	

USPN 6,977,328	Sunflower Seed having Low Saturated Oil Content (also high oleic)
USPN 6,956,156	Inbred Sunflower Line H1063R (also high oleic and imidazolinone resistant)

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification.

Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

EXAMPLES

EXAMPLE 1 – Agronomic Testing and Sample Results

This gene was originated by natural mutation in a sunflower breeding population. This gene was initially used to create hyper-early versions of early inbreds pursuing adaptation to short maturity regions. Later on its potential use to normalize hyper-late inbreds was understood and applied.

A set of experiments was carried out with the purpose to initiate characterization of the *em* gene in sunflower, using the following genotypes:

MG2 H757A*H120R Wild type

X223 H757A*EM229135R Early Mutant type

EM229135R = H120Rem (BC2F7 homozygous)

Some of the highlights are the following:

Table 1. Mean values for time to flowering (DtoFLW) and physiological maturity (DtoPhM), of two sunflower hybrids contrasting in earliness character. G: genotype, d.m.s: significant difference. *=note reduction in DTF, but same length of seed filling period

Attribute	Genotype		*P*	d.m.s
	X223	MG2	G	α= 0.10
DtoFLWB (day)	56*	67	0.00	1
DtoPhM (day)	89	99	0.11	11

Table 2. Mean values for leaf area index near of 13 (X223) and 16 (MG2) days after first anthesis (LAI13/16) and physiological maturity (LAIMF), onset of leaf (SLS), rate of leaf senescence (LSR) and proportion of the incident radiation which is intercepted by the crop near of 13 (X223) y 16 (MG2) days after first anthesis (Qd13/16) and physiological maturity (QdMF) of two sunflower hybrids contrasting in earliness character. *=note reduction in LAI, with consequent lower light interception ratio

Attribute	Genotype		*P*	d.m.s
	X223	MG2	G	α= 0.10
$LAI_{13/16}$	1.80*	3.40	0.01	0.53
LAI_{PhM}	1.63*	2.69	0.17	0.17
SLS (day)	32.5	30.9	0.53	7.1
LSR (LAI day-1)	-0.104	-0.198	0.14	0.130
$Qd_{13/16}$	0.848	0.959	0.01	0.041
Qd_{MF}	0.836*	0.903	0.24	0.132

Table 3. Agronomic characters and mean values for head diameter (HEAD_DIAM), petiole length (PET_LENG) and height (HEIGHT) of two sunflower hybrids contrasting in earliness character.

Attribute	Genotype		*P*	d.m.s
	X223	MG2	G	α= 0.10
HEAD_THICKNESS †	thin	thin		
HEAD_SHAPE ††	flat	flat		
NECK BENDING (degree) ‡	30	30		
HEAD_DIAM (cm)	16.2	18.9	0.05	2.2
PET_LENG (cm)	13.1	16.3	0.00	0.46
HEIGHT (m)	1.18	1.88	0.00	0.15

† Head thickness
†† Head shape
‡ Head orientation

Figure 2 shows the relationships between (A) leaf area index and (B) the proportion of the incident radiation which is intercepted by the crop (Qd) and the time from first anthesis for genotypes X223 (MG2em) and MG2. Vertical bars indicate standard deviation, when larger than the symbol.

Table 4. Mean values for oil yield (OIL YLD), grain number (#GRAINS), grain weight (GW), rate of grain filling (FR), time from anthesis to the end of grain filling (FD) and kernel percentage (%E) for the portions periphery (PER), intermediate (INT) and inner (CEN) of the head and grain oil concentration (OIL%) of two sunflower hybrids contrasting in earliness character; *=most significant difference

Attribute	Genotype		*P*	d.m.s
	X223	MG2	G	α= 0.10
OILYLD (kg/ha)	1049	1374	0.15	466
#GRAINS m-2	6078*	8109	0.10	2342
GW_PER (mg)	70.86	65.89	0.47	18.15
GW_INT (mg)	60.91	57.83	0.37	8.59
GW_CEN (mg)	48.40	50.79	0.33	7.73
FR_PER (mg d-1)	2.21	2.66	0.15	0.63
FR_INT (mg d-1)	2.16	2.61	0.53	1.97
FR_CEN (mg d-1)	1.71	1.43	0.61	2.25
FD_PER (day)	31.1	31.2	0.96	6.8
FD_INT (day)	33.6	32.7	0.80	10.6
FD_CEN (day)	35.2	40.0	0.32	15.2
OIL%	45.8	46.6	0.60	4.01

Figure 3 shows bi-lineal relationship between seed weight and time from first anthesis for genotypes X223 (MG2em) and MG2 planted in Colón 2002/03. Vertical bars indicate standard deviations, when larger than the symbol.

Table 5. Mean values for oil-corrected grain yield (YLD), oil-corrected biomass near of 12 (X223) and 15 (MG2) days after first anthesis (BMco12/15) and physiological maturity (BMCoMF), production of oil-corrected biomass (ΔBMco12/15-MF) and daily production of oil-corrected biomass between 12 (X223) and 15 (MG2) days after first anthesis and physiological maturity of two sunflower hybrids contrasting in earliness character. 12/15: days after first anthesis (12 days X223 and 15 days MG2), MF: physiological maturity.

Attribute	Genotype		*P*	d.m.s
	X223	MG2	G	α= 0.10
YLD corr by OIL (g m-2)	378	490	0.14	159
BMco12/15 (g m-2)	644	1277	0.00	200
BMcoMF (g m-2)	1014	1810	**0.00	251
ΔBMco12/15-MF (g m-2)	371	534	0.00	52
RBMco12/15-MF (g m-2 day-1)	21.3	33.7	0.02	5.05

**=note that YLD, Biomass, rate, and HI are different

Table 6. Mean values for harvest index (HI), rate of daily HI increase (HIR) and duration of the linear phase of HI increase (HID), of two sunflower hybrids contrasting in earliness character. * Determined as the ratio of oil-corrected grain dry matter to oil-corrected above ground dry matter.

Attribute	Genotype		*P*	d.m.s
	X223	MG2	G	α= 0.10
HI *	0.526	0.466	0.01**	0.020
HIR (IC day-1)	0.0163	0.0140	0.75	0.015
HID (day)	31.7	34.1	0.41	7.5

Figure 4 shows bi-lineal relationship between harvest index (corrected for synthesis costs) and time from first anthesis for genotypes X223 (MG2em) and MG2 planted in Colón 2002/03. Vertical bars indicate standard deviations, when larger than the symbol.

EXAMPLE 2 – Characterization of Gene Dominance, Gene Dosage, and Application of the ***em*** Gene for Reducing Phenophase Without Affecting the Filling Period

The subject mutation/mutated gene can be used to significantly reduce the phenophase "emergence-flowering" (V1-R5.1), without affecting the subsequent filling period (R5.5-R9), in the sunflower growing cycle; it could be used to convert very late "elite inbreds showing **reduced Genotype Environment interaction**" in earlier "iso-lines" for other geographies that require shorter maturity.

Two inbreds have already been converted (BC4+) and fixed (H840B and H418R), and one partially converted (BC2F7) but fixed (H120R). The *em* versions of both H840B and H120R fits perfectly the maturity normally used in North America. These *em* versions of inbred lines have even been useful to create hybrids of mg 3, being the recurrent inbreds mg 7 and mg 6, respectively. This hybrid performed earlier than 8377NS and near SF270.

H840B was used to make experimental hybrids with very good rust tolerance in the past. They were outstanding in performance but very late and tall. The new em version can be used to re-create those hybrids, and to include it in the "elite collection", once the cited problems have been removed by the effect of the em gene.

Based on various observations, the gene inheritance appears to be qualitative (single and incomplete dominant). The effect is seen as clearly dominant, but there are some indications of "gene dosage" effects. If this is true, it would allow creation of iso-hybrids for different maturity groups by using the gene in both hetero or homzygous form, which would expand even more the use of elite germplasm.

A series of experiments (RCBD) have been designed to prove/reject that hypothesis, with the purpose to clearly identify the inheritance mode and gene action, by the study of the following genotypes:

Table 7.

Entry Code	Gen	Genotype
H840B		Ee
H840Bem		EE
H840B/H840Bem	F1	ee/EE
(H840B/H840Bem)@	F2	EE;Ee;ee
H840B//H840B/H840Bem	BC1F1(-)	ee//ee/EE

Table 8.		
Entry Code	**Gen**	**Genotype**
H418R		Ee
H418Rem		EE
H418R/H418Rem	F1	Ee
(H418R/H418Rem)@	F2	EE, Ee, ee
H418R//H418R/H418Rem	BC1F1(-)	ee//ee/EE
H418Rem//H418R/H418Rem	BC1F1(+)	EE//ee/EE

Table 9.		
Entry Code	**Gen**	**Genotype**
H840B/H418R	F1	ee/ee
H840B/H418Rem	F1	ee/EE
H840Bem/H418R	F1	EE/ee
H418R/H840Bem	F1	ee/EE
H840Bem/H418Rem	F1	EE/EE

In addition to earliness related measurements, pleiothropic effects on traits such as PHGT, HDIAM, SDIAM, #LEAF, etc, will also be measured.

EXAMPLE 3 – Use of This Gene to Allow Expansion of the Sunflower Frontiers to Areas with Shorter Growing Seasons and More Limited Water Availability

Due to the significant reduction in that phenophase, the use of this gene can allow expansion of the sunflower frontiers to areas with shorter growing seasons and more limited water availability, by the combined use of elite iso-hybrids from other areas. Because of the pleiothropic effects of the gene on other traits, changes in the crop spatial distribution might be needed to maximize crop productivity. Antecedents such as Sunwheat and Sunola have been extensively tested, but with genetic background limitations.

An experiment has also been setup to study this kind of gene effects, and to quantify the effect of diverse spatial distribution in iso-hybrids, over the growth and developmental parameters and yield components.

Table 9.		
Entry Code	**Gen**	**Genotype**
H757A/H120R	F1	(ee/ee)
H757A/H120Rem	F1	(ee/EE)
H840A/H418R	F1	(ee/ee)
H840A/H418Rem	F1	(ee/EE)
Factorial design with: **Row spacing:** 0.70 cms & 0.35 cms **Plant Populations:** 35,000, 65,000, and 95,000 pl/ha		

EXAMPLE 4 – Use of the Subject Gene to Accelerate Introgression of Other Traits

Due to the inheritance of this gene, it is a very powerful tool to accelerate introgression of other traits, by keeping the *em* gene in heterozygous form along the backcross process. Gene must be introgress in donors or recurrents.

EE XX * ee xx

Ee Xx * ee xx

ee xx Ee Xx * ee xx

ee xx Ee Xx @ → ee XX recovered

EXAMPLE 5 - Further Characterization and Sequencing of the Gene

Due to the status of our conversions, the gene could easily be mapped, sequenced, and eventually transformed in a different crop (earliness is very easy to identify).

H840Bem*H840B F1

(H840Bem*H840B)@ F2

EXAMPLE 6 - Use of This Gene in Sunflower Hybrid Products

With the development of early mutant inbred isolines nearly complete, the next stage of testing was to determine practical use of this gene in sunflower hybrid products. Limited hybrid testing was done, comparing performance of the early mutant version of the hybrid MG2 against its normal group 6 maturity version and other hybrids of similar Group 3 maturity.

EXAMPLE 7 – Insertion of the gene into plants

One aspect of the subject invention is the transformation of plants with the subject polynucleotide sequences.

A heterologous promoter region capable of expressing the gene in a plant can be used. Thus, for *in planta* expression, the DNA of the subject invention is under the control of an appropriate promoter region. Techniques for obtaining *in planta* expression by using such constructs is known in the art.

A gene of the subject invention can be inserted into plant cells using a variety of techniques that are well known in the art. For example, a large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13mp series, pACYC184, etc. Accordingly, the heterologous sequence can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid is recovered. Sequence analysis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be cleaved and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids.

Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border, but often the right and the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted. The use of T-DNA for the transformation of plant cells has been intensively researched and sufficiently described in EP 120 516; Hoekema (1985) In: *The Binary Plant Vector System*, Offset-durkkerij Kanters B.V., Alblasserdam, Chapter 5; Fraley *et al.*, *Crit. Rev. Plant Sci.* 4:1-46; and An *et al.* (1985) *EMBO J.* 4:277-287.

Once the inserted DNA has been integrated in the genome, it is relatively stable there and, as a rule, does not come out again. It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G 418,

bleomycin, hygromycin, or chloramphenicol, *inter alia*. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA.

A large number of techniques are available for inserting DNA into a plant host cell. Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection, biolistics (microparticle bombardment), or electroporation as well as other possible methods. If Agrobacteria are used for the transformation, the DNA to be inserted has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the *vir* region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in Agrobacteria. The intermediate vector can be transferred into *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors can replicate themselves both in *E. coli* and in Agrobacteria. They comprise a selection marker gene and a linker or polylinker which are framed by the right and left T-DNA border regions. They can be transformed directly into Agrobacteria (Holsters *et al.* [1978] *Mol. Gen. Genet.* 163:181-187). The *Agrobacterium* used as host cell is to comprise a plasmid carrying a *vir* region. The *vir* region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be contained. The bacterium so transformed is used for the transformation of plant cells. Plant explants can advantageously be cultivated with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the transfer of the DNA into the plant cell. Whole plants can then be regenerated from the infected plant material (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a suitable medium, which may contain antibiotics or biocides for selection. The plants so obtained can then be tested for the presence of the inserted DNA. No special demands are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

The transformed cells grow inside the plants in the usual manner. They can form germ cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the normal

manner and crossed with plants that have the same transformed hereditary factors or other hereditary factors. The resulting hybrid individuals have the corresponding phenotypic properties.

EXAMPLE 8 – Additional Information Regarding the Early Flowering Mutation Gene

Characterization. Table 10 shows significant earlier flowering and shorter heights in the homozygous converted parents versus their normal recurrent parents. Table 11 conversions in various heterozygous backcross F1 stages of development indicate a mostly dominant gene action (incomplete dominance). The first 3 pairs of hybrid comparisons in Table 12 show additional supportive evidence of dominance conferred by the early mutation gene. The last two comparisons between homozygous and heterozygous iso-hybrids indicate a possible dosage effect of the gene – the gene in both parents may be earlier than the gene in one parent, difference depending on pedigree. Given the dominant nature of the gene, introgression into elite parents is easily accomplished by traditional backcrossing by selecting for early segregates in the BCnF1 generations for further backcrossing to the elite recurrent parents. Once fully introgresssed, the BCnF1 is selfed to select for individual homozygote EM segregates in the BCnF2 population. Presence of homozygosity can be observed for subsequent BCnF3 family rows.

Table 10. Parent days to flower and height comparisons between homozygous EM conversions and recurrent parent.

Name	DTF	Ht (in)
EM BC5 H840A	65	63
H840A	85	68
EM BC4 CN2922R	53	46
CN2922R	71	64
EM BC4 H120R	53	46
H120R	67	61
EM BC4 H535A	54	57
H535A	66	63
EM H1063R	44	35
H1063R	58	53
EM BC5 H418R	49	35
H418R	59	46

Table 11. Days to flower comparisons between heterozygous BCF1 conversions and recurrent parent line.

Name	DTF
EM BC2F1 ON2343B	54
ON2343B	61
EM BC1F1 HO00537B	58
HO00537B	71
EM BC1F1 CN2798B	63
CN2798B	68
EM BC1F1 HO00587B	59
HO00587B	65
EM BC1F1 CN1907R	56
CN1907R	73
EM BC1F1 ON1938R	59
ON1938R	71
EM BC1F1 ONN947R	56
ONN947R	62
EM BC3F1 HOU98510R	51
HOU98510R	59
EM BC3F1 MOC0666R	57
MOC0666R	62
EM BC2F1 H324B	38
H324B	49

Table 12. Days to flower and height comparisons between hybrids with one, two, or no EM parents. Two (consecutive) years.

Name	DTF	Ht (in)
H757A x EM H120R	60	na
H757A x H120R	78	na
H757A x EM H418R	57	na
H757A x H418R	65	na
CN1703A x EM H1063R	56	42
CN1703A x H1063R	65	68
EM 840A x EM CN2922R	61	73
EM 840A x CN2922R	66	84
EM 840A x EM H1063R	56	58
H840A x EM H1063R	57	60

Tables 13 and 14 show the additional pleiotropic effects of the early flowering mutant. Raw data in Table 13 show a general reduction in leaf number, width, and length; shorter petioles; smaller head size; and shorter plant heights. This appears to be the reason for Table 14 results which show significantly less leaf area index (for 13 and 16 days post flowering), light interception ratio (13 and 16 days post flowering), and biomass (13 and 16 days post flower, and at physiological maturity) for the EM hybrid compared to the normal hybrid. However, the harvest index ratio (grain matter/total above ground plant dry matter) is significantly more for the EM hybrid. This is good for higher population uses, which is discussed in point 3 below under gene utility.

Table 13. Plant trait measurement comparisons between EM and normal types.

plant type	leaf no.	leaf width (in.)	leaf length (in.)	petiole length (in.)	head width (in.)	plant ht (in.)
EM Inbreds (4)	25.5	8.5	9	5.2	5.2	50.5
Normal Inbreds	33	9.3	9.7	5.6	7	63.8
EM Hybrids (2)	28.5	12.1	12.1	5.6	10	55.9
Normal Hybrids	34.5	12	13.2	6.1	12	70.4

Table 14. Leaf area index (LAI), light interception ratio (Qd), and biomass (BM) for EM and normal hybrid 13 and 16 days after first anthesis and physiological maturity (MF); and harvest index (HI) comparison.

Hybrid		LAI 13/16	LAI MF	Qd 13/16	Qd MF	BM 13/16	BM MF	HI*
H757A x EM H120R		1.8	1.63	0.85	0.84	644	1014	0.526
H757A x H120R		3.4	2.69	0.96	0.90	1277	1810	0.466
	P	0.01	0.17	0.01	0.24	0.00	0.00	0.01

*Determined as the ratio of grain dry matter to above ground dry matter.

A major locus for the early flowering gene was mapped on one end of linkage group 5 using microsatellite or SSR (Simple Sequence Repeat) markers and flowering data of F3 families from the cross MOC0666R x CNE418.312. See **Figure 5.**

The maps in sunflower are usually referred to by linkage groups. Linkage group 5 corresponds to the maps published by Dr. Steve N. Knapp's group. See *See* Yu *et al.,* (2003)

"Towards a saturated molecular genetic linkage map for cultivated sunflower," Crop Sci. 43:367-387; and Tang *et al.* (2002) "Simple sequence repeat map of the sunflower genome," Theor Appl Genet 105: 1124-1136. Linkage group numbers of maps developed by European scientists are different from the ones developed by Dr. Knapp's group. The chromosome numbers have not been defined in sunflower yet.

Following are primer sequences and map positions of the SSR markers mapped on linkage group 5, where the early flowering locus (EF) is mapped.

Marker	Map Position (cM)	Forward Primer Sequence	Reverse Primer Sequence
HA1768	0	AAATCCACAAGGATGCTCAATC SEQ ID NO:1	GGAGATCATACAAAGCGTTATCGT SEQ ID NO:2
HA1620B	3	HEX-TTTCGTGATGGTGATTGATGATT SEQ ID NO:3	CAGCAACTCTGACCGTTTCATTA SEQ ID NO:4
HA1829	7	CATTGAGGACGAGAAGCCAGT SEQ ID NO:5	GTTCCGTACCCTGTTTGAGCTT SEQ ID NO:6
HA1102	24	TGTTCACAGCTCCCGTCTAA SEQ ID NO:7	CACACACACAACAACCTGACC SEQ ID NO:8
HA0694	26	GCCGTGAATAATGGGATTGA SEQ ID NO:9	GATTGGGTCAGCTTGTGTGA SEQ ID NO:10
HA0850A	27	CCCTGGAGTGTATGTCCGTTA SEQ ID NO:11	ATCCGTCTGCTGCCTAATCC SEQ ID NO:12
HA0729A	29	TGAAACGTAGTAACCTGCCAAA SEQ ID NO:13	TTGGACGACCTCGGTATCTT SEQ ID NO:14
HA1620A	30	HEX-TTTCGTGATGGTGATTGATGATT SEQ ID NO:15	CAGCAACTCTGACCGTTTCATTA SEQ ID NO:16
HA1489B	31	CTTATTCCAAGGACGCATAGTCG SEQ ID NO:17	CGATGGTATGATTCTCGACGTTA SEQ ID NO:18
HA1666	34	FAM-ATTTCACCCTCACTCCCACAC SEQ ID NO:19	TACCGGCTGGATATGGAGAAT SEQ ID NO:20
HA1485	34	GGGAAGTGGGCTTGTCTATGTAT SEQ ID NO:21	AACACACCGAAATCACCTATGAA SEQ ID NO:22
HA1838	34	AGAGGAATGAGATCGGGTTGAT SEQ ID NO:23	GTGGGACAACTCAGCAACGTC SEQ ID NO:24
HA0037	35	GAACATGGCCATAACTCATAGACG SEQ ID NO:25	CCTTCGACCCAACATC SEQ ID NO:26
HA0654	35	ACGCACATGAGAGAGAAAGAG SEQ ID NO:27	ACCTTCGACCCAACATCAAG SEQ ID NO:28
HA1779	35	ATTTGTTCCTGGTTCGGTATCC SEQ ID NO:29	CATGTCTGATCTTCGGGACTTC SEQ ID NO:30

Marker	Map Position (cM)	Forward Primer Sequence	Reverse Primer Sequence
HA0031	36	CTCACGAAACTCTTCATGCTG SEQ ID NO:31	CTCTCACACTTACTGAAC SEQ ID NO:32
HA1665	36	FAM-CCTAAGGGGATGAATTCTCTTTC SEQ ID NO:33	AACTTCCAATGTTCTCCAACCAT SEQ ID NO:34
HA0908	36	TTGTCTTCATCTGCGTGTGA SEQ ID NO:35	TTGCTGTTGTTGATCGGTGT SEQ ID NO:36
HA0870	38	GTGCGTTGGCTCTTATGGAT SEQ ID NO:37	AGTGATGGCATTCCCAATTT SEQ ID NO:38
HA1614	38	HEX-GTGATCCGAGTTGTGGATGTTC SEQ ID NO:39	GTTAGATGGCAACCCAAGTGAT SEQ ID NO:40
HA1242A	38	GGTGATGATGGAGGAGCAACTG SEQ ID NO:41	CACTCAACCATTGTTCTCCCAC SEQ ID NO:42
HA1667	41	FAM-GAACTCCGGTTAGTCTTCCGAC SEQ ID NO:43	GCAATTAAGTCTGCGTTTCAGTTT SEQ ID NO:44
HA0907	41	CATGAACATCGCCAATTCAG SEQ ID NO:45	TGCAAGGAACCATCAGAATC SEQ ID NO:46
HA0829	42	TTGTCATGTATGGGCTTTGG SEQ ID NO:47	ATCCAACAGGTGTGCGGAAT SEQ ID NO:48
HA0890	44	CACTTCATCCTCTCCCTCCTT SEQ ID NO:49	GGCGTGTGTGTTGGGTTATT SEQ ID NO:50
HA1756	44	ACACGAGTCCCAACCTGAATG SEQ ID NO:51	ACCTGAAATGCAAATCTCTACAGG SEQ ID NO:52
HA1930	44	TAGGCAATAACTTTGGGCGAAT SEQ ID NO:53	CCTGAAATGCAAATCTCTACAGG SEQ ID NO:54
HA1790	53	TCCCCAAACTTGCGTGTAGGT SEQ ID NO:55	CATTACAAACCACAGCTCCTTCC SEQ ID NO:56
HA1040A	55	CCTGGAACTGAACCGAGAAC SEQ ID NO:57	GCCGTGAAACAGAGAGAGGA SEQ ID NO:58
HA0041	62	CTAGCAACCAACCTCATTG SEQ ID NO:59	GTCTCCTTCTCTTTCTCGGC SEQ ID NO:60
EF	85		

Each primer pair corresponds to one marker on the map. These primers were used to amplify the DNA from two parents (one is early flowering, the other is normal flowering) of the mapping population. Each of them amplified the DNA fragments polymorphic between the two parents. Then these primers were used to amplify the individual plants of the mapping population, from which the map was constructed.

Gene Utility.

1) The gene could be used to convert later maturing elite inbreds to earlier iso-lines for other geographies or cultural practices requiring earlier maturing hybrids. Thus, one beneficial consequence is an expanded genetic base and versatility created for breeders. Table 6 results show utility of this concept. The female and male inbreds H840A and CN2922R are very late maturing lines adapted to central to north Argentina for development of group 6-8 hybrids. H535A is a group 6 female used to make late hybrids. H1063R is medium maturing male for group 2-5 hybrid development. Testcrosses of their EM conversions are provided in Table 6. Especially noteworthy, results are shown by the EM 2922R testcrosses - 5 of 6 EM 2922R hybrids made group 2 hybrids. Results show very competitive results with the ON3403A testcrosses against the normal group 2, 3, 4, and 5 checks.

Table 15. Early mutant and conventional hybrid comparisons from 2006. 3 locations, 6 reps.

Name	Fem Flr	Mal Flr	Hyb Flr	Hyb Mat	Ap (1-9)	Ht (in.)	Hlth (1-9)	H2O (%)	Yld (lb/ac)	Oil (%)	OPA (lb/ac)	18:1 (%)
8N510 (CN1703A / H1063R)	65	59	65	5	6	68	6	12.2	2625	46.4	1218	67.6
ON3403A / EM BC4 CN2922R	64	55	58	2	6	65	6	10.5	2618	46.0	1205	53.1
8H350DM (H251A / OND163R)	61	60	60	3	5	70	5	11.5	2538	46.7	1187	90.8
8N251 (H251A / CN1229R)	61	55	59	2	6	67	6	10.8	2478	51.2	1268	73.1
ON3403A / EM BC1 CN2922R	64	56	60	2	6	65	6	10.6	2371	46.1	1092	56.2
EM BC5 H840A / CN2922R	65	71	66	6	6	85	8	13.1	2327	39.4	918	19.4
EM BC5 H840A / HO207746R	65	57	63	5	6	76	8	12.8	2304	43.1	992	62.3
EM BC3 H535A / HOU98510R	57	63	58	3	6	62	6	11.1	2302	44.3	1020	72.4
8N453DM (CN2343A / OND163R)	65	60	61	4	6	65	6	11.9	2292	52.4	1202	73.4
EM BC5 H840A / EM BC4 CN2922R	65	55	61	4	7	73	7	11.5	2250	45.0	1012	19.8
H251A / EM BC4 CN2922R	61	56	57	2	6	56	5	10.4	2220	45.7	1014	67.0
EM BC3 H535A / CN1229R	57	71	57	3	5	61	6	10.9	2191	45.7	1000	27.7
EM BC3 H535A / OND163R	57	60	57	2	4	58	5	10.2	2119	48.7	1032	75.3
EM BC2 H535A / EM BC2 CN2922R	57	56	58	2	6	59	5	10.5	2108	43.4	916	26.1
H251A / EM BC1 CN2922R	61	56	57	2	5	53	5	10.9	2092	47.4	992	46.9
EM BC3 H535A / MOC0666R	57	65	59	3	6	64	8	11.6	1985	44.7	887	64.9
SF270 (C8283A / 687R)	58	59	58	2	5	54	5	10.8	1974	44.2	873	38.5
EM BC2 H535A / H1063R	57	59	58	2	4	63	6	10.8	1970	45.0	887	64.5
H535A / EM H1063R	68	46	56	2	4	53	5	10.6	1780	50.1	892	63.9
CN1703A / EM H1063R	65	46	56	2	5	42	5	10.4	1574	43.3	682	63.9
EM BC5 H840A / EM H1063R	65	46	56	2	4	58	6	10.8	1416	46.2	654	64.4
H840A / EM H1063R	85	46	57	3	4	60	5	11.6	1334	46.1	616	65.1
avg			59		5.6	62	5.6	11.2	2130	46.2	961	56.8
CV									20.6			

2) The gene could be used to make ultra early flowering/maturing plants for genetic studies due to short lifecycle. The BC2F1 conversion of H324B (see bottom of Table 11) shows this potential (flowering in 38 days relative to 49 days of its group 1 recurrent parent H324B).

3) The genes pleiotropic effects – reduced biomass (reduced leaf canopy, height) but higher harvest index – makes hybrids favorable to high density populations to improve yields and compete against normal later maturing hybrids. Table 16 shows this concept. All EM H1063R hybrids planted at 36,000 plants per acre yielded higher than the same hybrids planted at 18,000!. The EM hybrids are significantly earlier flowering and have less seed harvest moisture than 8N251 and 8N270 group 2 check hybrids. These very early hybrids could be marketed for late planting dates or double

cropping after wheat. Additional studies will be conducted using narrower rows with the higher plant densities.

Table 16. Performance results of EM hybrids and checks planted at 18,000 and 36,000 plants per acre in 30 inch rows.

Name	fem flr	FLR	MST	TW	YLD	OIL %	OPA	OLE
8N251-HPOP		56	24.8	27.6	1779	52.3	930	65.2
8N251		56	27.2	27.9	1778	52.3	930	65.2
ON3403A x EM H1063R-HPOP	64	48	19.8	28.5	1885	47.7	898	90.2
8N270		55	27.9	28.2	1978	44.2	874	81.6
8N270- HPOP		55	23.8	27	1955	44.2	864	81.6
CN3351A x EM H1063R-HPOP	62	47	15.4	27.5	1795	48.0	862	64.5
CN2343A x EM H1063R-HPOP	62	47	16.1	28.3	1829	44.5	813	63.9
H251A x EM H1063R-HPOP	61	45	13.9	25.5	1863	43.4	809	86.8
CN2343A x EM H1063R	62	47	21.5	30.5	1639	44.5	729	63.9
ON3403A x EM H1063R	64	48	20.4	26.4	1526	47.7	727	90.2
CN3351A x EM H1063R	62	47	19.4	27.9	1499	48.0	720	64.5
H251A x EM H1063R	61	45	17.8	26.6	1512	43.4	657	86.8
AVERAGE		49.7	20.7	27.7	1753	46.7	818	75.4
CV					14.6			

4) The gene can become a powerful tool to accelerate introgression of other traits by keeping the early mutant gene in heterozygous form during the backcross process. In the example below, the EM gene has been introgressed into the donor parent with desired gene – indicated by the underlined genotype. The recurrent parent is indicated in bold.

Start: EE XX * **ee xx**

BC0 Ee Xx * **ee xx**

BC1 *ee xx* *Ee xx* Ee Xx * **ee xx**

BC2 - BCn *ee xx* *Ee xx* Ee Xx * **ee xx**

BCnF2 self to recover ee XX

Another scheme is indicated in Figure 8, where the desired gene is called "YFG." As illustrated by Figure 8, the Clearfield gene (for example) in the Clearfield donor is crossed to EM mutant parent, giving a heterozygous EM/CL F1. The F1 progeny (used as the donor for the CL trait) can be crossed to an elite recurrent parent. At each of 3 backcross stages, progeny of each cross is then crossed to the recurrent parent (with each backcross, selecting for EM/CL from EM, CL and EM /CL progeny) using molecular markers to recover the recurrent parent. By third backcross

using molecular markers, one can recover most of the genome of the recurrent parent which will contain the gene of interest (the Clearfield gene).

The same can be accomplished after 5 rounds of backcrossing using visual selection (without molecular markers). However, molecular markers and the subject early gene greatly speed the cycle. For example, each cycle can be reduced by 20 days, for example. Thus, three to four generations, for example, can be obtained per year by practice of the subject invention.

In summary, a cross can be made between the 'Donor' and 'Recurrent' parent. Then the F1 and subsequent generations are crossed (backcrossed) to the recurrent parent. The backcross generations converge on a single genotype. The genetic contribution of the 'Donor' parent will be halved each generation.

A satisfactory recurrent parent is usually from an established cultivar. A donor parent typically provides a desirable characteristic. There are a sufficient number of backcrosses to reconstitute the recurrent parent.

These backcrossing methods can provide the breeder a high degree of control. The traits to be improved can be described in advance. These methods are repeatable. Extensive field trials are not required. In addition, there is a reduced need for taking notes and record keeping.

5) Utility of the sunflower early flowering mutant gene offers exciting possibilities for known prior disclosure of transgenic development in other crops by broadening the adaptability of economically superior genetic combinations. There is no known prior disclosure of a similar dominant gene action occurring in other plant species. The gene can also be further mapped and sequenced. Gene optimizations can also be made for additional transformation. A TaqMan or an invader assay can also be developed to assist introgression.

EXAMPLE 9 – Additional Marker Development

Materials and methods

A strategy for marker development is summarized in this Example and is depicted in Figure 6. Markers were selected and developed for the lower telomere region of linkage group 5 (LG 5) and were screened for polymorphisms between parental lines MOC0666R and CNE418R of the MOC0666R x CNE418R mapping population, which was previously used to map the early

flowering (EF) mutant gene. Polymorphic markers were then mapped in the MOC0666R x CNE418R mapping population. For markers monomorphic between MOC0666R and CNE418R, primers were designed to amplify their genomic loci. Amplicons from both MOC0666R and CNE418R were cloned and sequenced to identify single nucleotide polymorphisms (SNPs), if any, between the two parental lines. TaqMan MGB Allelic Discrimination assays were developed to map identified SNPs. JoinMap 4.0 (Van Ooijen, 2004) was employed to map newly developed markers.

Results

SSR marker development

Three SSR markers were screened for polymorphisms between MOC0666R and CNE418R (Table 17). One SSR marker (HA1805) was polymorphic, and amplicons from MOC0666R and CNE418R were 240 bp and 235 bp, respectively. Correspondingly, the MOC0666R x CNE418R mapping population was genotyped with HA1805 using the following PCR primers and reaction conditions. PCR products were resolved on ABI 3730 Sequencer.

HA1805 Forward Primer: 5'-6FAM-GAAGTTGGGAGGGTTGTTCAAG-3' (SEQ ID NO:61)

HA1805 Reverse Primer: 5'- CCTCCTGTTGGAACACCAAAT-3' (SEQ ID NO:62)

PCR components:
- 4 ng gDNA
- 1X PCR buffer (Qiagen, Valencia, California)
- 0.25 μM Forward primer
- 0.25 μM Reverse primer
- 1 mM $MgCl_2$
- 0.1 mM of each dNTP
- 0.4%PVP
- 0.04 Units HotStar Taq DNA polymerase (Qiagen, Valencia, California)
- Total Volume: 4.8 μl

Thermocycler setup:
- Step 1: 94 °C for 12 minutes

Step 2: 94 °C for 30 seconds
Step 3: 55 °C for 30 seconds
Step 4: 72 °C for 30 seconds
Step 5: repeat steps 2, 3 and 4 for 35 cycles
Step 6: 72 °C for 30 minutes

SNP marker development

Five pairs of primers were used to amplify five genomic loci from both MOC0666R and CNE418R to develop SNP markers (Table 17).

Table 17: Markers tested.

F Name	Sequence	R Name	Sequence	Note
1) SSR				
HA1659F-NED	GGTCTTTTGTTTAGAGGCGT GAT (SEQ ID NO: 68)	HA1659R	CGTTTCCCCATTTACATCATCTT (SEQ ID NO:70)	
HA1805F-FAM	GAAGTTGGGAGGGTTGTTC AAG (SEQ ID NO:61)	HA1805R	CCTCCTGTTGGAACACCAAAT (SEQ ID NO:62)	polymorphi c
ZVG24ssr F-FAM	AAGCTTTGATCCGGGTTTCT (SEQ ID NO:69)	ZVG24ssr R	GCCTTTCTTCCCAGCA (SEQ ID NO:71)	
2) SNP				
ZVG23sn pF	CTGAATTCGAACACGAGCA A (SEQ ID NO:72)	ZVG23snp R	TCTCCAGCCTTCAGCGTTAT (SEQ ID NO:77)	monomorph ic
ZVG24sn pF	TGAGTCTTACGTGGCAAAC G (SEQ ID NO:73)	ZVG24snp R	TGTCGCACAGGAAGTTTGAG (SEQ ID NO:78)	monomorph ic
HT120F	TACAAAGAAAGAGGGCGA GA (SEQ ID NO:74)	HT120R	AACATAAGAAAACCATATTCAA ATCA (SEQ ID NO:79)	SNPs
HT137F	TCCGTCTGGACTCAAAACT C (SEQ ID NO:75)	HT137R	CCAGAAGCACTTCAAGAGGA (SEQ ID NO:80)	SNPs
HT151F	GTACGTCAACGATGCATTT G (SEQ ID NO:76)	HT151R	TATCATTCCTCCACCGAGAA (SEQ ID NO:81)	monomorph ic

Two primer pairs (ZVG23snpF/R and ZVG24snpF/R) were designed based on sequences from restriction fragment length polymorphism (RFLP) probes ZVG23 and ZVG24 (Kolkman et al. 2007). Primer sequences for HT120F/R, HT137F/R, and HT151F/R were from Lai et al. (2005). SNPs were found in the amplicons from HT120F/R and HT137F/R. A TaqMan MGB Allelic Discrimination assay was developed for one SNP locus in the HT120F/R amplicon (see below), and the MOC0666R x CNE418R mapping population was genotyped using this assay.

There were two SNP loci (underlined) in the HT120F/R amplicons from MOC0666R/CNE418R.

TACAAAGAAAGAGGGCGAGAATTGCGGATAAAAAGAAAAGAATTGCGAAGGCG
AAATCCGAGGCY(T/C)GCAGAGTATCAGAAACTTCTTGCTACGAGATTGAAGGA
ACAGAGAGAAAGGCGGAGCGAR(A/G)AGCTTAGCAAAGAAAAGGTCGAGACTTT
CTGCTGCTTCGAAACCTTCTATTGCAGCATAAGTTAACAAGTTTTCAGGGTAATT
TCACAATGATTTGAATATGGTTTTCTTATGTT (SEQ ID NO:63 (wild-type, where Y=T and R=A) and SEQ ID NO:82 (early mutant, where Y=C and R=G))

The TaqMan Assay was developed for the R-locus, and the SNPO marker was designated DAS HA SNP 2008. The following sequences were used as indicated:

5'-ACGAGATTGAAGGAACAGAGAGAAA-3' (Forward Primer (SEQ ID NO:64),

5'-GCAGCAGAAAGTCTCGACCTTT-3' (Reverse Primer (SEQ ID NO:65),

5'-6FAM-CGGAGCGAGAGCT-3' (Probe 1; SEQ ID NO:66), and

5'-VIC-AGCGAAAGCTTAGC-3' (Probe 2; SEQ ID NO:67).

Real-Time PCR components:
- 25 ng gDNA
- 1X Taqman Universal PCR Master Mix
- 22.5 μM Forward Primer
- 22.5 μM Reverse Primer
- 5 μM Probe 1
- 5 μM Probe 2
- Total Volume: 25 μl

Bio-Rad iCycler setup:
- Step 1: 95 °C for 15 minutes
- Step 2: 94 °C for 30 seconds
- Step 3: 60 °C for 1 minute
- Step 4: repeat steps 2 and 3 for 65 cycles
- Step 5: 4 °C forever

Mapping new markers

JoinMap 4.0 (Van Ooijen, 2006) was used to map HA1805 and DAS HA SNP 2008 (Figure 7).

Both HA1805 and DAS HA SNP 2008 were tightly linked to the EF mutant gene, 1.4 and 1.8 cM below the EF mutant gene, respectively. Both markers are good-quality, co-dominant markers that can be readily used to, for example, facilitate the selection for early flowering in breeding programs.

References for Example 9:

Kolkman, J. M., S. T. Berry, A. J. Leon, M. B. Slabaugh, S. Tang, W. Gao, D. K. Shintani, J. M. Burke, and S. J. Knapp. 2007. Single nucleotide polymorphisms and linkage disequilibrium in sunflower. Genetics **177**: 457-468.

Lai, Z., K. Livingstone, Y. Zou, S. A. Church, S. J. Knapp, J. Andrews, and L. H. Rieseberg. 2005. Theor Appl Genet **111**: 1532-1544.

Van Ooijen, J. W. 2006. JoinMap 4.0, Software for the calculation of genetic linkage maps in experimental populations. Kyazma B. V., Wageningen, Netherlands.

Claims

1. An early maturing sunflower plant comprising a mutated, dominant single gene that confers early flowering and/or early maturation phenotype on said sunflower plant, wherein a major locus for said gene can be mapped via DNA markers to one end of linkage group 5.

2. The early maturing sunflower plant of claim 1 comprising an early maturity gene as present in ATCC #PTA-8715.

3. A seed produced by the plant of claim 1, wherein said seed comprises said gene.

4. A progeny plant of the plant of claim 1, wherein said progeny plant comprises said gene.

5. A method of determining if a genomic test sample comprises a gene capable of conferring an early flowering and/or early maturity phenotype on a plant, said genomic test sample being obtained from a test plant or tissue, seed, or a part of said test plant, said test plant comprising a genome, and said method comprising assaying said test sample for presence of at least one single nucleotide polymorphism (SNP) in SEQ ID NO:63 or SEQ ID NO:82 in said genome, wherein presence of said at least one SNP indicates presence of said early flowering gene in said genome, and absence of said at least one SNP in said genome indicates a wild-type plant lacking said gene.

6. The method of claim 5, said method comprising obtaining genomic DNA from said test plant, amplifying a segment of said genomic DNA to form an amplicon, and determining if said amplicon comprises said at least one SNP.

7. The method of 6, said method comprising sequencing said amplicon.

8. The method of claim 6, wherein primers comprising SEQ ID NO:64 and SEQ ID NO:65 are used to amplify said genomic DNA to form said amplicon.

9. The method of claim 5 wherein said test plant comprises SEQ ID NO:66 if said plant comprises said early flowering gene, and said plant comprises SEQ ID NO:67 if said plant lacks said SNP and is wild-type.

10. The method of claim 5 wherein said method comprises determining if the genome of said test plant comprises Guanine at position 9 of SEQ ID NO:66, which is indicative of said early flowering gene.

11. The method of claim 5 wherein said method comprises determining if the genome of said test plant comprises Adenine at position 9 of SEQ ID NO:66, which is indicative of wild-type.

12. The method of claim 5 wherein said method comprises determining if the genome of said test plant comprises Guanine at residue 6 of SEQ ID NO:67, which is indicative of said early flowering gene.

13. The method of claim 5 wherein said method comprises determining if the genome of said test plant comprises Adenine at residue 6 of SEQ ID NO:67, which is indicative of wild-type.

14. The method of claim 5 wherein said method comprises determining if the genome of said test plant comprises SEQ ID NO:82, with Cytosine at residue 65 of SEQ ID NO:82, which is indicative that said test plant comprises said early flowering gene.

15. The method of claim 5 wherein said method comprises determining if the genome of said test plant comprises SEQ ID NO:63, with Thymine at residue 65 of SEQ ID NO:63, which is indicative of wild-type.

16. A method of producing an early maturing plant, said method comprising the steps of claim 5, selecting an early maturing plant comprising said gene, and growing and propagating said early maturing plant.

17. A method of selective breeding, said method comprising the steps of claim 5, further comprising selecting a plant that tests positive for SEQ ID NO:82, and further breeding said positive plant.

18. The method of claim 17, said method comprises crossing said positive plant with another plant.

19. The method of claim 17 wherein said plant is a sunflower.

20. The method of claim 18 wherein said plant is crossed with a sunflower plant of a line selected from the group consisting of an ornamental line and a confectionary line.

21. A method of propagating a plant, said plant having an early flowering phenotype, said method comprises the steps of claim 5, said method further comprising growing said positive plant, and self-crossing said positive plant.

22. A method of selecting for an early maturing plant, said method comprising the steps of claim 5, and selecting said early maturing plant for further breeding and/or propagation.

23. A plant comprising a genome comprising SEQ ID NO:66 in said genome.

24. The plant of claim 7, said plant further comprising SEQ ID NO:82 stably incorporated into said genome.

25. A plant comprising an early flowering gene, said plant comprising a genome, said genome comprising at least one single nucleotide polymorphism (SNP) in SEQ ID NO:82 in said genome, said genome comprising a Cytosine at residue 65 of SEQ ID NO:82.

26. The plant of claim 25 wherein said genome comprises two polymorphisms in SEQ ID NO:82.

27. The plant of claim 25 wherein said plant is a sunflower.

28. The plant of claim 25 wherein said plant exhibits an early flowering phenotype as compared to said wild-type plant.

29. The plant of claim 25 wherein said plant is a sunflower capable of flowering in 35 days.

30. A sunflower plant identified according to claim 5.

31. A part of a plant of claim 25, said part comprising said gene.

32. The plant part of claim 31, wherein said part is a seed or pollen.

33. A method for identifying presence of a marker locus that is associated with early flowering, said method comprising obtaining a polynucleotide sequence from the plant of claim 25, said sequence being unique to and discriminating of plants comprising said gene.

34. The method of claim 33, wherein said method comprising using a set of primers.

35. An isolated polynucleotide that hybridizes with a sequence (or the complement of said sequence) selected from the group consisting of SEQ ID NO:82, SEQ ID NO:66, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65, and SEQ ID NO:67, wherein hybridization is maintained under conditions of 55° C and 0.2X salt (SSPE or SSC).

36. The polynucleotide of claim 35, wherein said polynucleotide is a probe.

37. The polynucleotide of claim 35, wherein said polynucleotide is a primer.

38. An isolated polynucleotide comprising at least one single nucleotide polymorphism present in SEQ ID NO:82 as compared to SEQ ID NO:63.

39. A method of determining if a test plant comprises an early flowering gene, said test plant comprising a genome, and said method comprising assaying said plant for presence of SEQ ID NO:66 or SEQ ID NO:67 in said genome, wherein presence of SEQ ID NO:66 in said genome indicates presence of said early flowering gene in said genome, and presence of SEQ ID NO:67 in said genome indicates a wild-type plant.

40. A method of accelerating introgression of a gene of interest into a sunflower plant, said method comprising:

crossing a donor plant containing a gene of interest with a sunflower plant comprising an early flowering gene to obtain an F1 sunflower plant;

backcrossing the F1 plant to an elite sunflower parent plant having a genome;

and backcrossing one or more subsequent generations of progeny of the backcrosses to recover at least one new elite parent sunflower plant comprising the genome of the elite sunflower parent and the gene of interest.

41. The method of claim 40, wherein said new elite parent comprises both the early flowering trait and the gene of interest.

42. The method of claim 40, wherein said method further comprises segregating out said early flowering gene from said gene of interest in the new elite parent.

43. The method of claim 40, wherein said method comprises using at least one molecular marker for said early flowering gene.

44. A plant produced by the method of 40.

45. The plant of claim 44, wherein said plant is an ornamental or confectionary sunflower.

46. An early maturing sunflower plant comprising a mutated, dominant single gene that causes early flowering and/or maturation, as compared to plants comprising the wild-type version of the gene, wherein a major locus for said gene can be mapped via microsatellite or SSR markers to one end of linkage group 5.

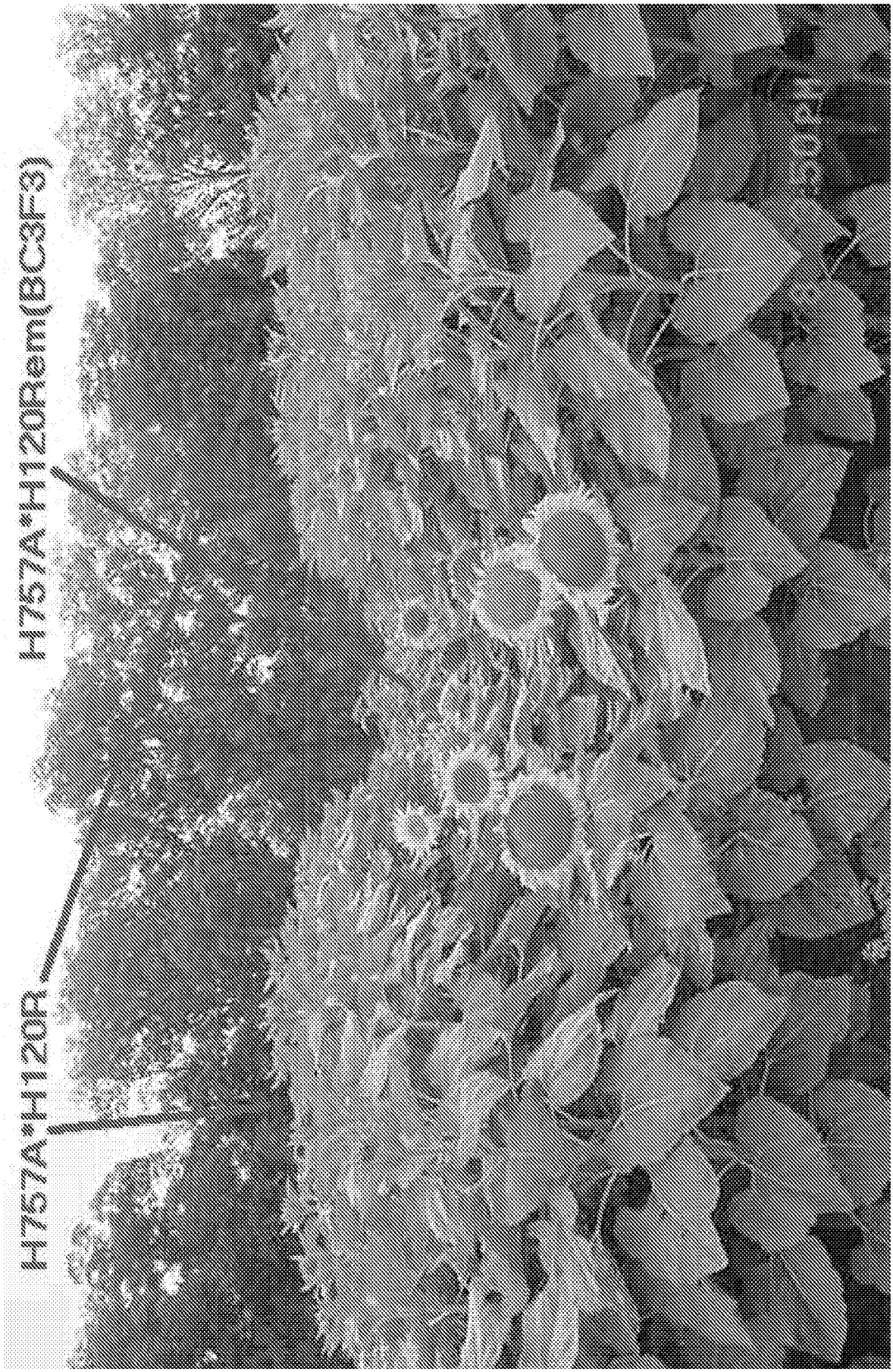


FIGURE 1

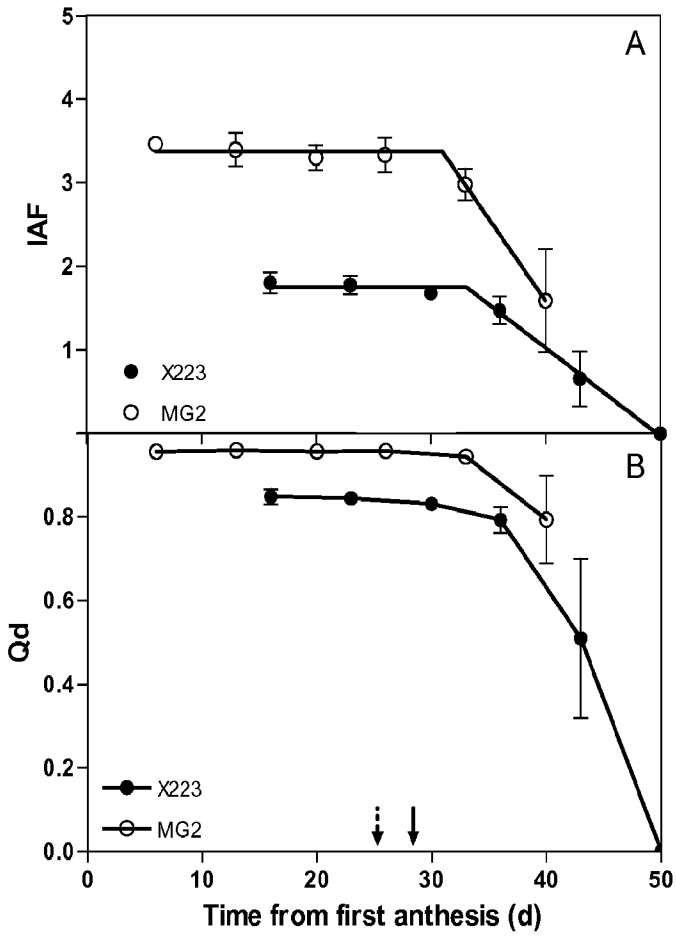

A
B
IAF
Qd
X223
MG2
X223
MG2
Time from first anthesis (d)
0
10
20
30
40
50
0.0
0.2
0.4
0.6
0.8
1
2
3
4
5

FIGURE 2

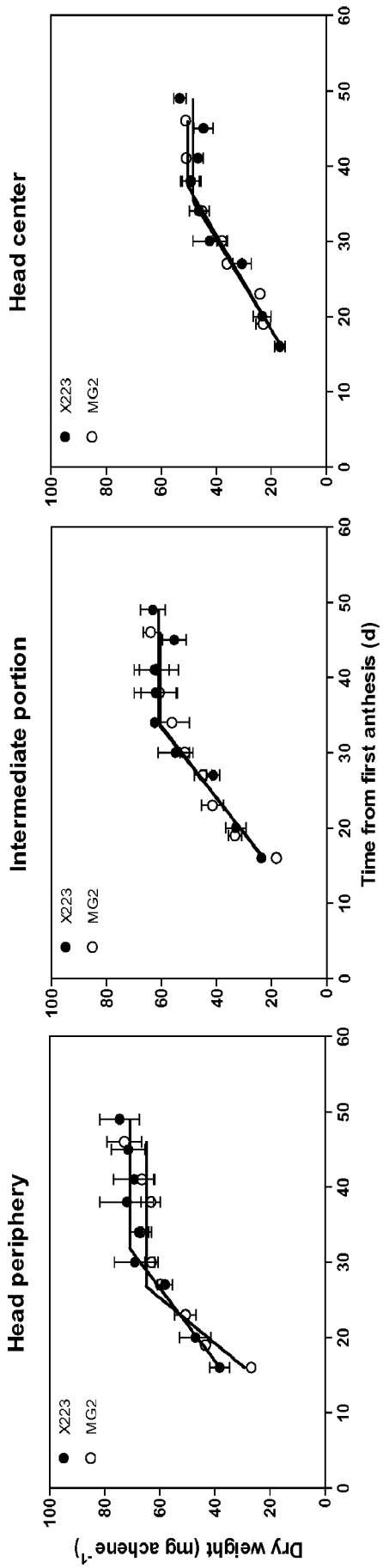

Head periphery
Intermediate portion
Head center
X223
MG2
Dry weight (mg achene-1)
Time from first anthesis (d)
0
20
40
60
80
100
0
10
20
30
40
50
60

FIGURE 3

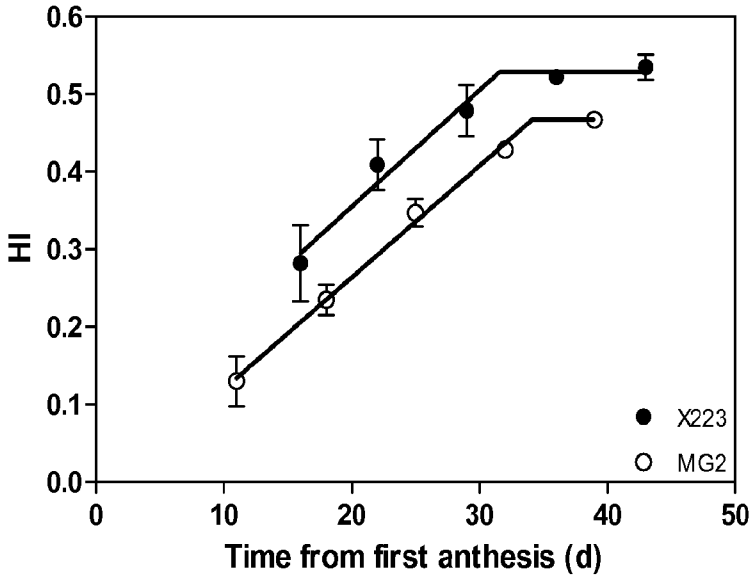

0.6
0.5
0.4
0.3
0.2
0.1
0.0
HI
0
10
20
30
40
50
Time from first anthesis (d)
X223
MG2

FIGURE 4

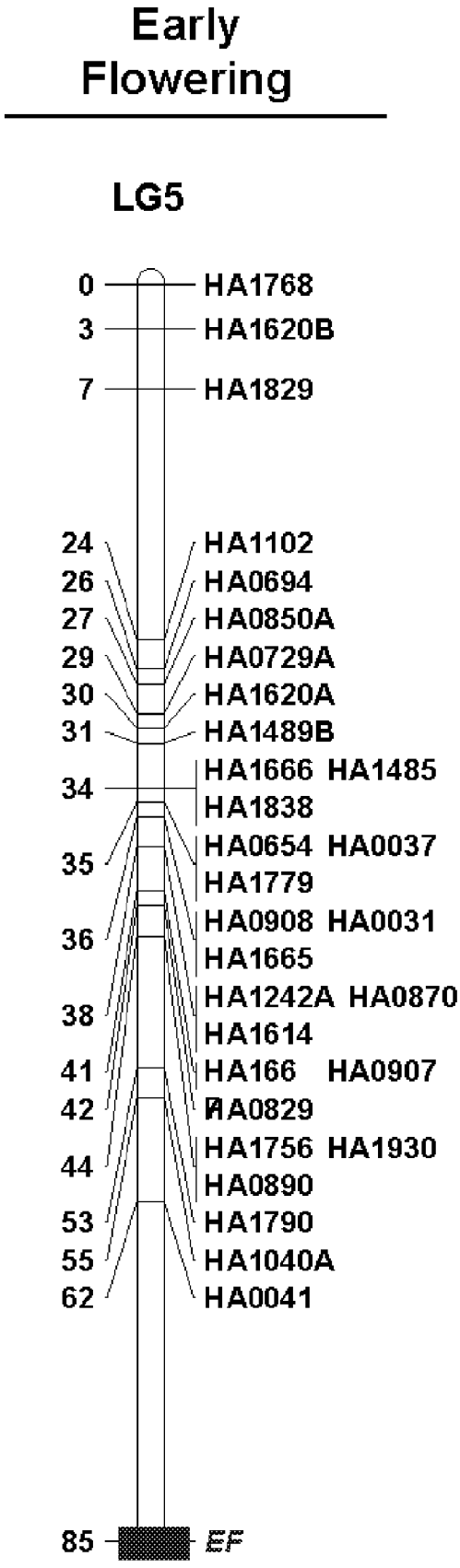

Early
Flowering
LG5
0
HA1768
3
HA1620B
7
HA1829
24
HA1102
26
HA0694
27
HA0850A
29
HA0729A
30
HA1620A
31
HA1489B
34
HA1666 HA1485
HA1838
35
HA0654 HA0037
HA1779
36
HA0908 HA0031
HA1665
38
HA1242A HA0870
HA1614
41
HA166 HA0907
42
HA0829
44
HA1756 HA1930
HA0890
53
HA1790
55
HA1040A
62
HA0041
85
EF

FIGURE 5

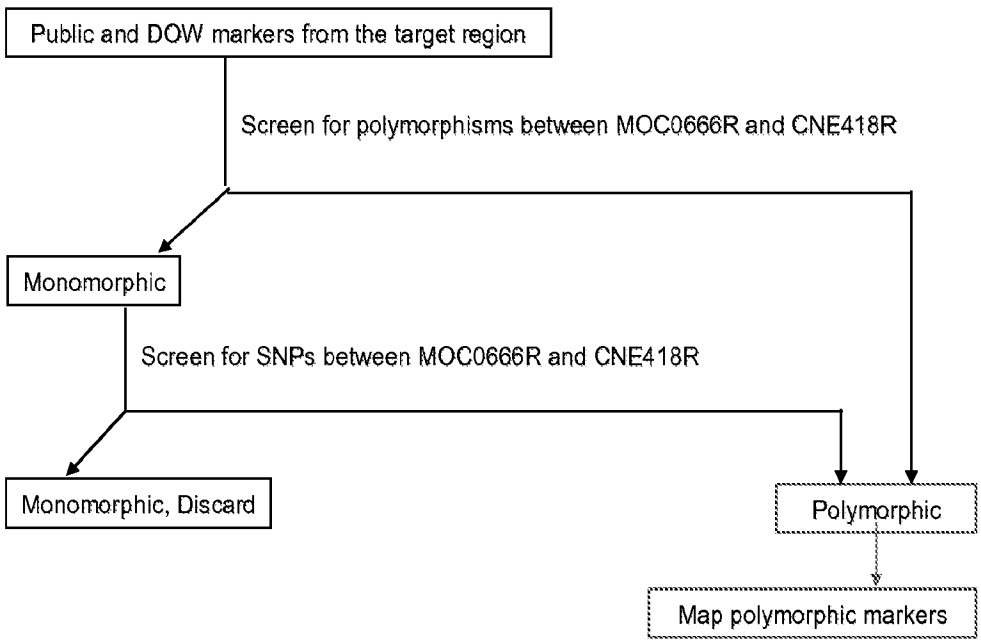


Figure 6: Strategy for marker development.

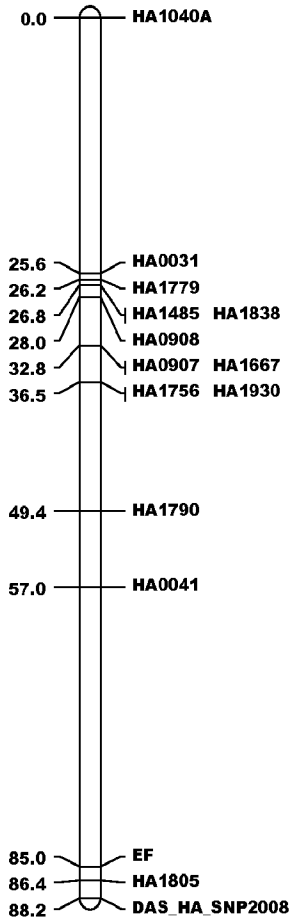
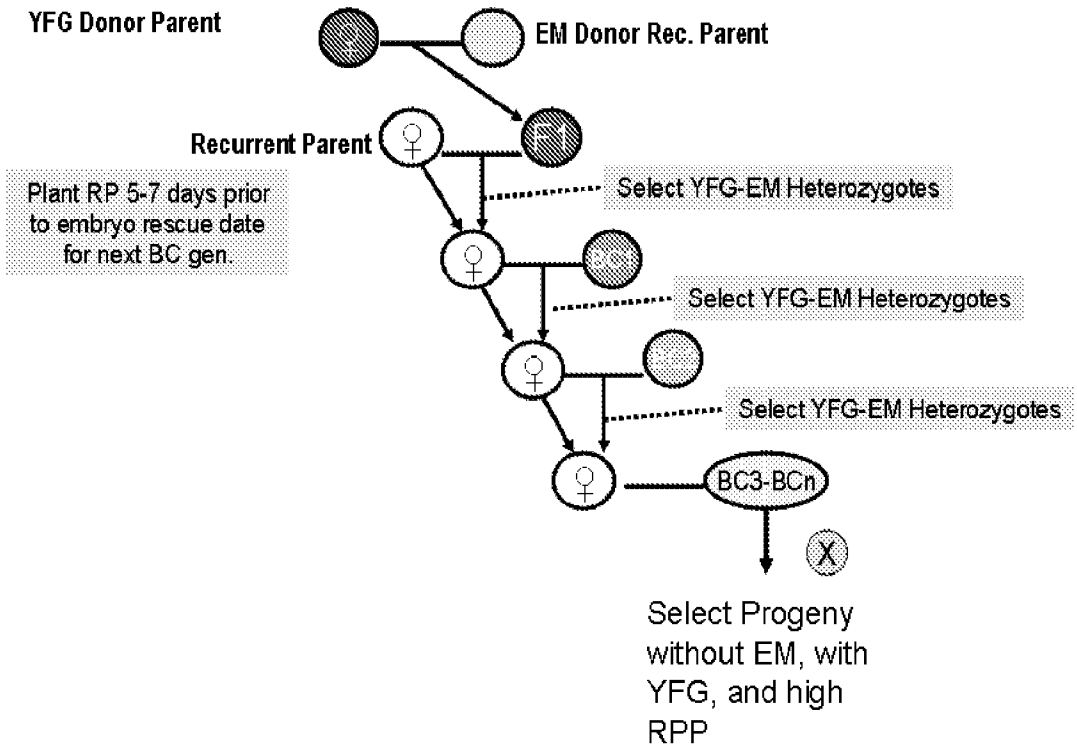


Figure 7: Markers HA1805 and DAS_HA_SNP2008 tightly linked to the early flowering (EF) mutant gene.

Figure 8


YFG Donor Parent
EM Donor Rec. Parent
Recurrent Parent
Plant RP 5-7 days prior to embryo rescue date for next BC gen.
Select YFG-EM Heterozygotes
Select YFG-EM Heterozygotes
Select YFG-EM Heterozygotes
BC3-BCn
Select Progeny without EM, with YFG, and high RPP