# SELECTION ASSISTED MARKERS TO INTRODUCE PERVENETS MUTATION IN A WIDE SUNFLOWER GENETIC BASIS: A NEO DOMESTICATION PROGRAM

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**Short running title: Selection assisted markers for the Pervenets mutation**

**Abstract:** in sunflower the Pervenets mutation is used to release commercial varieties for a high oleic oil for nutrition and industrial uses. In the future, higher yielding and rustic varieties with a high oleic oil will be required namely for the production of bio fuel. Rusticity and broadening of the genetic basis are brought by seventy-six wild sunflower ecotypes chosen based on their diversity and some evaluated agronomic traits. To perform selection assisted markers of the high oleic trait the Pervenets mutation was characterised and sequenced. Markers were defined in the mutation enabling its efficient dragging. Associated with markers for other characterised domesticated traits (branching, oil content, mildew resistance) all progenies (wild x crop) enables to us to handle three wide basis sunflower populations to improve sunflower crop. One is oleic and the two others are classic. The markers for the Pervenets mutation combined with the phenotype information of the seed allow to identify the genotypes that brought the Pervenets suppressor and other minor modifiers of the oleic content.

**Résumé:** la mutation Pervenets chez le tournesol permet la production commerciale de variétés donnant une huile à haute teneur en acide oléique pour l'alimentation et les utilisations industrielles. Pour le futur, des variétés plus productives rustiques et à haute teneur oléique seront nécessaires notamment pour la production de bio carburants. La rusticité et l'élargissement de la base génétique proviennent de soixante seize écotypes du tournesol sauvage choisis pour leur diversité et après évaluation de quelques caractères agronomiques. Pour réaliser la sélection assistée par marqueurs du caractère à haute teneur oléique de l'huile la mutation Pervenets a été caractérisée et séquencée. Des marqueurs définis dans la mutation permettent de la sélectionner efficacement. Associée à d'autres caractères domestiqués marqués (ramification, teneur en huile, résistance au mildiou) l'ensemble des descendances (sauvage x cultivé) permet de disposer de trois populations à base large pour l'amélioration du tournesol. L'une est oléique et les autres sont classiques. L'une est oléique et les autres sont classiques. Les marqueurs de la mutation oléique combinés à l'information du phénotype

de la graine, permettent d'identifier les génotypes qui portent le suppresseur de Pervenets et d'autres allèles modificateurs mineurs du phénotype LO.

Spanish

III Key words : Genetic basis, High oleic, Neo-domestication, Pervenets, Selection assisted markers

**Introduction**

Vegetable oil nutritional qualities depend mainly on their fatty acid composition. Saturated fatty acids induce an increase of the cholesterol atherogenic fraction and consequently, the risk for cardiovascular diseases (Hegsted et al. 1965, Keys et al. 1965). Polyunsaturated linoleic (18:2) and linoleic (18:3) fatty acids reduce both cholesterol atherogenic and anti-atherogenic fraction levels.

**Interest for high oleic acid**

On the other hand, the monounsaturated oleic acid (18:1) reduces only the cholesterol atherogene fraction level and consequently, preserves the cholesterol anti-atherogene fraction which is beneficial for cardiovascular disease prevention. Thus, diets containing vegetable oil with high oleic acid content have been reported to be the most effective to prevent cardiovascular diseases (Delplanque et al. 1997, 2000; Broun et al. 1999). The high oleic oil may also use for bio lubricant and bio fuel and the free oleic acid is also at the basis of many industrial products and is highly valorised.

**Sunflower oil composition**

Sunflower oil is naturally rich in linoleic acid (55-70 %) and consequently poor in oleic acid (20-25 %). Varieties are qualified as Low Oleic (LO). Until the 1970s, mutagenesis programs were conducted in order to produce varieties with an increased oleic acid content compared to the LO varieties. The Pervenets sunflower population was obtained by chemical mutagenesis (Soldatov 1976).

**The oleic source**

It displays oleic acid content in seed oil higher than 65 % (Soldatov 1976). New varieties with oleic acid content higher than 80 % (HO varieties for High Oleic) were then obtained from the Pervenets population through breeding programs. This fatty acid composition modification is located specifically in embryo tissues (Garcés et al. 1989). Due to the increased health interest and industrial uses of oleic acid and the similar agronomic performance of the HO compared to the LO varieties, HO

varieties are now widely used in the world covering about 1.2 million ha (Evrard 2003, Mestries 2003).

**The difficulties to manage Pervenets mutation**

As previously reported breeders have faced difficulties to recover high oleic phenotype after crosses and backcrosses of the Pervenets sources with classical sunflower to convert the best LO lines into HO lines (Lacombe and Bervillé 2000). The behaviour of the mutation depends on the background of the classical sunflower lines crossed onto the Pervenets source. The HO trait may behave as a dominant, recessive or semi-dominant trait. In the subsequent generations, depending on the progenies, the HO trait may disappear, segregate as a Mendelian factor, or submitted to strong distortion in the segregation pattern either as an excess of LO or an excess of HO individuals

**Requirements to unravel the inheritance and the structure of the Pervenets mutation**

In the high oleic varieties, the oleate-desaturase mRNA accumulation is reduced compared to the LO genotypes leading to a decrease of oleate-desaturase activity in the seeds during lipid reserve elaboration steps (Garcés and Mancha 1989, 1991, Kabbaj et al. 1996, Hongtrakul et al. 1998 a, b). Using candidate gene approach in diversity analysis, linkage disequilibrium was reported between the Pervenets mutation and a HO specific oleate desaturase allele. This allele was not found in any of the LO genotypes tested (Lacombe and Bervillé 2001). Genetic studies performed on F2 and Recombinant Inbred Line (RIL) populations revealed that this linkage disequilibrium is due to a closely genetic linkage between the Pervenets mutation and the HO specific oleate desaturase allele (Lacombe et al. 2001, Lacombe and Bervillé 2001). However, these approaches could not determine if the HO specific oleate desaturase allele carries or is genetically linked to the Pervenets mutation. Consequently, the nature of the mutation is still unknown.

**Combination of Pervenets and a wide genetic basis**

For the future, we have forecast that the areas for HO sunflower will increase for both nutrition and industrial uses. However, each use has its specific constraints and for industrial uses the fist requirement is a low cost of the harvested seeds. Because the genetic basis of the classical sunflower is narrow we decided to enlarge the basis using a wide range of wild Helianthus annuus ecotypes that spread in USA.

**Why sunflower is important for South Europe?**

Sunflower is naturally one of the most drought tolerant crop. Wild *H. annuu*s in the state are still much more drought tolerant and those introduced in Europe are witnesses of the expected progresses.

Because, sunflower also could break mono-culture of cereals, especially with durum wheat, it is required in the blocking plan. Sunflower crop has been proven to increase the organic matter in the soil, it helps the bio-diversity of micro-organisms and finally the crop is ideal for a sustainable cropping system. How could be possible? Each research centre have some good inbreed who have promising characters like drought tolerance and disease resistance, oil content, combining ability, but we though that it is important to enlarge the genetic basis with wild sunflowers that displays much more diversity for disease resistance and stress resistance that the sunflower crop. Yes, if we could be work on the principals factors limiting the yield as in particular downy mildew, *Sclerotinia sclerotiorum*, drought and the parasitic plant *Orobanche cumana* if in the some time we could increase the oil content in the seed and oil quality (Vannozzi 2005).(Je ne comprends pas la phrase !!!)

**Aims of the work**

We therefore pursued two aims: on the one hand we prepare the molecular tools to manage the Pervenets mutation by marker assisted selection and on the other hand we constructed several sunflower gene pools based on a wide genetic basis to enhance the rusticity of the future crop. It is obviously a long term program. We will discuss the first milestones.

**Materials and methods**

**Plant materials**

*Sunflower lines*

As sources for domesticated alleles we used several sunflower lines (H1=89HR2, H2=90R19 and H3= RT1B11) as pollen donors and female lines with the PEF CMS source (*PEF- HA89 PEF-D34, PEF-83HR4).*As Pervenets sources we used RHA345 and lines derived at INRA or from Monsanto (BD for classical and BE for HO)

*Wild sunflower*

The Wild *H. annuus* accessions were chosen based on their geographical origin and phenotypic evaluation, with contrasted behaviours for growth, oil content, and other ecological adaptation traits. They carry at a high frequency *Rf* genes, namely for Pet1, as well as resistance factors to downy mildew: 60 accessions on 80 displayed resistance to race 710 in a previous experiment.

Crosses were hand made and flowers were wrapped under paper bags. Genetic analyses were performed in a greenhouse during the next winter after seed harvest. Oil content was determined with a RMN and oil composition was determined using with a Gas Chromatograph.

## Molecular methods

Molecular methods were described in Lacombe and Bervillé (2001). Sequencing was performed by Qiagen (Courtaboeuf, France).

### Strategy to sequence the Pervenets mutation

A series of seven primer pairs was designed on the oleate desaturase cDNA to amplify about 240 bp long fragments that slightly overlapped. Those primer pairs revealed six fragments with the expected size according to the cDNA sequence and one of about 1700 bp suggesting the presence of an intron (Lacombe et al. 2002). The intron starts 83 bp downstream of the coding sequence. The fragment was cloned and sequenced and revealed the characteristics of the intron sequence ends. It is 1588 bp long and it carries an *Hind*III restriction site. This explains the faint *Hind*III fragment appearing on RFLP since it carries only 83 bp of the oleate desaturase cDNA. However, since the 8 kb *Hind*III fragment carrying most of the cDNA oleate desaturase coding sequence lengthens to 16 kb when the Pervenets mutation is present, we deduced that it also carries an insertion with oleate desaturase homologous sequence. Assuming that the insertion could be direct or in opposite sense we combined one primer F8 with one primer from the seven primers pairs, which could match on the direct or the opposite sense of the repeated oleate desaturase sequence. Thus we applied several pair-wise combinations such as F1-R8 supposing a direct repeat and F1-Fx supposing the repeat in the opposite direction.

## Results

### The oleate desaturase region in sunflower

A12kb fragment containing an oleate desaturase gene was sequenced (Figure 1). It carries all the specificity for a functionnal gene and the encoded protein is targeted in the microsomes according to the peptide signal sequence. The intron was confirmed. Moreover, in the intron a 16 TTA motives was identified. Two primer pairs were designed to verify whether it is polymorphic in sunflower. Four alleles of 14, 15, 16 and 17 motives were detected among elite lines on a fragment of about 240 bp. It was coded HassrOD1 (Bervillé et al. 2004, Patent).

H, E *Hind*III and *Eco*RI

The Oleate desaturase gene:

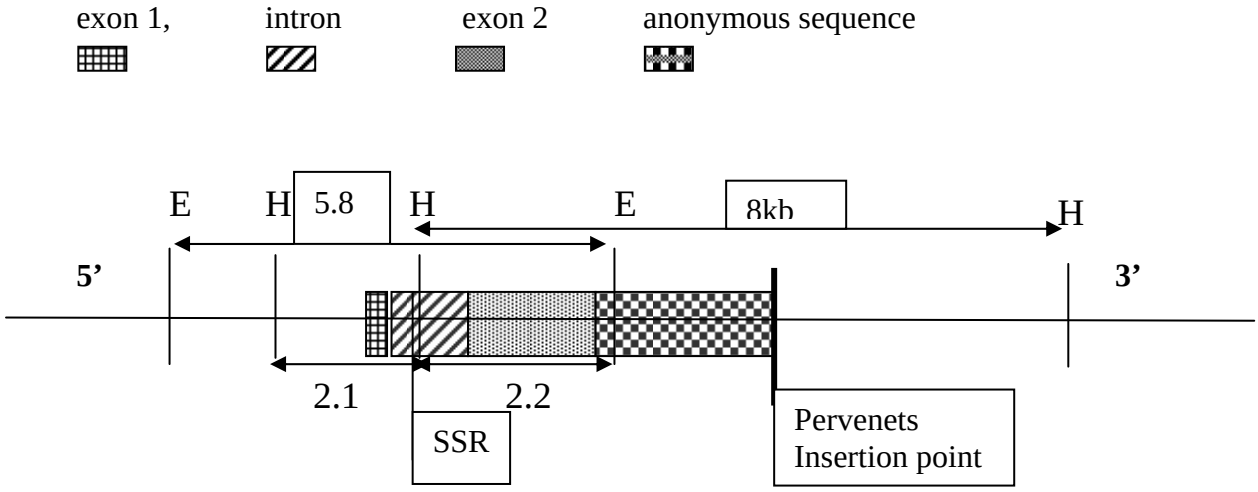


Figure 1: Organisation of a microsomal oleate desaturase region in sunflower

From genotypes carrying the Pervenets mutation, we obtained a 2.9 kb fragment with the primer pair F1-R8 suggesting that the oleate desaturase repeat is in the direct direction. We obtained no amplified product when the mutation is absent in LO genotypes.

Sequencing

The 2.9 kb amplified fragment was cloned and sequenced (Figure 1). It displays as expected oleate desaturase sequence. A portion of the oleate desaturase sequence was duplicated and inserted downstream of the end at about 1,200 bp. The insertion starts by a part of intron sequence until almost the end of the oleate desaturase cDNA (Figure 3).

H, E *Hind*III and *Eco*RI

The dashed line is the PCR 4.6 kb fragment amplified with a long PCR technique.
Pervenets 8 kb insertion ◄··►
The Oleate desaturase gene:

exon 1, intron exon 2 anonymous sequence

The represents the length and the position of the PCR 952 bp pervenets specific fragment

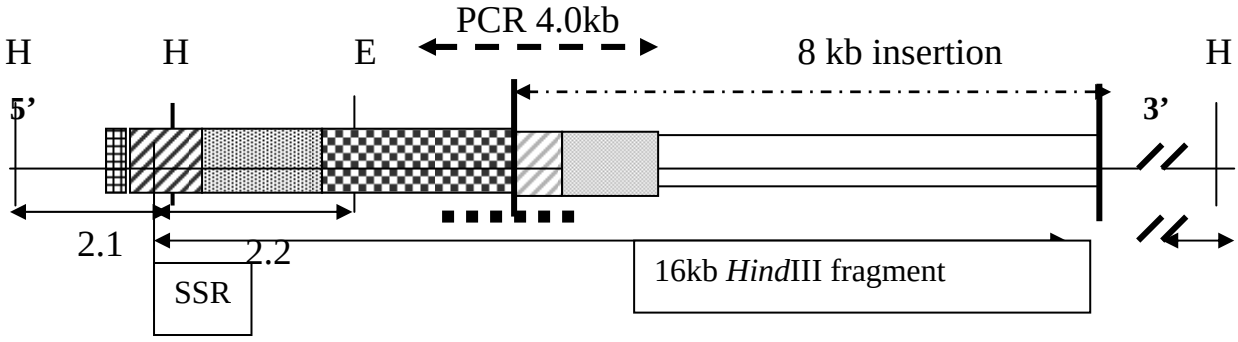


Figure 2: Organisation of the Pervenets mutation and some features for regulation of the expression according to Lacombe and Bervillé (2001) and Bervillé et al. (2004).

We defined primers to amplify a fragment of about 1 kb across the insertion point to release a PCR test for the presence of the Pervenets insertion. N1 is a fragment upstream of the insertion point, N2 is the Pervenets insertion fragment and N3 is downstream of the insertion point. N1 and N3 are contiguous when the insertion is absent. We chose the primer pair N1-4F N2-1R that leads to a 952bp fragment (Bervillé et al. Patent). At this level we lack of a positive control for the LO region since the sequence downstream of the insertion point is not known yet. All lines carrying the Pervenets mutation revealed the 952 bp fragment with the N1-4F N2-1R primer pair, whereas it was absent in all lines that did not carry the mutation.

Genetic studies on the Pervenets mutation

In a set of 174 recombinant inbred lines (RI Lines) from the cross between 83HR4 and RHA345 (Lacombe et al. 2002) at the F6 generation we determined the alleles for the oleate desaturase RFLP profiles with *Eco*RI and *Hind*III, for the HassrOD locus and we compared the polymorphisms to the phenotype of each 174 seeds. The oleic acid content of the 174 RI lines is given and are indicated the SSR and the 952 bp fragment segregation. Half of the RI lines carrying the insertion did not display HO content.

Construction of the gene pools

The following scheme was pursued to construct the HAS1 pool (Figure 3). The plants were maintained in isolation plots to enhance mixing (panmixy) and at each generation seeds were harvested and stored for each mother plant to maintain maternal lineages.

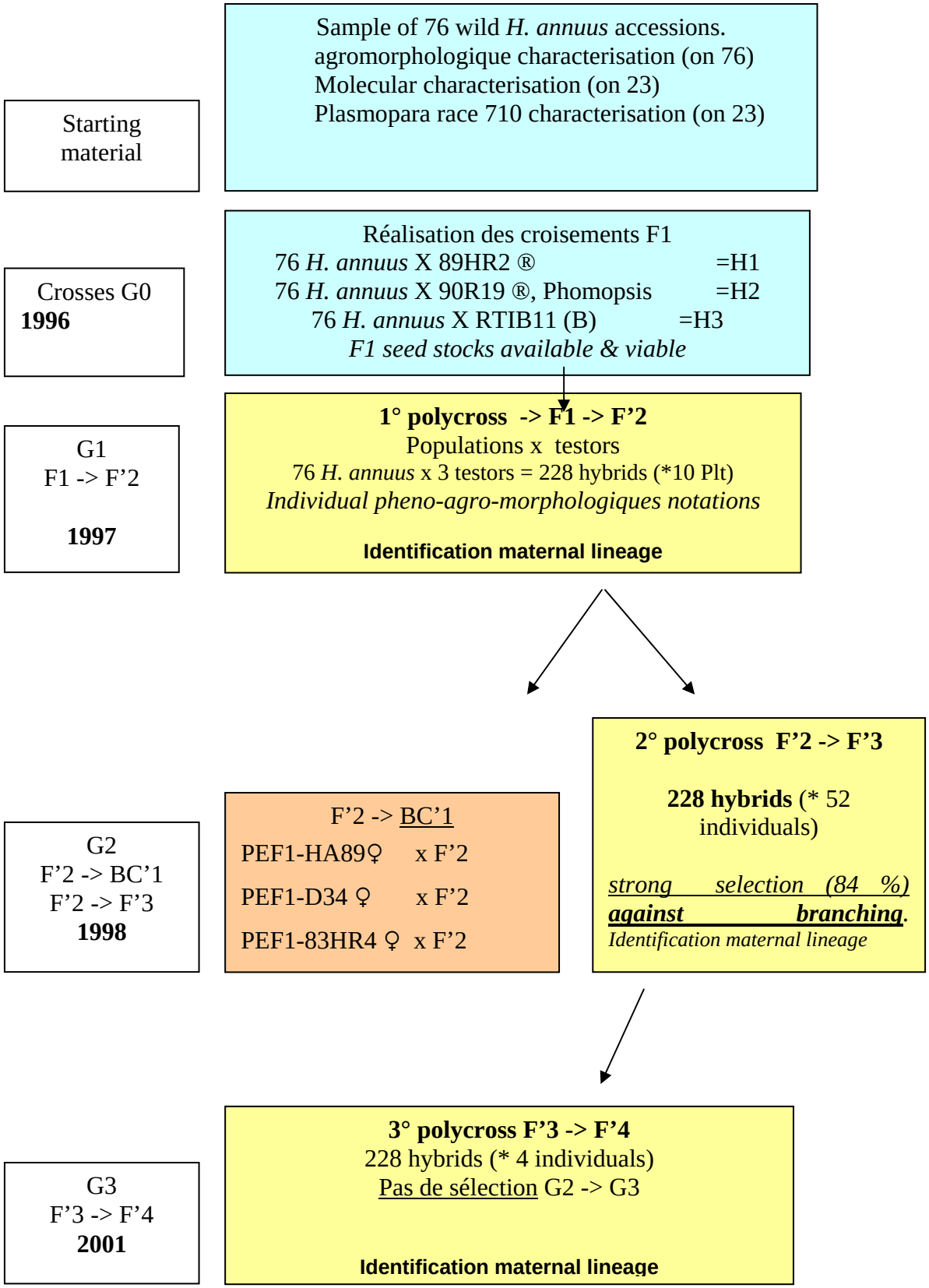

Figure 3: Steps to construct the HAS1 Pool on a wide basis population using 76 wild sunflower accessions. Three panmictic mixing cycles were performed and a strong selection pressure for domestication traits was applied in G2.

After the G3 we obtained 2,100 progenies enabling us to apply a strong, a moderate or a weak selection pressure for the domesticated traits (monohead, high oil content, seed size) to recover in the following generations, as fast as possible, domesticated sunflower or to maintain mixing of wild and domesticated alleles for the next generation (Table 1).

| | Minimum | Mean | Maximum |
|---|---|---|---|
| Branching % | 0 | 52 | 100 |
| Plant height cm | 108 | 151 | 192 |
| Thousand seed weight | 29.2 | 39.2 | 57.5 |
| Oil content % | 16.7 | 27.9 | 34.4 |

Table 1: Characteristics and range of variation of the germplasm resources based on 2,280 progenies.

These wide-based sunflower gene pools are in experiment to introduce the Pervenets mutation and to produce seeds for next year to determine their agronomic performances under different experiments with stress conditions (rain-fed, watered) and with watering. Disease pressure will be experimented at different locations (Toulouse, Montpellier) for downy mildew and other fungal diseases. Any domesticated trait lacking in our progenies can be introduced by cross in a wide basis gene pool but only a part of the gene pool will contain the trait. Several strategies can be achieved to introduce any trait in the gene pool.

**Discussion**

Pervenets mutation organisation

The Pervenets mutation organisation corresponds to a rearrangement of several sequence parts in a fragment of 8 kb. It includes a part of a oleate desaturase genomic fragment, and inserted sequence from another locus or several other loci.

Silencing model

The duplication of a part of the oleate desaturase gene probably causes silencing of the normal gene that is still present besides the Pervenets mutation. The exact type of silencing mechanism has

not been unravelled yet. Silencing mechanism is dominant, it acts in trans in F1 hybrid plant heterozygous for the Pervenets mutation. The absence of oleate desaturase transcript explains the absence of the oleate desaturase activity and consequently the high oleic traits. This mutation mimics a GMO construction although it was natural. As in GMO's, suppressors may affect the expression of the silencing that may cause to save oleate desaturase transcript and thus to restore the LO trait. The suppressor locus in the RI lines populations was not mapped efficiently since only the lines carrying Pervenets can be used to map it.

Genetic of the mutation

In a F2 population BD x BE the oleic acid content and the RFLPs specific to the Pervenets mutation (8 kb *Eco*RI and 16kb *Hind*III fragments) strictly co-segregated and any suppressor was detected (Lacombe and Bervillé 2001). Silencing suppressors are spread in sunflower and their presence can be detected only in the presence of a DNA structure causing silencing. This explains that only some lines crossed on a Pervenets knock out the effect of the mutation and the progeny display LO phenotype. Conversely, any line which crossed onto Pervenets will lead to a LO progeny should carry a suppressor, but suppressor may be different and should be tested to determine its location. We cannot exclude that some lines could carry several suppressors leading in a cross with Pervenets to a complex segregating pattern of LO / HO in the progeny.

Markers for dragging mutation

Markers such as the SSR in the intron of the gene and the Pervenets-specific 952 bp fragment are sufficient to warrant that the mutation is dragged in a progeny. For the next generation seeds are randomly chosen and half a cotyledon are checked for the oleic acid content whereas all the plantlets are tested with the Pervenets markers to be sure that the Pervenets mutation will be homozygous (Conte et al 1989). In the RI lines population the presence of the Pervenets mutation is not sufficient to determine the high oleic level. However, none RI line without the Pervenets mutation display the HO trait. Thus the mutation is required. The markers are sufficient to drag the mutation but not to warrant the HO trait because of the presence of suppressors or other modifiers.

Sunflower wild wide genetic basis

The constructed gene pools required still selection and mixing to display sunflower plants as the present crop. Several years to experiment them in various locations and years are also needed. However, we can plan that they exhibit a much higher diversity than the sunflower crop. Moreover, introgression of any new domesticated traits required for breeding is still possible whatever the level of the generation.

**Conclusions**

We provide through this programme an opportunity to enlarge the genetic basis of the classic sunflower domesticated from a single ecotype (Burke et al 2002) with the diversity of seventy-six wild sunflower benefiting of the domesticated trait already improved in the crop (Figure 4).

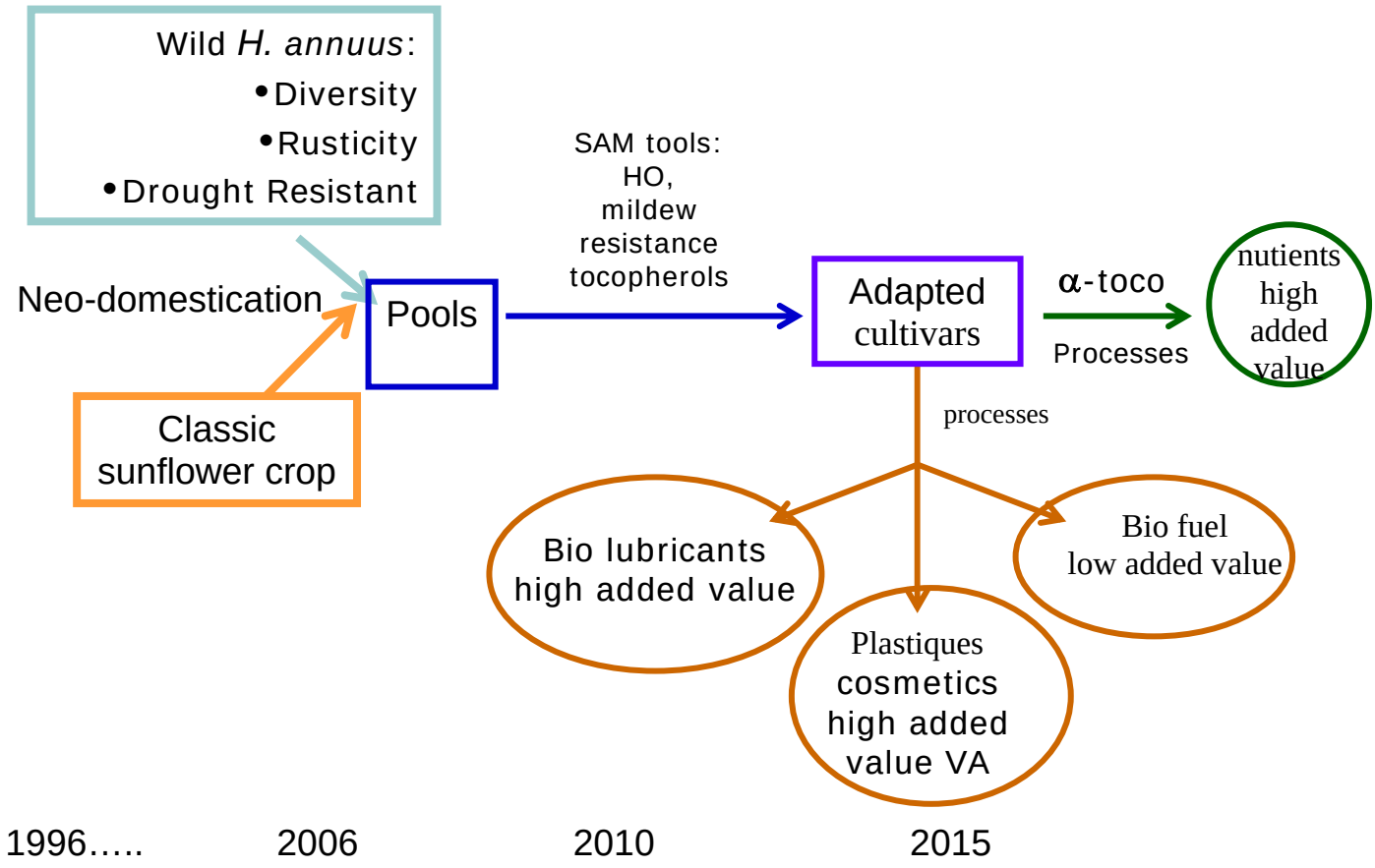


Figure 4: global scheme for the introduction of the Pervenets mutation in wide sunflower genetic basis.

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