Functional genomic tools for *Arabidopsis* available at the MPIZ

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Summary

This article summarizes the activities at the Max Planck Institute for Plant Breeding Research (Max-Planck-Institut für Züchtungsforschung, MPIZ) in the area of "Arabidopsis genomics".

We describe the status of three *Arabidopsis thaliana* genomic projects at the MPIZ: 1) The Gene Knock-Out Facility ZIGIA (Zentrum zur Identifikation von Genfunktionen durch Insertionsmutagenese bei *A. thaliana*, Center for Functional Genomics in *A. thaliana*) using lines tagged with the maize transposon En/Spm, 2) the GABI-Kat project that provides sequence indexed T-DNA tagged lines and 3) the GABI-MASC project that creates mapping tools based on single nucleotide polymorphisms (SNP) for efficient assessment of natural diversity in *A. thaliana*. The materials and tools developed by these projects are publicly available and used worldwide by scientist to explore the frontiers of plant sciences.

Key words:
functional genomics, plants, Arabidopsis, transposon, T-DNA, SNP.

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1. Introduction

Techniques of crop plant breeding have been very important in ensuring the steady improvement in efficiency of agricultural systems. Knowledge of the chromosomal locations of relevant genes, and their alleles, allows directed approaches to the assembly of new combinations of traits by recombination. Plant breeding is a time-consuming process that can now be accele-

rated by the use of molecular methods. Moreover, highly precise and innovative improvements of plants are possible if detailed knowledge about the gene function is available.

For many years, the weed *Arabidopsis thaliana* has been the model organism of choice for studying molecular genetics in higher plants. International coordination of efforts culminated in the availability of the first genome of a flowering plant sequenced in the year 2000 (2). An evaluation by Meinke et al. (18) listed 620 published descriptions of cloned genes with a loss-of-function phenotype. Currently, an ambitious plan is underway to obtain information about the function of all 26,500 identified genes until 2010 (25). Given the scientific interest in the investigation of the biological roles of *A. thaliana* genes, and their agricultural potential, the MPIZ early initiated several projects devoted to functional genomics of this model plant.

Unfortunately, so far no efficient methods for targeted gene disruption are available in flowering plants, and even though methods for silencing genes post transcriptionally have been improved (29), RNAi frequently results in off-target gene regulation as revealed by expression profiling with microarrays (10). Therefore, random insertional mutagenesis in which the foreign DNA does not only disrupt a gene, but also provides a tag for the characterization of the mutation is the method of choice for large scale reverse genetics. In *A. thaliana* two types of inserts have been used to establish large mutant populations: transposons (3,17,19) and T-DNAs (7,14). In both cases, co-segregation of phenotypes and insertions forms the basis of gene function predictions. The MPIZ offers populations from knock-out mutagenesis using the En/Spm transposon of *Zea mays* and from T-DNA based activation tagging as resources to isolate mutations in genes of known DNA sequence.

An additional source for identifying functionally important genes is the naturally occurring genetic variation that can be studied by quantitative trait locus (QTL), linkage disequilibrium (LD) or association mapping approaches (1). Traditionally, most genetic studies in *A. thaliana* involved crosses and mapping populations derived from the genetically distinct Columbia (Col-0) and Landsberg *erecta* (*Ler*) accessions (e.g. 16). More recent work has shown that different accessions of *A. thaliana* harbor large amounts of heritable genetic variation that can lead to marked phenotypic differences among individuals (1). Such genetic mapping approaches and, in addition, the map-based positional cloning of chemically induced mutants (11) require large sets of genetic markers that are polymorphic between individuals or populations. As a class of markers SNPs have recently attracted much interest, because they are abundant in the genome and suitable for high-throughput genotyping (5). The GABI-MASC project, a collaboration of four institutes of the Max-Planck-Society (MPI of Chemical Ecology, Jena, MPI for Molecular Genetics, Berlin, MPI of Molecular Plant Physiology, Golm, MPI for Plant Breeding Research, Cologne), carried out a large-scale identification and characterization of a genome-wide set of SNP markers by sequencing from up to 12 accessions of the model plant *A. thaliana*.

This article outlines the objectives and the realization of three *A. thaliana* genomics projects at the MPIZ:

- The Gene Knock-Out Facility ZIGIA
- Flanking Sequence Tags in GABI-Kat
- Single Nucleotide Polymorphisms in GABI-MASC

The materials and tools developed by these projects are publicly available and are used worldwide by scientist to explore the frontiers of plant sciences.

2. The gene knock-out facility ZIGIA

2.1. The ZIGIA populations

The use of transposons for random insertional mutagenesis builds on many years of work by many people at the institute. Seminal work on the molecular analysis of the En/Spm (hereafter referred to as En) transposable element system of *Zea mays* was performed in the 1980's (20), which leads to evidence for the mobility of the maize transposable element in *A. thaliana* (4) and finally its application for insertional mutagenesis in *A. thaliana* in the 1990's (30).

The history of T-DNA tagging goes equally far back in time. Experiments on the transformation of plants with the Ti-plasmid from *Agrobacterium tumefaciens* in the 1980's (12), resulting in the isolation of genes by T-DNA tagging in *A. thaliana* in the 1990's (13) and the production of an insertional mutagenesis population with an elegant selection system (9).

ZIGIA was set up in 1998 with the aim of expanding and exploiting these two types of insert-tagged mutant populations of *A. thaliana* for the discovery of new gene-phenotype relationships. Either the autonomous maize transposon En-1 or T-DNA were used to disrupt the genes and, in addition, provided a tag for the characterization of the mutation. The final ZIGIA populations consist of 16.000 En-lines, each carrying 5-20 copies of the transposon, representing on average six independent insertions per plant or ca. 96.000 integration sites for the whole En-population, and an additional 7.000 T-DNA lines with on average 1,5 insertions per plant. Throughout the project, this artificial genetic diversity was successfully used by four screening groups at the MPIZ (supervised by Drs. Dario Leister, Bernd Reiss, Heinz Saedler and Imre Somssich) and a technology center that provided an array of services, most notably the screening facility.

2.2. The screening facility

The ZIGIA knock-out screening facility used the En-lines to set up a reverse genetics screening service. The use of large scale T-DNA transformation to establish

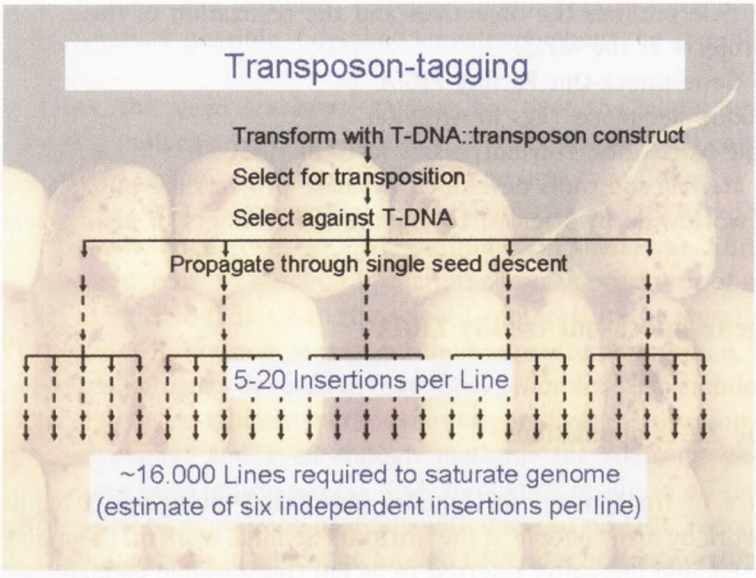

Fig. 1. Strategy used for the production of a transposon-tagged population. Transposon tagging involves a few transformations and 6-12 rounds of propagation through single seed descent. The background image shows spots in corn kernels caused by a somatic reversion characteristic for a transposon. The estimation of the number of lines required to saturate the genome assumes random distribution of the insertions and a 5 kb gene. In practice, larger numbers are likely to be needed.

populations was not yet fully developed, and we knew that the En transposon had a wide range of secondary insertion sites, making it possible to start with 5 original insertion events and to saturate the genome with insertions through 6 to 12 generations of selfings (3, Fig. 1). In addition, there is the unique feature of transposons that confirmation of a gene-phenotype relationship is possible by monitoring the reversion to wild type upon transposition of the active transposon out of the original site (Fig. 2). Usually, this results in restoration of the original sequence, but it is also possible to obtain a footprint that created a stable allele which then can be used for further studies.

Initially, candidate knock-out mutants were identified by PCR analysis of DNA isolated from pools of plants, using gene-specific primers and primers directed against the transposon, at that time a common approach also used to identify candidate mutant plants in other populations (14,28). By using PCR on DNA pools, our En-1 mutagenised population of the Col-0 accession was screened for insertion mutations in 718 genes. However, using our population of formerly 8.000 individuals and the pooling strategy described in Baumann et al. (3) this approach required 200 PCRs and two Southern blots per gene. In order to achieve a higher throughput, a more efficient approach to reverse genetics had to be developed.

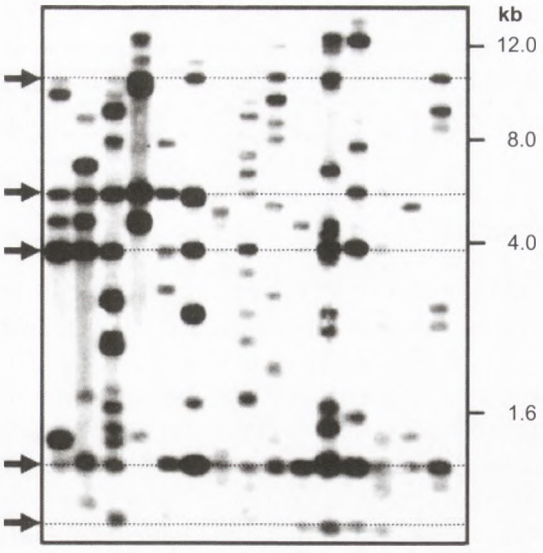

Fig. 2. Southern blot analysis of 15 individual SSD lines of generation 4 which were derived from a single line carrying 8 copies of En-1 illustrating the behaviour of the maize transposable element En/Spm in Arabidopsis. A labeled En-fragment was used as the probe. The arrows and dashed lines mark five insertion positions which were present in the parent. Different intensities of bands can be the result of hemizygous inserts or somatic insertion events.

Since the *A. thaliana* genome sequence was not available at that time, and since our population contained individuals with up to 20 copies of the transposon, a hybridization-based approach was selected for screening. This approach allowed us to avoid pools and to screen directly for mutants in specific genes of interest without being dependent on the output of the sequencing project. As a first step, the isolation of DNA from individual lines was automated by the use of pipetting stations and 96-well blocks. All other reactions could be performed also in 96-well format. For the amplification of flanking regions, we modified a method based on linker-mediated PCR (31) to one suitable for high throughput (26). The linkers which are double stranded at one end but consist of non-matching single strands at the other end are ligated to DNA ends produced by cleavage with restriction enzymes, followed directly by two successive DNA amplifications. The first, linear amplification is performed with En-specific primers directed against the 5´-end and the 3´-end of the transposon, respectively, while in the second PCR nested En-specific primers are combined with a linker-specific primer. The amplification products obtained from any one plant were pooled and spotted at high density in duplicate on nylon membranes. In total, 8.640 plants were represented on two filters of 8 cm × 12 cm, with each pair of dots containing the insertion-flanking sequences from one individual plant. A simple hybridization with a non-radioactive gene-specific probe is sufficient to identify directly the candidate plants that contain a mutation in the re-

spective gene. The ZIGIA screening service performed this identification of En-tagged knock-out mutants for 3.465 genes, with a success rate of about 50%. These results are available through a public database (**www.mpiz-koeln.mpg.de/zigia/publicdb/**) where identified candidates for a particular gene are described.

2.3. Impact of the project

The scientific contribution of this project is best underscored by the number of publications in which insert tagged lines from the ZIGIA populations have featured. For example, the collaborations that have used insertion tagged lines of the ZIGIA populations between July 1996 and April 2003 resulted in 15 publications from forward genetics, 20 publications from reverse genetics and 4 publications from combined approaches. The time from discovery till publication was on average two years.

Moreover, by combining forward screening and reverse genetics, the four ZIGIA screening groups discovered a total of 30 gene-phenotype relationships, of which 21 relationships had not been described before. Upon request from the industrial partners in the project, BayerCropScience, Deutsche Saatveredelung, KWS SAAT and Norddeutsche Pflanzenzucht, a patent application was filed for six of these relationships. After completion of this project in 2003, the established resources from ZIGIA were transferred into the German plant genomics initiative by integration of ZIGIA and GABI-Kat.

3. Flanking sequence tags in GABI-Kat

By the end of 2000, the activation-tagged T-DNA-line approach along with the insert-flanking-sequence amplification technique from the En-approach were used as a starting point for the separate GABI-Kat project. This project is building a large T-DNA mutagenized *A. thaliana* population (accession Columbia, the sequenced genotype) of finally 70.000 plants with sequence-characterized insertion sites.

The availability of almost complete *A. thaliana* genome sequence generated by The Arabidopsis Genome Initiative (2), the advances in high throughput isolation of DNA from plants as well as of techniques for the amplification of insert flanking sequences and the development of sequencing in 96-well format enabled large scale recovery from plant genomic DNA sequences flanking the insertion site (flanking sequence tag, FST). Since it is possible to locate the exact position of insertions in the *A. thaliana* genome, GABI-Kat is one of the main resources (other large collections are e.g. SALK, FlagDB (21) or SAIL (24) that currently supply the community with insertion tagged lines. End users can identify the candidate lines without experimental work, and the approximate insertion site relative to the gene of interest can be deduced from the sequence of the FST connected to a candidate line.

3.1. About the T-DNA lines

The sequenced *A. thaliana* accession Col-0 was used for transformation by *Agrobacterium tumefaciens* mediated transfer of usually pAC161 (Genbank AJ537514) that contains one full length CaMV 35S-promoter for possible activation tagging. Two innovations in particular had much impact on the odds of really producing 70.000 independent T-DNA lines with limited resources (Fig. 3). In 1998, Clough and Bent (6) published their paper about the efficient transformation of *A. thaliana* by simply dipping in a suspension of *A. tumefaciens*. A modification of this procedure turned out to be very successful in our hands. Very helpful was also the development of a method that allows simple selection of *A. thaliana* transformants in greenhouse conditions by using sulfadiazine resistence as a selection marker (9). Genetic analysis of these lines indicated an average of 1,3 insertion sites per line. We note that the actual number of insertions is higher, because not all copies, such as insertions at genetically linked sites, are detected by genetic analysis.

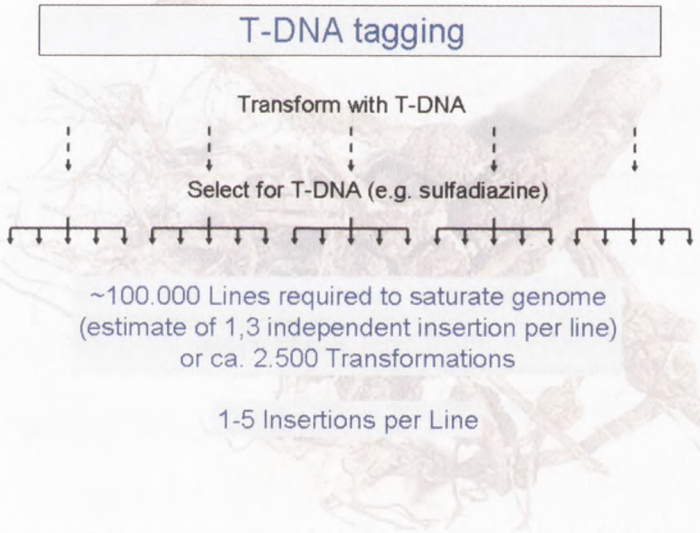

Fig. 3. Strategy used for the production of a T-DNA-tagged population. T-DNA tagging requires efficient large-scale transformation- and selection systems, followed by one or two propagations through single seed descent. The background image shows a root infected by *Agrobacterium tumefaciens*, the microorganism from which the T-DNA vectors were derived. The estimation of the number of lines required to saturate the genome assumes random distribution of the insertions and a 5 kb gene. In practice, larger numbers are likely to be needed.

3.2. Generation of sequence indexes

For economic high-throughput production, a pipeline for the characterization of *A. thaliana* mutants by generating FSTs had to be created and optimized. A PCR walking procedure similar to the one used for the En-population required two nested amplifications and one cycle sequencing reaction for each border (Fig. 4). When applied to the T-DNA population on both the Left and Right borders (4 amplifications plus 2 sequencing reactions), about 66% of the lines yielded at least one hit.

However, several factors were encountered that had a negative effect on efforts to characterize the T-DNA-tagged population by sequencing. T1-seeds and plants generated from these seeds are still habitated by *A. tumefaciens* used for transformation. Therefore, DNA extracted from T1 leaves can still contain the T-DNA plasmid, and such plasmid-derived sequences may amplify preferentially during recovery of plant genomic fragments flanking the T-DNA insertions. Another reason for poor quality sequences can be truncated, tandem or multiple insertions that often occur in complex patterns (27). Separation of unwanted sequences by gel-electrophoresis is tedious, while low success rate in FST production is expensive.

The final GABI-Kat pipeline included robotized extraction of genomic DNA in 96-well format, an adapter-ligation PCR method for amplification of plant sequences adjacent to T-DNA borders, automated purification and sequencing of PCR frag-

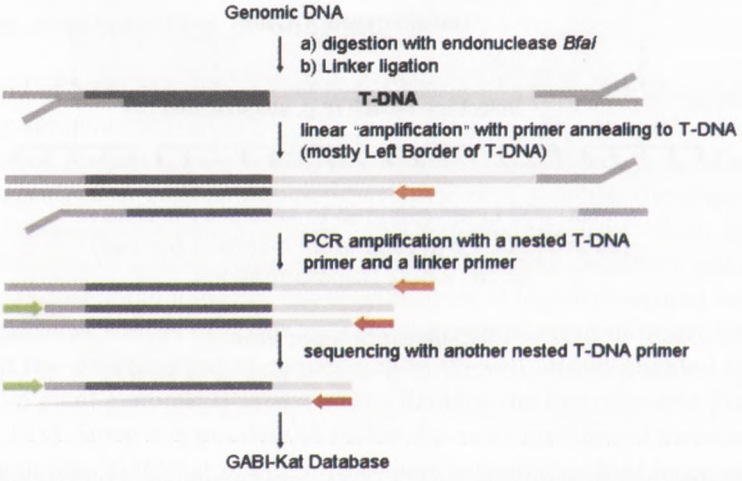

Fig. 4. Molecular biological procedure used in GABI-Kat to generate flanking sequence tags (FSTs) from the T-DNA tagged lines. Normally, only the genomic DNA flanking the left border of the T-DNA is amplified so that the procedure involves three rounds of DNA amplification. All reactions are performed in 96-well plates. Only sequences of more than 30 nucleotides with BLAST expect values lower than 5e-4 are accepted.

ments, and computational trimming of generated sequence files. Data quality was significantly improved by the addition of one extra digestion to reduce trivial sequences from Ti-vector derived fragments present, and the design of the adaptor primers for the second PCR step to enhance selective amplification of single insertion-fragments. By including these steps, gel-purification was avoided, the number of amplification reactions per line was reduced from 4 to 3, and the percentage of lines that yielded at least one FST increased from 66 to 86% using only the Left-border.

3.3. GABI-Kat SimpleSearch: a flanking sequence tag database

GABI-Kat SimpleSearch (15) is the database of FSTs that was generated by the GABI-Kat project (**http://www.mpiz-koeln.mpg.de/GABI-Kat/**). The sequence trace files derived from PCR-generated DNA fragments spanning insertion sites were processed using Phred (8). T-DNA vector sequences in the candidate FSTs were masked using cross match, and sequences shorter than 30 nucleotides were discarded. Candidate FSTs passing this filter were aligned to the *A. thaliana* genome sequence (MIPS *Arabidopsis thaliana* Database, (23)) by BLASTN and the expected insertion site were calculated. Only sequences with BLAST expect values lower than 5e-4 were considered as good FSTs. MAtDB gene annotation data were used to determine if a given insertion site was within a gene. A FST qualifies as a 'gene hit' when the insertion site is located between 300 bp upstream of the ATG and 300 bp downstream of stop codon of an annotated gene. We use the term 'CDSi hit' when an insertion site is located between ATG and stop codon (insertions in CDS and included introns).

SimpleSearch allows quick access to the T-DNA insertion mutants generated by GABI-Kat. Two ways to start a search are available: 1) a sequence-based search using BLASTN or TBLASTN against all FSTs; and 2) a text-based search to find 'gene hits'. The text-based search accepts either AGI gene codes (e.g. At1g23450) or a keyword as input. In addition, FST data is also available from the GSS division of EMBL/GenBank/DDBJ.

The July 2003 release of SimpleSearch was based 36.206 T-DNA transformed *A. thaliana* lines with genome hits. A total of 12.382 different genes (47% coverage of all *A. thaliana* genes) have been hit, of which 8.425 qualify as CDSi hits (32% coverage). GABI-Kat SimpleSearch will be updated regularly with new FSTs until the final goal of 70.000 analyzed lines is reached.

4. Single nucleotide polymorphisms in GABI-MASC

The four Max Planck Institutes (MPI of chemical ecology, MPI for molecular genetics, MPI of molecular plant physiology, MPI for plant breeding research) consti-

Fig. 5. Two commonly used accessions from *Arabidopsis thaliana* to illustrate some of the phenotypic differences. Shown are accession Columbia-0 (Col-0) on the left and accession C24 on the right. Both lines were grown for 10 weeks in standard greenhouse conditions.

tuting the `Max-Planck-Arabidopsis-SNP-Consortium´ (MASC) aim to support an efficient use of the natural diversity in *A. thaliana* by the establishment of high-efficiency SNP-based mapping tools (22).

A. thaliana occurs naturally in Asia, Europe and Africa and has been found in North America, Australia and Japan as well. Its broad geographic distribution coincides with adaptation of the local populations to a wide range of different growth environments (Fig. 5). Accordingly, about 250 available accessions show very considerable diversity in adaptive traits such as resistance to biotic stresses (interactions with various pathogens) or abiotic parameters such as high or low temperatures, drought or salt conditions and different day length regimes (1). Therefore, studying natural variation in *A. thaliana* offers opportunities for dissecting more

complex traits, such as QTLs, in comparison to the single gene oriented approaches that were mentioned above.

The main project goal was to identify a genome-wide set of SNP markers for high-throughput mapping in *A. thaliana* in order to accelerate positional cloning and QTL mapping of genes influencing important phenotypic traits. Two approaches were followed: 1) the sequencing of ESTs from 6 accessions and 2) the amplification and sequencing of evenly spaced regions in the *A. thaliana* genome of 12 varieties.

4.1. SNP detection in EST sequences

A total of 10.706 ESTs were sequenced from 6 different accessions of *A. thaliana*: L*er*, Ei-2, Cvi-0, Nd-1, C24 and Ak-2. SNPs were detected by pairwise sequence comparison of clustered ESTs with the Col-0 genome sequence (22). To filter out potential paralogs, sequences with a divergence of more than 3% from the genomic reference sequence were discarded. The vast majority of ESTs showed only little sequence divergence to the reference sequence. Only ESTs with at least 80 bp of high-quality sequence (phred score > 30) were used for SNP detection (N = 4,176). Using these sequences, it was possible to identify 4.327 SNPs and 18 InDels.

4.2. SNPs derived from sequence tagged sites (STS)

In this approach, 12 accessions were compared: Col-0, L*er*, Cvi, C24, Nd-1, Ei-2, CS22491, Gü-0, Lz-0, Wei-0, Ws-0 and Yo-0. Different sets of primers were designed to amplify genomic segments of about 600 bp length from regions that are spaced on average 100 kb apart. The use of 595 primer sets resulted in 2.2 million non-redundant basecalls that were compared with the Col-0 genome sequence. The comparison led to the identification of 3.773 SNPs and 619 InDel polymorphisms (22).

4.3. Access to the data

MASC SNPs are made publicly available at **http://www.mpiz-koeln.mpg.de/masc/** (Fig. 6). The polymorphic sites and their alleles can be retrieved by location (pseudochromosome, bac, annotated genes), polymorphism type and accession. Additional information about restrictions sites and primers for various genotyping methods (e.g., CAPS, primer extension, Pyrosequencing) is given, which is expected greatly foster the design of mapping experiments.

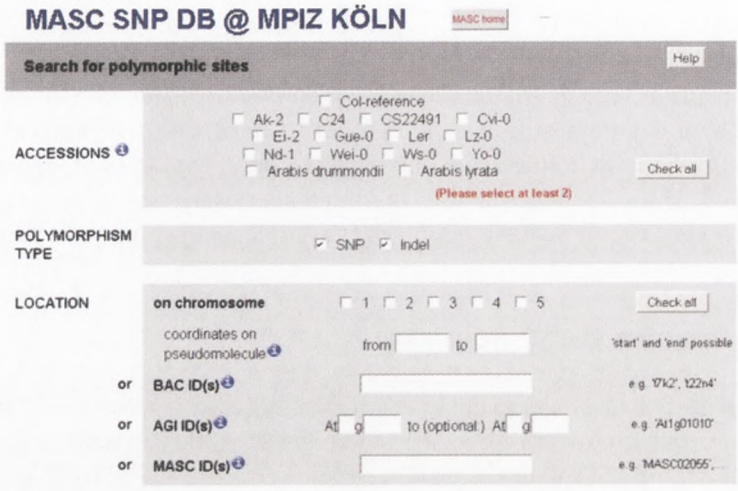

Fig. 6. Screen shot from the GABI-MASC Arabidopsis SNP-database (**www.mpiz-koeln.mpg.de/masc/**) illustrating accessions for which SNPs are available and the options that the end-user can select to suit his specific purpose.

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