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Development of a Transposon-based Activation Tagging system in *Arabidopsis thaliana*: A tool to study root development and P uptake

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RESUMEN

Si bien se conoce la secuencia casi completa de Arabidopsis, la tarea de asignar funciones a genes específicos queda por hacer. El estudio de mutantes, naturales o generadas experimentalmente, facilita dicha tarea.

Existen actualmente poblaciones de mutantes obtenidas por diversos métodos, y aquellas generadas con inserciones como T-DNA o transposones tienen la ventaja de requerir tiempos menores para conocer la identidad del gen afectado que aquellas generadas con agentes químicos, por ejemplo EMS. Sin embargo, en la mayoría de las poblaciones de inserciones se obtienen mutaciones por interrupciones de genes, siendo la mayoría recesivas. Dada la presencia de grupos de genes con funciones similares y redundantes, la interrupción de un gen no necesariamente da como resultado un fenotipo alterado, visible en la planta. Una estrategia que ha permitido descubrir funciones de genes que son redundantes, o que tienen funciones translapadas con otros genes, es la Activación Etiquetada de genes.

La Activación Etiquetada consiste en el uso de secuencias activadoras dentro de un inserto, que pueden actuar aumentando la expresión de genes aledaños. Las poblaciones generadas con este sistema hasta ahora reportadas utilizan T-DNA como inserto. Desgraciadamente, el T-DNA al insertarse en el genoma puede crear configuraciones y rearreglos no favorables para la estrategia de Activación, por ello, en este trabajo se han empleado transposones como inserto. Usando transposones, se construyó una población de aproximadamente 13,000 plantas de Arabidopsis conteniendo en total un estimado de 8,300 insertos activadores diferentes, con una (la mayoria) a tres inserciones por planta. Los resultados obtenidos con el uso de transposones para la Activación Etiquetada han sido muy alentadores, pues el número de mutantes de fenotipo aéreo alterado ha sido considerablemente más alto (cerca del 1% contra 0.1%) que en las poblaciones que emplean T-DNA (Weigel D., *et al.,* 2000). En el presente trabajo se detallan con mayor profundidad las características de algunas de las mutantes encontradas, algunas de las cuales presentan fenotipos nuevos.

En la parte final del trabajo, se emplearon partes de la población de activación con transposones para realizar búsquedas de mutantes con alteraciones en el desarrollo postembrionario de la raíz. Asimismo, se buscaron mutantes que fueran capaces de crecer mejor que controles silvestres en medio con fosfato insoluble. Se identificaron mutantes candidatas para ambos escrutinios.

En conclusión, se construyó y probó un sistema de activación con transposones de gran utilidad para la obtención de mutantes y el subsecuente aislamiento de genes. Se identificaron mutantes tanto visualmente como en escrutinios específicos, indicando que este sistema puede ser usado para estudiar una amplia variedad de funciones de genes. Además, el sistema de activación con transposones puede ser usado en plantas en las que la carencia de métodos de transformación efectivos haría extremadamente difícil la aplicación de activación con T-DNA.

SUMMARY

Although the almost complete genomic sequence of Arabidopsis is know, functions must still be assigned to many genes and the study of natural or induced mutants aids in the task.

At present, many mutant populations, obtained using diverse methods, are available. From these populations, those made with insertions like T-DNA or transposons have the advantage of requiring shorter times to know the identity of affected genes than those mutagenized with chemical agents, like EMS. However, in most of the populations mutagenized with insertions, gene disruption causes altered phenotypes. Given the presence of genes with similar functions, gene disruption does not always leads to visible phenotypic alterations. A strategy that has allowed the study of redundant or overlapping gene functions is Activation Tagging.

Activation Tagging is featured by the use of inserts containing enhancer sequences that can increase expression of neighboring genes. Until now, Activation Tagging populations reported in the literature use T-DNA as a tag. However, T-DNA can create configurations and DNA rearrangements that are unfavorable for Activation Tagging. Therefore, in this work transposons are used for Activation Tagging.

Using transposons, an *Arabidopsis* Activation Tagging population of around 13,000 plants was built, containing near 8,300 independent insertions in total, with one (the majority) to three inserts per plant. The population has been screened for visible morphological mutants and the results obtained have been very encouraging. The number of identified mutants with altered aerial phenotype has been considerably higher (near 1% vs. 0.1%) than in populations that employ T-DNA as a tag (Weigel D., e*t al.,* 2000). The features of some of the aerial mutants found are described in the present work, some of them showing novel phenotypes.

In the last part of this work, the transposon activation tagging population was used to screen for mutants with postembryonic root development alterations and for mutants with enhanced growth in medium supplemented with insoluble phosphate. Candidate mutants were identified in both screens and their initial characterization is described.

In conclusion, the transposon activation tagging system here described proved to be useful for mutant obtention and for subsequent gene isolation. Mutants were identified both visually and under specific screening conditions, indicating that this system can be used to study a broad variety of gene functions. Moreover, the Transposon Activation Tagging system can be used for plants where the lack of effective transformation methods would make extremely difficult to use T-DNA Activation Tagging.

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ABBREVIATIONS

AIE. Activating *I* Element.
***En*.** Named also *Spm*. Autonomous element of the *En/Spm-I/dSpm* maize transposon system, encoding a functional transposase. In this work, it refers to an inmobilized transposase driven by the CaMV 35S promoter.
EDS. Empty donor site. The sequence that remains in the T-DNA after transposon excision.
FDS. Full donor site. The transposon is present still in the T-DNA.
I. Also named *dSpm*. Non-autonomous element of the *En/Spm-I/dSpm* maize transposon system that requires a functional transposase to transpose. In this work, it refers to the mobile transposon that contains the CaMV 35S enhancer tetramer.
ITF. Independent transposition frequency. Ratio of the number of different AIE insertions observed to the number of plants analyzed.
Kb. Kilo base pairs.
Mb. Mega base pairs.
n. Nucleotides.
PPT. Phosphinotricin. This compound has the same toxicity as glufosinate, the active compound of the herbicide Basta.
STF. Stable transposition frequency. Ratio of the number of surviving plants to seeds sown.
TAT. Transposon Activation Tagging.
TIR. Terminal inverted repeat(s) of the *I* transposon.

Other abbreviations used inside the figures are explained in their respective legends.

CHAPTER I.

INTRODUCTION

Part I. TRANSPOSONS AND ACTIVATION TAGGING IN ARABIDOPSIS

Arabidopsis thaliana

Arabidopsis thaliana or Thale cress belongs to the mustard or crucifer (Brassicaceae or Cruciferae) and to the Sisimbriae tribe. It grows naturally in temperate regions from Asia, Europe and North Africa, and has been introduced in other areas like North America and Australia. The natural habitat of Arabidopsis is sandy soils where water filters easily (Anderson and Wilson, 2000, Price, R. A. *et al.*, 1994). Arabidopsis is a small sized plant, around 30 cm. tall, depending on the ecotype and the conditions of growth. This reduced size allows the growth of a large number of plants in a greenhouse of reduced space.

Although literally a "weed", Arabidopsis is used as a model plant by many plant scientists. It has a relatively small genome when compared with other angiosperms. Compared with other organisms, its genome is around 15 times the size of *E. coli* and 8 times the size of the genome of *S. cerevisiae*. Almost completed, the Arabidopsis genome sequence is estimated to be 146 Mb long, predicted to code for around 25,500 to 30,000 genes (Hosouchi T. *et al.*, 2002, The Arabidopsis Genome Initiative, 2000). Additionally, the average density of genes has been estimated to be around 1 gene each 5 kb. The small genome and the availability of almost all the genomic sequence make Arabidopsis a very attractive plant for mutagenesis and gene cloning. Moreover, many mutant collections and bioinformatic tools are

available. The haploid number of chromosomes in Arabidopsis is five. In contrast with other plants, that are polyploid, Arabidopsis is a diploid plant. Being diploid, the study of recessive traits can be easier, and the effects of gene dosage are less than in polyploid plants.

The life cycle of Arabidopsis lasts around six to eight weeks from seed to seed, and is "season independent". Many generations can be obtained in one year, in contrast to other plants, like maize. The relatively short time of generation and the possibility to grow many generations in one year can speed genetic analysis. Moreover, a single plant can produce thousands of seeds, making Arabidopsis an ideal organism to make mutagenesis experiments. Intensive labour is not required to maintain homozygous lines, because Arabidopsis self-fertilizes. However, crosses with other lines can be also done when required (Anderson and Wilson, 2000; Weigel and Glazebrook, 2002).

The transformation methods of Arabidopsis have been simplified in the past 5 years, and nowadays, transformation can be done by just dipping flower buds in a solution of *Agrobacterium tumefaciens* with the desired construct, followed by seed harvesting and selection some weeks after (Clough and Bent, 1998).

Arabidopsis is also an excellent model for developmental studies. For instance, the root of Arabidopsis can be well studied when grown *in vitro*. It is a transparent organ that allows all cellular layers to be seen. Additionally, as a brassicaceae, Arabidopsis is adapted to grow in low phosphorous conditions, and some ecotypes show a well characterized P deficiency phenotype. In those ecotypes, root architecture shows distinguishable characteristic changes in low and high P (Lopez-Bucio, J. *et al.*, 2002).

Mutagenesis strategies

The study of natural variation and mutants has been of great value to address biological questions in different organisms. Alterations in the genome of the organisms are invaluable in genetic analysis. Mutants can be present in nature already. However, mutations in organisms can also be induced experimentally by different means.

Mutagenic agents

Three classes of mutagenic agents have been used to cause mutations in the laboratory: physical, chemical or biological. Physical agents include gamma radiation, UV light, fast neutrons, and ionizing radiaton among others (Cecchini, E. *et al.*, 1998). Chemical mutagens are, for example, EMS (ethylmetanesulfonate) (Feldmann, K. A. *et al.*, 1994). Finally, biological mutagenic agents are usually insertion elements. In plants, there are two kinds of insertion elements that are frequently used: the T-DNA from *Agrobacterium tumefaciens*, and transposons. Either endogenous or heterologous transposons have been used successfully to tag genes (Lightner and Caspar, 1998; Martienssen, R. A., 1998; Pereira, A., 2000). Recently, the use of retrotransposons to tag genes has also been reported (Kumar and Hirochika, 2001).

The use of each mutagen will depend on certain considerations. These considerations include the desired kind of mutation (for example, point mutations vs. deletions), feasibility of different gene cloning methods, and the desired mutation frequency. In many cases, these factors can be contradictory (Lightner and Caspar, 1998).

Chemical and physical mutagens

Chemical agents can cause many mutations in a high rate. These mutations are normally due to single nucleotide modifications, and not to large genetic changes. Because of the number and the small size of the modifications, gene cloning might be difficult, and is mostly done by gene mapping. Mutations caused by chemical agents can be recessive or dominant. Moreover, chemical agents can cause not only null alleles, but also mutations with different degrees of funcionality, or temperature-sensitive alleles. Because of the functionally diverse range of mutations found in them, mutant collections generated with chemical mutagens are very valuable.

Ethylmethanesulfonate (EMS), an alkylating agent, is the most used chemical mutagen and it causes mutations because it can donate an ethyl group to a nucleotide. This modification induces the breakage of the DNA chain or an erroneous coupling. Analysis of alleles induced by EMS showed that mainly GC->AT transitions are produced. This kind of alkylating agents are very powerful carcinogens, and should be treated carefully.

Physical mutagens, like ionizing radiation, produce mainly deletions or chromosomal rearrangements that can facilitate gene cloning. Some physical mutagens, like fast neutrons, produce a mutation frequency comparable to chemical mutagens. Fast neutrons generally produce small deletions (around 1 kb deletions) that cause null mutations (Weigel and Glazebrook, 2002). Both chemical and physical mutagens are mainly used to mutagenize seeds. Treated seeds are named M1. The next generation, M2, is used to screen for recessive homozygous mutations. (Lightner and Caspar, 1998).

When starting a new screen aiming at novel mutations, it can be practical to begin using chemical mutagens because the type of mutations produced by these agents give broader possibilities to find new mutants. Chemical induced mutations give a good idea of the kind of mutants that can be found, and their genetic relationship (Lightner and Caspar, 1998). Gene mapping can be used to clone the genes of interest. However, gene mapping can be laborious, and a common practice is to use insertional mutants for gene cloning when possible because it can be done in less time and with less effort than gene mapping (Weigel and Glazebrook, 2002).

Insertional mutagens

Insertions are biological mutagens that interrupt the DNA sequence. In most cases, insertions in promoter or coding regions lead to mutant phenotypes. These mutagenic agents cause mainly recessive, null mutations.

Insertional mutagenesis was first used with well-characterized transposable elements in *Drosophila melanogaster* (Bingham, P. M. *et al.*, 1981). In plants, the same strategy was used when transposable elements of maize and snapdragon were characterized (Walbot, V., 1992). Different kinds of inserts besides transposons have been used. For example, T-DNA from *Agrobacterium tumefaciens*, or retrotransposons like the *Tnt*1 element from tobacco are functional in Arabidopsis (Feldmann, K. A., 1991; Grandbastien, M.-A., 1992; Lucas, H. *et al.*, 1995).

A high number of genes have been isolated using populations of insertional mutants, either generated with T-DNA or transposons (reviewed in Parinov and Sundaresan, 2000; Pereira, A., 2000; Sussman, M. R. *et al.*, 2000). However, there are different benefits or drawbacks associated with each one. Stability is an advantage of T-DNA over transposons as a tag. Once integrated in the plant genome, T-DNAs behave as stable insertions. They are believed to integrate relatively randomly in the genome. Unfortunately, they can have complex integration patterns. Another problem of T-DNA is that integration can cause rearrangements of adjacent chromosomal DNA. On the other hand, transposons are suitable to perform targeted tagging (Speulman, E. *et al.*, 2000). Transposon reversion can be used to confirm that a mutation is indeed caused by transposon insertion. Moreover, transposons provide a way to disperse insertions in the genome. If unmodified transposons are used, a problem of transposon tagging can be transposon instability. However, transposons can be modified to produce stable insertions. **Insert 1** explains the advantages of transposons over T-DNA.

Many collections generated using insertion elements bear only a few mutations per individual, in contrast to EMS generated collections (Lightner and Caspar, 1998). Because of the fewer number of mutations per individual, larger numbers of plants are necessary to perform a screen. Furthermore, in contrast to EMS mutagenized collections, a drawback of populations made by means of insertional mutagenesis is that they are more laborious to generate, particularly those where T-DNA or heterologous transposons are used (Lightner and Caspar, 1998). Frequently, the characterization of chemically induced mutants leads to the design of screens that are more adequate to work with a higher number of individuals. Populations of plants mutagenized with insertions (T-DNA or transposons, for example), in which fewer mutations per plant are present, are then screened using those simpler screens. These insertional mutants can ease gene cloning in many cases (Weigel and Glazebrook, 2002). Depending on the nature of the insert used to generate the mutants, different techniques can be used to obtain the sequence adjacent to the insertion. These techniques, if succesful, take less time and can be less laborious than gene mapping, used for chemically induced mutations. Examples of those techniques are inverse PCR (Deng, X.-W. *et al.*, 1992; Pereira and Aarts, 1998), thermal asymmetric interlaced or TAIL-PCR (Liu, Y.-G. *et al.*, 1995; Liu and Whittier, 1995; Tsugeki, R. *et al.*, 1996), plasmid rescue (Yanofsky, M. F. *et al.*, 1990), or

"walk PCR" (Balzergue, S. *et al.*, 2001; Siebert, P. D. *et al.*, 1995). The availability of nearly all the genome sequence of Arabidopsis allows positioning of the insert in the genome.

Advantages of transposons over T-DNA in insertional mutagenesis

- The *Agrobacterium* mediated transformation process to introduce T-DNA, can cause mutations that are not related to the insertion. Endogenous transposons are already in the plant genome, and do not need to be delivered to it. However, heterologous transposons need to be delivered. They can be introduced to the plant using T-DNA, that requires an *Agrobacterium* transformation. The difference between T-DNA and the delivered heterologous transposons is that a transposon can cause new mutations when it moves to new places in the genome. In this way, if new mutations are identified in the progeny of transformed plants that were not present in the original transformant, they are very likely to be caused by the new transposon insertion. In contrast, in plants where T-DNA is the mutagen itself it becomes difficult to discern *"a priori"* if a mutant phenotype is due to the insert or to another mutation ocurred during transformation when both are linked.

- T-DNA rearrangements leading to inverted configurations can also be problematic for activation tagging purposes. They could lead to enhancer silencing. Moreover, some configurations, like tandem repeats, may difficult the isolation of adjacent sequences to the insertion using inverse PCR or TAIL-PCR. When using transposons, and using a system that would allow elimination of the original T-DNA, "cleaner" insertions could be obtained after transposition.

- Reversion, or the excision of the transposable element from its original locus, can be used to prove that a mutant phenotype is caused by the transposable element.

- Transposons can be used for targeted tagging. Inserts near an interesting gene can be remobilized in order to obtain insertions inside the gene of interest. Transposition frequency to linked loci varies for different transposon systems. For the *En/I-Spm/dSpm* maize transposon system, it has been estimated to be 30% in Arabidopsis (Aarts, M. G. M., 1996). For the *Ac-Ds* system, also from maize, the *Ds* transposition frequency to linked positions was estimated to be around 68%. However, the distribution of transposed elements varied in different donor sites (Bancroft, I. *et al.*, 1993).

- Transposons give the possibility of having multiple inserts in a plant. Multiple inserts would decrease the total number of plants to find a specific mutation. However, for activation tagging having more inserts may complicate mutant analysis.

Insert 1. Advantages of transposons over T-DNA in insertional mutagenesis.

Forward and reverse genetics screenings

Mutagenized populations in general can be used in two ways: "Forward" and "Reverse" genetics. "Forward genetics" implies the initial identification of a phenotype, followed by gene cloning. On the other hand, "Reverse genetics" involves the prior knowledge of a gene sequence. The sequence is then used to look for plants with mutations in it, normally using PCR and sometimes in combination with other techiques. When an individual carrying a mutation in the desired sequence is identified, the phenotype is then evaluated (Das and Martienssen, 1995).

"Forward genetics" approaches to identify mutants can be very similar regardless of the mutagenic agent but they differ afterwards, in gene cloning strategies. In contrast, the techniques used to search for mutations in the "Reverse genetics" approach vary according to the nature of the mutagen employed to generate the mutant collection.

An example is a technique named TILLING (Targeting Induced Local Lesions IN Genomes), that combines chemical mutagenesis (EMS) with sensitive mutation detection systems. In the original TILLING technique, DNA from a collection of EMS-mutagenized Arabidopsis plants was pooled, subjected to PCR amplification and screened for mutations in specific genes using denaturing HPLC (DHPLC). DHPLC detects mismatches in heteroduplexes created by melting and annealing of heteroallelic DNA (Mccallum, C. M. *et al.*, 2000). Recently, modifications were done to this method that allows a less expensive high-throughput application. In this modified version, amplification products are incubated with an endonuclease, CEL I, that preferentially cleaves mismatches in heteroduplexes between wild type and mutant (Oleykowski, C. *et al.*, 1998). Cleavage products are electrophoresed using an automated sequencing gel apparatus, and gel images are analyzed with an image-processing program (Colbert, T. *et al.*, 2001).

Another reverse genetics method employs fast neutron bombardment and PCR to identify and isolate deletion mutants for targeted plant genes. DNA samples extracted from deletion mutant libraries are screened by PCR using specific primers flanking the targeted genes (Li, X. *et al.*, 2001). Similarly, "Reverse genetics" for insertional collections also make

use of PCR. Specific primers for the desired gene and for the ends of the insertion are used. The PCR products are blotted and hybridized to confirm the sequence identity (Mckinney, E. C. *et al.*,1995, Speulman, E. *et al.*, 1999). Some laboratories with large insertional collections offer this kind of screens as a service (Sussman, M. R. *et al.*, 2000). Even screens for more than one gene family member at a time have been done, by using degenerate primers (Young, J. C. *et al.*, 2001). Recently, Iida and collaborators reported a method for efficient gene targeting by homologous recombination in rice (Terada, R. *et al.*, 2002). If this method proves to be as efficient for other plant species, such as Arabidopsis or maize, it would be very useful to addresss gene functions.

In "Forward genetics" screens, the use of insertions as a mutagen eases gene cloning in many cases (Lightner and Caspar, 1998). However, gene cloning is not always easier when using insertions. Gene cloning might be hindered by complex structures of the insert, particularly when using T-DNA. Furthermore, the induction of DNA rearrangements during transformation can lead to mutations that are not directly caused by the insertion itself. Those rearrangements can make gene cloning difficult.

Insertional Mutagenesis with Transposons

Transposons

A transposable element, or transposon, is a DNA fragment that can move in the genome of an organism. Transposons can cause mutations by insertion, excision or chromosome break. For example, the insertion of a transposon in a functional gene or its regulatory sequences can cause a change in the phenotype of the organism. In a genome, transposons can also cause deletions, duplications, inversions, and translocations when they mobilize taking a part of the host genome.

Transposons seem to have been associated with genomes for a long time. In the eukaryotic genomes, they form part of the "repetitive" DNA. Different theories have been

formulated to explain the prevalence of transposons in genomes. One theory postulates that their only function is to replicate themselves. Because of that only selfreplicative function they are termed "selfish DNA". However, another theory proposes that transposons might act as a complement of the genome, making it more diverse and adaptable. They might even play a role in temporal and spatial patterns of gene expression. In many cases, transpositions have been shown to be influenced by developmental and environmental stimuli. If transposons are indeed a complement of the genome, a genome with more active transposition would adapt better or faster to changes in the enviroment (Ferl and Paul *et al.*, 2000).

In plants, transposons can move in two ways:

1. Transposons can move by a DNA-mediated "cut and paste" mechanism. This "cut and paste" mechanism involves the excision of the transposon from its original location, and its insertion in a new position in the genome. A model for transposition has been proposed where the formation of a stem/loop structure is involved. Because of their "cut and paste" DNA-mediated mechanism of transposition, these elements are normally present in low or moderate numbers. Examples of this kind of transposons are the *Ac-Ds* and *En-I/Spm-dSpm* elements of maize.
2. Retrotransposons have a different mechanism of transposition. They move without excision. In contrast with transposons, they use an RNA intermediate and a reverse transcriptase that synthetizes DNA from the RNA template. Retrotransposons are thought to derive from RNA viruses. One example of this kind of transposable elements is the mutator element (*Mu*) of maize. *Mu* transposes very frequently: so frequently that mutated progeny is produced at a rate 50 times higher in a genome containing it than one without it (Ferl and Paul, 2000).

Barbara McClintock described transposons for the first time in the 1940s. The transposons that she described belong to the first type of transposons. This kind of transposons contain terminal inverted repeats that are recognized by the enzyme *transposase*. The enzyme binds to the terminal inverted repeats and it is thought to create a stem/loop structure that

excises from that region of the genome. The excised loop can integrate again in the genome, in a different region (Ferl and Paul, 2000).

The transposable elements that code for a functional transposase are named Active or *Autonomous*, because they can promote their own transposition. However, there are elements that have a defective transposase and can not transpose autonomously. Elements with defective transposase are named *Non-autonomous*, and require the presence of an autonomous element in the genome to transpose (Fedoroff, N. V., 1983).

Barbara McClintock discovered transposable elements in maize (*Zea mays)*, that is now known to bear many different transposon families. She studied chromosome breakage and fusion that led to loss of genetic markers along the chromosome. Commonly, she observed that the markers that were lost first were those farthest from the centromere. However, she also worked with a strain that lost activity of markers closer to the centromere. This latter strain seemed to have a weaker region, that McClintock denominated "*Dissociation" (Ds)* or breaking point in the chromosome. Her observations led her to realize that the breaking point seemed to "move" its position in some lines. In some lines, the breaking point *Ds* moved to a genetic marker producing a mutant phenotype. However, in rare cases *Ds* moved out of it again. Phenotypically, this was observed as variegated maize kernels [1]. Interestingly, she also observed that the *Ds* breaking point was stable in some lines and unstable in others. Barbara McClintock proposed then the existence of "controlling elements" that explained the behaviour of *Ds* in the unstable lines. She gave the name *"Activator" (Ac)* to those controlling elements (Mcclintock, B., 1947; 1951).

With time, more transposable elements were identified in maize, and were shown to belong to different families. An element that can induce transposition of a second element belongs to the same family. On the other hand, different transposon families are unable to induce transposition among each other. This genetic criterium helped to determine if a transposon system is unique and allowed to define more than 10 different systems or families in maize (Peterson, P. A., 1987). Generally, one family is formed by an autonomous active

[1] Somatic transposition cause the appearance of mutated sectors. For example, spots in a maize kernel. The size of the sector will indicate the timing of the transposition. Early transpositions give rise to larger sectors. On the other hand, when an insertion is germinal the whole plant will have the mutation.

element and many non-autonomous elements. A non-autonomous element is assigned to the family of the autonomous element capable of activating it *in trans*.

The isolation and molecular characterization of the *Ac-Ds* (Fedoroff, N. *et al.*, 1983) and other transposon systems showed why they belong to different families. Their sequences are different and code for transposases, the enzyme that mediates transposition, that are specific for each element. Therefore, one does not induce tranposition of the other.

Members of the *Ac-Ds* system have an 11 base pair terminal inverted repeat. In addition, when the element inserts in a new genome location, a "footprint" is created. This footprint is an 8 bp direct repeat generated from the host genome, located to both sides of the transposon. After excision of the element, the footprint stays in the genome in the place where the transposon used to be (Ferl and Paul, 2000).

The *Ac* element is 4.6 kb long. A 3.6 kb messenger RNA is transcribed from it, and codes for a transposase, an 807 aminoacid protein (Kunze, R. *et al.*, 1987). *In vitro* experiments showed that the transposase binds to the transposon subterminal sequences. These sequences are necessary for transposition to occur (Kunze and Starlinger, 1989).

Ds elements, in contrast, lack a functional transposase, and can vary from a minor change in the transposase gene to a total lack of the coding sequence. In some cases, the only thing in common between *Ac* and *Ds* is the terminal inverted repeats.

Several factors regulate transposon activity. Epigenetic changes like methylation seem to play an important role in transposition regulation. For example, the *ddm1* Arabidopsis mutant with reduced methylation levels shows increased transposition of endogenous transposons (Hirochika, H. *et al.*, 2000; Martienssen, R., 1998; Miura, A. *et al.*, 2001). Moreover, "self-regulating" controls seem to be important mechanisms to regulate transposition. For example, an increase in the number of *Ac* elements in the maize genome decreases the frequency of transposition (Lewin, B., 2000).

En/Spm maize transposon system

In the progeny of the plants where McClintock studied the *Ac* and *Ds* elements, she also discovered another transposon system in 1954. She named this new system *Suppressor-*

Mutator (Spm). Similarly to the members of the *Ac-Ds* system, *Spm* also seemed to show the "controlling element" phenomenon (McClintock, B., 1956). On the other hand, the transposon system *Enhancer-Inhibitor* (*En-I),* was also found in maize. This system was found in progeny of plants exposed to the "Bikini" trial of the atomic bomb (Peterson, P. A., 1953). After some years, it was demonstrated that the *En-I* and *Spm-dSpm* transposon systems were genetically equivalent (Peterson, P. A., 1965). As in the case of *Ac* and *Ds* elements, there are mutant *En/Spm* elements that are uncapable of transposing by themselves. These non-autonomous elements were named *Inhibitor (I)* or *defective Suppressor/mutator (dSpm)* elements.

Molecular characterization of the *En/Spm* system revealed that members of the *Spm-dSpm* or *En-I* family have a 13 base pair terminal inverted repeat (Pereira, A. *et al.*, 1985). The sequence of the terminal inverted repeat of this family is different in sequence to *Ac-Ds*. The terminal inverted repeat sequence of *En-I/Spm-dSpm* elements begin with the consensus sequence CACTA. For transposition, other subterminal regions with smaller inverted repeats are required *in cis*. The autonomous element, *En / Spm* , is 8,287 kb long and codes for a functional transposase. Due to alternative splicing, different messenger RNAs are produced from the transposase gene. They have been termed *tnpA, tnpB, tnpC* and *tnpD,* in increasing size order.

tnpA is a 2500 bases messenger that codes for a 621 aminoacid protein. The function of *tnpA* is necessary for excision, but may not be sufficient. The fourth messenger RNA, *tnpD,* contains two additional open reading frames located in the first, long intron of *tnpA*. This messenger is 6000 bases long. Interestingly, it is present at only 1% of the level of *tnpA* mRNA.

The protein that *tnpD* encodes might be involved in the binding and cleavage of the transposon terminal inverted repeats. Some studies indicate that both mRNAs are necessary and sufficient for transposition in tobacco (Frey, M. *et al.*,1990; Masson, P. *et al.*, 1989). There are mutants with deletions of the additional ORFs that show weaker transposition activity (named *Spm-w*).

TnpA is thought to bind DNA while TnpD interacts with TnpA, stabilizing the TnpA-DNA complex. This interaction, highly specific, might allow the alignment of the element ends for transposition (Raina, R. *et al.*, 1998).

Like *Ac-Ds* elements, *En-I/Spm-dSpm* elements produce a footprint upon insertion in the genome. The footprint is the duplication of only 3 base pairs, in contrast with the 8 base pair duplication of *Ac-Ds*.

An interesting feature of *En/Spm* transposons is that they can control the expression of a gene at the site of insertion. A gene with an insertion of an element responding to *Spm* can be also controlled by *Spm*. This control over gene expression can be negative or positive. A recipient locus that suffers inhibition of expression is named *Spm-suppressible* locus. In contrast, a *Spm-dependent* locus requires *Spm* for expression. Interestingly, if the inserted element is a non-autonomous (*I* or *dSpm)* element, suppression or dependence will depend on the presence of a *trans*-acting *En/Spm* (Lewin, B., 2000).

A *dSpm-suppressible* allele can be explained by a *dSpm* insertion in a gene exon. Oddly, in some cases this kind of insertion does not inhibit totally gene expression because sometimes the element can be spliced out from the mRNA. Therefore, the presence of the defective element sometimes reduces but not eliminates gene expression totally. However, when *tnpA* binds to the repeated termini it can block the transcription of the gene.

On the other hand, a *dSpm-dependent* allele can be explained by an insertion outside the coding region of a gene. An insertion near a gene seems to provide an enhancer that can activate the promoter of the gene when *En/Spm* is *in trans*. Both effects, suppression and dependence are the result of the *tnpA* binding to the *cis*-acting terminal sequences of the element. The position of the elements is what makes the effect different (Lewin, B., 2000).

Autonomous *En/Spm* elements are also subject of modifications. *En/Spm* elements can be methylated. Methylations of sequences near the startpoint of transcription cause the inactivation of the element. Inactivated elements are named "cryptic". Because of these modifications, *En/Spm* elements can range from fully active to cryptic. Transient reactivation or conversion to the active state of a cryptic element is possible by interactions with fully active *En/Spm* elements (Lewin, B., 2000).

Homologous members of these two maize families exist in many plant species, though not all have been demonstrated to be mobile. In *Antirrhinum majus* (snapdragon), the *Tam3* elements are quite similar in structure and function to maize *Ac* elements, and code for a 60% aminoacid identical transposase. Likewise, *Tam1* elements, also from snapdragon, show high

similarity to *En/Spm* elements. Their terminal repeats are only different in a single nucleotide. Moreover, they also generate a 3 base pair direct repeat upon insertion and the exons of both elements are similar. Interestingly, *Tam* elements seem to be environmentally regulated. When plants are propagated at 15ºC instead of 25ºC, *Tam* transposition frequency can increase 1000 fold (Ferl and Paul, 2000).

Transposons in Arabidopsis

Transposons are naturally present in all living organisms. Arabidopsis has different transposon families, as shown in **Table I** (Kapitonov and Jurka, 1999). Repetitive sequences in Arabidopsis have been compiled and categorized into 142 groups of putative transposons. Some of them belong to superfamilies that have been studied in other plants and organisms, like those with or without long terminal inverted repeats, miniatures and so on (Le, Q. H. *et al.*, 2000). An example is the transposon family termed *Atenspm1*, that belongs to the maize *En/SpmI* transposon family. This family share the CACTA motives and the 3bp direct repeats that characterize *En/Spm* elements. Interestingly, there is 99% nucleotide identity between the consensus sequence and different *Atenspm* copies. This indicated that these elements have been active relatively recently.

Moreover, there is a second *Atenspm* subfamily, *Atenspm2*, 60-70% identical to *Atenspm1*. Even a third subfamily, *Atenspm3*, has been observed, more similar to *Atenspm2* than to *Atenspm1* (Kapitonov and Jurka, 1999). Interestingly, in the hypometylated *ddm1* mutant, a member of the CACTA family, named *CAC1*, has been shown to transpose and increase copy number at high frequencies (Miura, A. *et al.*, 2001).

Recently, a new transposon family was discovered in Arabidopsis. This new transposon was named *Basho* (after the japanese haiku poet). The site http://soave.biol.mcgill.ca/clonebase/ provides more information.

Interestingly, not all families of transposable elements are equally distributed among different *Arabidopsis* ecotypes. For example, *Tag1*, an element of the family *Ac* or hAT, has been identified in the ecotype Landsberg *erecta*. However, *Tag1* is not present in the ecotypes

Columbia, Ws, neither in some No-0 isolates. Also interesting, the introduction of the non-autonomous element (*dTag*) via *Agrobacterium* in an ecotype containing an autonomous element (*Tag)* causes the appeareance of transposase transcripts (Liu and Crawford, 1998).

Superfamily	Autonomous families	Nonautonomous families	Target site duplication	Terminal inverted repeats
Tc1/IS630	*Atpogo*	*Atpogon1*	TA	CAGTAAAACCTCTATAAATTAATA
		Atpogon2	TA	CAGTAGAAACTCTATAAATTAATA
	?	ATTIRTA1	TA	38-bp: CTCCCTCTGTTTCTRAATAT-
		DT1	TA	38-bp: CTCCCTCTGTTCCTTTTTAT-
hAT	*Tag1* (Tsay et al., 1996)		8-bp	CAATGTTTTCACGCCCGACCCGAA
	Atac1		8-bp	TAGAGtTGTCAagCGGGCCG
	Atac2	*Simplehat1*	8-bp	TAGaGaTGTcAAaCGGgCTg
	Atac3		8-bp	TAGAGATGTTaaTGaTGGGT
En/Spm	*Atenspm1*		3-bp	CACTACAAGAAAA
	Atenspm2		3-bp	CACTACAAGAAAA
	Atenspm3		3-bp	CACTACAAGAAAA
	Atenspm4		3-bp	CACTACAAGAAAA
Harbinger/Is5	*Harbinger*	*Simplegny1*	3-bp	GGTCCTGTTTGTTTGTCCATTTGGA
MuDR	*ATMU1*		10-bp	280-bp: GGGAAAAAACCCTAA-
	ATMU2		10-bp	290-bp: GGGGAAAAAGCTTAA-
	AT9MU1	*AT9NMU1*	9-bp	150-bp: GGGATTTAATGAAAA-
		Brodyagal	10-bp	350-bp: GGGAAAAATGTCAAA-
		Bomzh1	10-bp	500-bp: GGGAAAAATGTCATT-
Arnold	*Arnold1-Arnold4*	*Arnoldy1*	9-bp	–
		Arnoldy2	9-bp	–
	Vanda11		9-bp	–
	Vanda12		9-bp	–
		ATN9_1	9-bp	–
ATREPs	?	ATREP1	–	–
		ATREP2	–	–
	Surzycki and Belknap (1999)	ATREP3	–	–
	Kapitonov and Jurka (1999)	ATREP4	–	–
		ATREP5	–	–
		ATREP6	–	–
		ATREP7	–	–
		ATREP8	–	–
		ATREP9	–	–
		ATREP10	–	–
?	?	ATTIRX1A	TAA	GGGGATGTATTCAATC
		ATTIRX1B	TAA	GAGGGTGTATTCAATC
		ATTIR16T3A	TWA	GGGGGTGTTATTGGTT

Transposons without references have been identified by Kapitonov and Jurka (1999). Unknown autonomous elements are marked by '?'. Transposons without target site duplications and terminal inverted repeats are marked by '–'. TIRs longer than 25 bp are not complete for the lack of space, but their lengths are indicated.

Table I. Transposons present in Arabidopsis (Kapitonov and Jurka 1999).

Further study might render the different transposons in Arabidopsis suitable for insertion mutagenesis (Le, Q. H. *et al.*, 2000).

Heterologous Transposon Tagging

Insertional mutagenesis with transposons is named Transposon Tagging. For Transposon Tagging purposes, the transposons to use can be endogenous –belonging to the plant in study-. For example, one could choose *Tag1* or *Basho* in Arabidopsis.

However, transposons from different organisms, normally from other plants, can be also used. The use of heterologous transposons offers some advantages over endogenous transposons. For example, heterologous transposons can be engineered to carry marker genes. The use of marker genes inside the transposons makes them easier to follow genetically. Additionally, different promoters can be fused to the transposase gene. By using different promoters, the expression of the transposase could be conveniently modulated or enhanced. Interestingly, though a few years ago the use of those inducible (heat-shock for example) or specific (pollen) promoters to regulate transposase activity was proposed by different scientists, none has been reported to be effective to date (Feldmann *et al.*, 1994).

Moreover, engineered heterologous transposons provide a way of obtaining stable mutations, as described later in the text (Tissier, A. *et al.*, 1999).

Two very commonly used heterologous transposon systems for insertional mutagenesis are the maize transposon families *Ac/Ds*, and *Spm-dSpm* or *En-I*. These two maize transposon systems have been extensively studied and used in different plants for insertional mutagenesis. With these transposon systems diverse genes have been succesfully isolated in petunia, tomato, potato, tobacco, flax, and Arabidopsis, among other plants.

Initially, the development of heterologous transposon systems for transposon tagging in Arabidopsis began with the use of the *Ac-Ds* system[2]. When introduced for the first time, it was found to have a transposition frequency of 0.2-0.5% (Dean, C. *et al.*, 1992; Schmidt, R. *et al.*, 1995; Van Sluys, M. A. *et al.*, 1987). Because this transposition frequency is low, the *Ac* system was not suitable for gene isolation. However, the elimination of a *Nae* I restriction fragment from the untranslated 5' end of the transposase gene increased transposition frequency 10 fold. The *Nae* I restriction fragment had a CpG rich sequence that was sensitive

[2] For example, using unmodified autonomous *Ac* elements, either alone or in combination with *Ds* elements.

to methylation and its elimination made the system useful for tagging purposes (Bhatt, A. *et al.*, 1996).

Additionally, the frequency of germinal excision was increased by using stable transposase sources to mobilize *Ds* elements. These stable transposase sources were literally immobilized autonomous elements without terminal inverted repeats (Bancroft, I. *et al.*, 1992; Greveldings, C. *et al.*, 1992; Honma, M. A. *et al.*, 1993; Long, D. *et al.*, 1993b; Swinburne, J. *et al.*, 1992). Transposition frequences over 30% could be obtained by using the strong CaMV 35S promoter to express the transposase gene. However, such an increased transposition frequency had a major drawback: it led to early transposition and only a few independent – different- *Ds* insertions were observed in the next generation (Long, D. *et al.*, 1993b). Nevertheless, the *Ac-Ds* system was then used to generate insertional mutants (Bancroft, I. *et al.*, 1993; Long, D. *et al.*, 1993a). Further modifications to the *Ds* elements have also been done. One example of these modifications was the addition of a CaMV 35S promoter to one of the transposon ends. The promoter pointed outwards, in such a way that it could promote transcription of genes adjacent to the insertion. This "read-out" promoter system could cause dominant mutants (Wilson, K. *et al.*, 1996). Another example of *Ds* modifications is the addition of a marker gene inside the element. Promoterless marker genes inside the insertion become promoter traps. The addition of splicing sites to the promoterless marker gene is used to build gene traps. Similarly, a minimum promoter-marker gene fusion inside the element becomes an enhancer trap (Fedoroff and Smith, 1993; Springer, P. S. *et al.*, 1995; Sundaresan, V. *et al.*, 1995; Tsugeki, R. *et al.*, 1996; and Martienssen, R. A., 1998).

En/Spm elements have also been employed for transposon tagging purposes in Arabidopsis (Pereira, A., 1998). Initially, experiments based on the use of unmodified *En/Spm* elements in tobacco led to the use of *En/Spm* in Arabidopsis (Cardon, G. H. *et al.*; 1993a, Pereira and Saedler, 1989). These experiments showed that the *En/Spm* transposition frequency in Arabidopsis was higher than in other host plants such as tobacco and potato (Cardon, G. H. *et al.*, 1993b; Frey, M. *et al.*, 1989). Moreover, *En/Spm* transposition frequency was also higher than the transposition frequency of the *Ac* element in Arabidopsis.

The transcription level and relative abundance of the two *En* mRNAs, *tnpA* and *tnpD*, seemed to be the reason behind this higher frequency. In Arabidopsis, *En* mRNAs transcription level and relative abundance were more similar to maize than in solanaceous

species. Likewise, *En/Spm* transcripts were closer to the levels in maize than *Ac* in Arabidopsis. Another advantage of *En/Spm* is that it has an average germinal transposition frequency of 7.5% over many generations. Additionally, and quite desirable for tagging strategies, independent *En/Spm* transpositions are often observed (Aarts, M. G. M. *et al.*, 1995).

With heterologous transposons, the number of elements used can vary. For a "one element" system, a single autonomous, unstable, element is used. On the other hand, a "two component" system employs a non-autonomous element in combination with a transposase source (Aarts, M. G. M. *et al.*, 1995). Even a "three element" system has been described. In that system, based on the *En/Spm* transposons, the two *En* transcripts *tnpA* and *tnpD* were fused to the CaMV 35S promoter. The third component was an *I/dSpm* non-autonomous element that could transpose due to the presence of *tnpA* and *tnpD* in the genome (Cardon, G. H. *et al.*, 1993a).

The "two component" system seemed to be convenient for transposon tagging in Arabidopsis. Aarts *et al.* used an immobile *En* autonomous element fused to a CaMV 35S promoter as part of the system. By eliminating the terminal inverted repeats, the element becomes incapable of self-mobilization, though still coding for a functional transposase. The *I/dSpm* non-autonomous element was the other component of the system. Like in the "three element" system, the non-autonomous element required the presence of the functional transposase, in this case in the immobilized autonomous element, to transpose. This system allows the stabilization of the non-autonomous element when the immobile autonomous element is not present (Aarts, M. G. M. *et al.*, 1995).

The "two component" system developed by Aarts *et al.* proved to be an efficient gene tagging tool. *CER1* and *MS2* were some of the first Arabidopsis genes isolated using this "two component" *En/Spm* system (Aarts, M. G. M. *et al.*, 1993; 1995). The system showed a frequency of unique insertions (independent transposition frequency) of 4.4 to 29.2% in different populations. Additionally, 30% of the elements were estimated to transpose to linked and 70% to unlinked chromosomal positions (Aarts, M. G. M. *et al.*, 1995). The high frequency of independent transpositions is a highly desirable feature for gene tagging.

Moreover, the ability to transpose to unlinked loci, rendering selection for segregated transpositions efficient, make the *En-I* system particularly suitable for the selection of stable transposants.

The establishment of the *En/Spm-I/dSpm* system as an efficient tool for heterologous transposon tagging in Arabidopsis led to the development of large mutant populations, some made using full-size autonomous *En* elements, producing unstable insertions, and some, based in "two component" system, bearing stable insertions (Wisman, E. *et al.*, 1998; Speulman, E. *et al.*, 1999; Tissier, A. *et al.*, 1999). The number of total insertions per individual varies among the developed populations. For example, some populations have more than 10 elements per plant (Speulman, E. *et al.*, 1999; 2000). Additionally, as for other kind of insertional populations (Ito, T. *et al.*, 1998), insertional databases have been developed for some *En/Spm* populations (Speulman, E. *et al.*, 1999, Tissier, A. *et al.*, 1999).

One of these *En/Spm* populations represents a significant development in transposon technology. It uses greenhouse positive and negative selectable markers to select for stable transpositions, which are also employed in the research reported here (Tissier, A. *et al.*, 1999). This system makes use of two selectable markers. The positively selectable marker is the *bar* gene conferring resistance to the herbicide Basta (De Block, M. *et al.*, 1987; Thompson, C. J. *et al.*, 1987). Conversely, the negatively selectable marker is the *su1* gene that converts the pro-herbicide R7402 (DuPont) into the herbicide sulfonylurea inhibiting or reducing the growth of plants that contain it (O'keefe, D. P. *et al.*, 1994). The selection for stable transposition can be performed in soil in the greenhouse, avoiding the need for media and seed sterilization. Both markers are carried on a single construct that also bears the non-autonomous element and a source of transposase (an *En*/*Spm* element that lacks the terminal inverted repeats, thus immobile). The selectable *bar* gene, is placed inside the non-autonomous *I/dSpm* element. On the other hand, the negatively selectable marker *su1* is in the adjacent T-DNA allowing selection against the transposase source. The application of both Basta and R7402 to the progeny seedlings of transformants containing both elements leads to selection of plants bearing stable germinal insertions. In **Table II**, the effects of the sprayed compounds in each progeny genotype can be observed.

	I (bar)	En (su1)	=
A	✓	☠	☠
B	-	☠	☠
C	✓	-	☺
D	-	-	☠

Table II. Effect of spraying seedlings of different genotypes with Basta and R7402. A, the presence of the *bar* and *su1* genes cause the death of the seedling. B, *bar* absence and *su1* presence result in death. C, the absence of both genes cause death. D, *bar* presence and *su1* absence is the only possibility that allows the seedling to survive.

In the present work, the double selection system was applied to develop and generate *I/dSpm* inserts containing a multiple enhancer element for large-scale activation tagging.

Activation Tagging

The Arabidopsis genome sequence revealed about 25,500 genes at least that have been predicted by a variety of bioinformatics tools (Arabidopsis Genome Initiative, 2000). However, the exact biological role for most genes is still unknown, requiring specific biological experiments to uncover their function. Changes in expression of the genes brought about by forward as well as reverse genetics methods are expected to provide tools to describe their function. Gene knockouts abolishing gene function, using diverse mutagens, are classically employed to reveal the biological role of specific genes. However, recent reverse genetics analysis has shown that most gene knockouts do not reveal informative phenotypes under "standard" conditions, although some knockouts can reveal phenotypes under specific environmental conditions or when analyzed at different growth stages (Bouché and Bouchez, 2001; Boyes *et al*., 2001).

A possible explanation for this 'phenotype gap' comes from the analysis of the Arabidopsis genome (The Arabidopsis Genome Initiative, 2000). About two-thirds of the genome is duplicated in the form of large chromosomal segments, although during evolution some of these duplicated genes have diverged to produce new functions. In addition, about 4000 genes are tandemly repeated as two or more copies. These duplicated and "redundant" genes would therefore not be expected to display a knockout mutant phenotype.

A strategy to uncover the function of "redundant genes" involves the use of activating sequences, or "enhancers", inside the insertion. Enhancers positively influence gene expression even when located at a considerable distance to the target promoter (Lewin, B., 2000). They can promote the overexpression of endogenous genes, generating dominant mutations in contrast with knockout recessive mutations. The use of enhancers inside a tag is denominated "Activation Tagging". **Table III** shows the advantages of Activation tagging over classic gene disruption approaches.

Activation tagging in plants was first proposed as a novel gene isolation method (Walden, R. *et al.*, 1994) and has been effectively applied using T-DNA inserts (Kakimoto, T., 1996; Kardailsky, I. *et al.*, 1999; Borevitz, J. *et al.*, 2000; Lee, H. *et al.*, 2000; Ito and Meyerowitz, 2000; van der Graaff, E. *et al.*, 2000; Weigel, D. *et al.*, 2000; Huang, S. *et al.*, 2001; Zhao, Y. *et al.*, 2001) and the *Ac-Ds* transposon system (Wilson, K. *et al.*, 1996). These inserts are designed to carry strong activating sequences that can act on genes adjacent to the insertion site, and modify their expression. The well-characterized CaMV 35S enhancer or promoter sequences (Odell, J. T. *et al.*, 1985) have been used as transcriptional activators in this type of insertion system. Their primary function has been to over-express the tagged genes to reveal dominant gain-of-function phenotypes. In a number of cases, the CaMV 35S enhancer used in the T-DNA inserts acts by quantitatively increasing the original expression pattern of a gene, rather than by ectopic or constitutive over-expression of genes (van der Graaff, E. *et al.*, 2000; Neff, M., *et al.* 1999).

The most recent developments include transactivation systems. A report in the 2002 Arabidopsis meeting in Sevilla by Scheres and collaborators is an example. Their transactivation system uses cell or tissue specific promoters to regulate the expression of a

transactivator (GAL4:VP16). By placing a T-DNA carrying UAS (Upstream Activating Sequences, to which GAL4 binds) randomly in the genome, target genes are overexpressed in a pattern determided by the transactivator. Additionally, the UAS carrying T-DNA vector acts also as a promoter trap because it carries a promoterless *gus* marker gene. Because of this last feature, the system has been named "Promoter Trap based Activation Tagging" or "PoTATo tagging". This system shares some features with the previously available transactivation system developed by Haseloff. An animated explanation of that system is available at http://www.plantsci.cam.ac.uk/Haseloff/GAL4/GALtrapscheme.html.

Knockout tagging	• Gene tagging causes recessive phenotypes mostly. Therefore, homozygous plants are required to observe mutant phenotypes. • Knock-out of genes with overlapping functions with other genes ("redundant" genes or genes belonging to the same family) might not display an evident mutant phenotype. • Gene knockout may lead to lethal phenotypes, making impossible the study of gene function in different developmental stages.
Activation tagging	• Activation tagging causes dominant mutations. However, because in this strategy the activating sequences are in a tag that inserts in the genome, gene knock-outs can also be obtained. • Gene overexpression may possibly lead to evident phenotypes of genes with overlapping functions. • Gene overexpression may possibly lead to viable individuals where gene knockout leads to lethal phenotypes. • Activation tagging populations are particularly suited to perform positive selection screens. For example, resistance or tolerance to chemical, physical or biological stresses. • Activation tagging can also be successfully used for the study of metabolic pathways (Van Der Fits and Memelink, 2000). • For biotechnological purposes, activation tagging has a high potential for the direct application of interesting genes. In contrast to knock-out phenotypes where the inactivation of an endogenous gene is necessary, genes found by activation tagging require to be overexpresed.

Table III. Advantages of Activation tagging over knockouts.

Haseloff's system comprises a T-DNA that includes the GAL4:VP16 transactivator. Acting as a promoter trap, the transactivator expression is determined by adjacent plant regulatory sequences. Aditionally, the T-DNA contains a UAS:*GFP* marker gene that will reveal the expression of the transactivator. Plants with interesting GFP specific expression patterns can be selected. Another vector containing a gene of interest fused to the UAS can be introduced in the selected plants to obtain the same specific expression patterns for the gene of interest (Kiegle, E. *et al.*, 2000).

A refined activation tagging system was reported by Zuo and colleagues. It is a chemically induced transactivation system, and consists of two main components. One is a fusion of three parts: a DNA binding domain (Lex A bacterial repressor, X), an activator (VP16, V), and the regulatory region of the human estrogen receptor (E). This fusion, named VXE, is controlled by the strong constitutive promoter G10-90. The second component is an octamer of the Lex A operator fused upstream of a -46 35S minimal promoter. Both components are in one single T-DNA. Located in the T-DNA border and oriented outwards, the second component can be used to cause the overexpression of target genes. Upon treatment with estrogens, the VXE fusion protein is activated and can promote such overexpression (Zuo, J. *et al.*, 2002).

Activation tagging possesses inherent advantages that can overcome some of the limitations of gene disruption, since this technique generates gain-in-function dominant mutations. With a strong constitutive enhancer such as that derived from the CaMV 35S promoter, gene expression can be increased above normal levels (Neff, M. *et al.*, 1999), or even ectopically. In this way even redundant genes might display an overexpression phenotype if their product is limiting or a change in concentration of gene products creates an imbalance that is manifested as a phenotype. These phenotypes can either directly reveal the gene function or provide a clue to the pathway in which the gene is involved. Moreover, it might be possible to study the function of essential genes that display lethal knockout phenotypes by using activation tagging strategies. The frequency of lethal mutations, essential to the haploid gametophyte and/or the early diploid embryo, is predicted to be around 1 gene every 100 kb (Martienssen, R.A., 1998) amounting to about 1300 genes, some of which might be revealed by gain-in-function phenotypes. For biotechnological applications, the controlled

overexpression of genes with specific functions could be easier to employ than gene suppression or knockouts in crops of interest (Huang, S. *et al.*, 2001).

Most activation tag collections have been generated using the *Agrobacterium tumefaciens* T-DNA as a tag (Ito and Meyerowitz, 2000, van der Graaff, E. *et al.*, 2000, Weigel, D. *et al.*, 2000). Detailed analysis of a number of mutants identified using T-DNA activation tagging has recently contributed to a better understanding of this gene activation system and also resulted in the identification of novel genes. One such example is the discovery of the role of members of the family of flavin monooxygenases in auxin biosynthesis (Zhao, Y. *et al.*, 2001), which had not been characterized previously. In Madagascar periwinkle (*Catharantus roseus*), a positive selection system was used to isolate the ORCA3 gene, which codes for a jasmonate-responsive transcriptional regulator of primary and secondary metabolism (Van Der Fits and Memelink, 2000). Kakimoto could identify genes whose overexpression mimicks cytokinin activity by using activation tagging (Kakimoto, T., 1996). Furthermore, this strategy has also been employed as a novel approach to isolate suppressor mutants of known mutant phenotypes in Arabidopsis (Neff *et al.*, 1999a). The use of activation tagging has also been reported to aid in the discovery of uncharacterized structural and regulatory genes of flavonoid biosynthesis (Dixon and Steele, 1999). Additionally, the floral inducer *FLOWERING LOCUS T* and a MYB regulator of phenylpropanoid biosynthesis were isolated in Arabidopsis also using this strategy (Kardailsky, I. *et al.*, 1999) (Borevitz, J. *et al.*, 2000). Similarly, a gene affecting petiole development, *LEAFY PETIOLE*, was isolated using activation tagging as well (van der Graaff *et al.*, 2000).

Moreover, by using their inducible XVE activation-tagging system, Zuo et al. (Zuo, J.*et al.*, 2002) were able to find an unknown function of the previously studied *WUSCHEL* gene. The new *WUSCHEL* gain-of-function phenotype was characterized for the formation of somatic embryos without exogenously applied hormones. The inducibility of their system allowed this particular phenotype to be studied, because constitutive *WUSCHEL* overexpression gave raise to impaired development.

Further studies and developments are encouraged, as all these examples demonstrate the versatility of the applications of activation tagging systems in the study and discovery of gene functions.

Because it causes dominant mutations, activation tagging is particulary suited to perform positive screens. For example, looking for genes for which overexpression may result in resistance or tolerance to environmental stresses. Environmental stresses can include those of chemical, physical or biological nature. Not only tolerance to stresses, but new interactions and responses to diverse stimuli (again, chemical, physical or biological) might be found. As shown by the examples indicated before, this system can also be an important tool in the search and study of genes involved in basic or "secondary" metabolic pathways (Van Der Fits and Memelink, 2000). In short, gene functions involved in development, signaling, regulation, tolerance or resistance, metabolism and so on may be discovered with this system.

One of the major disadvantages of T-DNA-based activation systems is the creation of complex integration patterns and chromosomal rearrangements near the insertion site (De Neve, M. *et al.*, A., 1997; Gheysen, G. *et al.*, 1990; Nacry, P. C. *et al.*, 1998). This could affect either the activity or the interaction of the T-DNA integrated enhancer sequences with the adjacent plant DNA. The use of single stable transposon insertions as the activation tags could overcome this problem and would also allow targeted tagging of genes near the original insertion site. This could give more information about specific members of linked gene family clusters.

The purpose of this work was to develop and test a transposon system that could produce plants containing low numbers of stable insertions. If functional, it could open the possibility for activation tagging strategies in plants lacking efficient transformation methods.

An advantage of the activation tagging system is the potential for direct application of an overexpressed gene that causes a desired trait. Given the broad spectrum of applications that this system can have, one application example would be to use the system to search for genes that can enhance the growth and hence the yield of plants (and desirably crops) in poor soils. Therefore, the developed system was used to make preliminary searches for genes

involved in postembryonic root development and phosphate uptake, both important features involved in plant nutrient utilization. The following section provides information about current knowledge in the field of root development and phosphate uptake studies.

Part II. POSTEMBRYONIC ROOT DEVELOPMENT AND PHOSPHORUS NUTRITION STUDIES

ROOTS

Whereas animals and unicellular organisms lack them, plants rely on their roots to absorb water and mineral ions from the soil. Moreover, roots provide anchorage to plants. Other root functions are storage and conduction. Roots also synthesize hormones, for example cytokinins and gibberellins. Furthermore, some secondary metabolites of human interest are produced in roots, like nicotine (Raven, P. H. *et al.*, 1999).

Many root studies in the last years have been done using Arabidopsis. This is due mainly to the ease of Arabidopsis genetic and molecular analysis, and to Arabidopsis root size that allows growth of many plants in petri dishes and makes root studies amenable. Arabidopsis root studies have led to the isolation of a number of interesting developmental mutants. Root development in this model plant is similar to other angiosperms. However, in contrast to other dicots like alfalfa, dandelion or carrot, a taproot is not formed in Arabidopsis. Instead, lateral roots develop from the primary root within eight to ten days after germination (Schiefelbein and Benfey, 1994).

The embryo of Arabidopsis can be divided in 3 regions: apical, central and basal portion. Root develops from the basal portion. Determination of the lower portion of the apical-basal axis depends on the *MONOPTEROS* and *GNOM* genes (Aesbacher, R. A., 1994). The root apical meristem is formed during embryogenesis. When the seed is mature, all the main root layers are already established. Following seed imbibition, the root apical meristem is activated after radicle emergence from the seed coat (Schiefelbein and Benfey, 1994).

The primary root has a very simple organization. Interestingly, differenciated cells in roots are found in files. These files can be traced back to a small number of cells, named *initials*. Moreover, there are cells at the beginning of the files that divide infrequently. These cells were termed the *"quiescent center"* and they do not participate actively in forming the

differenciated cells that emanate from the apex. In contrast, *initials* are actively dividing and are the earliest detectable progenitors of more differenciated cells. However, quiescent center cells are required to organize a newly proliferating root, as surgical dissection experiments indicate. Together, the initials and quiescent center form the root meristem (Aesbacher, R. A., 1994). Mutations in the *HOBBIT* gene affect specifically the formation of the root meristem while leaving the rest of the embryonic root intact (Willemsen, V. *et al.*, 1998).

The root meristem has fewer cells and a simpler structure than the shoot apical meristem. In Arabidopsis, the cell division pattern in the root meristem has been well characterized. A defined lineage is followed in Arabidopsis root development, and an almost nearly complete fate map for every cell type can be drawn. The quiescent center seems to play an importat role in maintaining meristematic activity. It seems to maintain the identity of the surrounding initial cells by inhibiting their differenciation (**Figure 20**). Surrounding the quiescent cells are initials for vascular bundle, pericycle, cortex, endodermis, epidermis, columella and root cap.

Interestingly, root cap meristematic cells give raise to cell layers that progressively differenciate through specialized stages, and finally separate from the cap periphery. These detached cells are termed "border" cells, and they produce specific metabolites, like antocyanins and antibiotics in some plants. They also have been proposed to protect the root tip (Hawes, M. C. *et al.*, 2000).

The mature part of the root of Arabidopsis has a radial pattern of cell arrangement. Normal cell divisions in the meristem give rise to this fixed radial cell pattern. From inside to outside, the cell layers are vasculature, pericycle, endodermis, cortex and epidermis. The pericycle and vasculature constitute the stele. Cells in each layer originate in the initial cells in the root meristem, and follow a stereotyped sequence of divisions. From two daughter cells, one remains as an initial, and the other differentiates. Some cell files of the root have the same clonal origin. Epidermis and lateral root cap cells share the same initial, as endodermis and cortex do (**Figure 1,** Nakajima and Benfey, 2002).

Interestingly, in fern species, like *Azolla pinnata*, root development originates and is maintained by a single apical cell. Moreover, cell-cell interactions do not guide cell differentiation in *Azolla pinnata*, but rather cell-autonomous genetic programming. In contrast, cell differentiation is mainly directed by cell-cell interactions in Arabidopsis (Aesbacher, R.

A., 1994). This cell-cell communication allows respecification of cell fate when initial cells are injured. It has been suggested that the correct pattern formation requires a "top-down" flow of positional information form mature cells to the initial cells. However, also radial flow of information during root patterning has been shown to occur (Nakajima and Benfey, 2002). This radial flow was observed in studies with *scr (scarecrow)* and *shr* (*short root)* mutants. These mutants have a modified root pattern, where only one layer is formed instead of two. *shr* mutants lack endodermis while *scr* mutants have only one layer with both cortical and endodermal features. Both genes belong to the GRAS family of transcription factors and are required for the longitudinal division of the cortex/endodermis initial daughter cell. Using SHR - *GFP*, and the *SCR* promoter fused to the *SHR* gene, Nakajima and colleagues showed that SHR moves from the stele to the nucleus of the adjacent layer of cells. Moreover, they showed that SHR acts as a signal from the stele and as an activator of endodermal cell fate and SCR-mediated division (Nakajima, *et al.*, 2001)

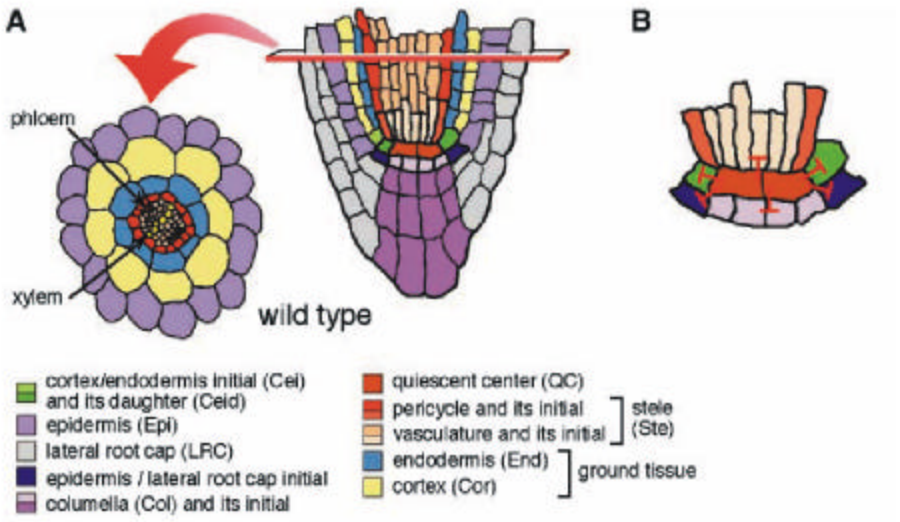


Figure 1. Schemes of wild type Arabidopsis root structures (Taken from Nakajima and Benfey 2002). A, wild type root (Dolan *et al.*, 1993). Cell types are given in the key at bottom. Abbreviations shown in parentheses are used in all subsequent figures. B, QC cells function as an organizing center of the RM by inhibiting the differentiation of surrounding initial cells (stem cells) (van den Berg *et al.*, 1997; Umeda *et al.*, 2000).

In a growing root, besides the meristematic zone, where the basic cell types are produced and organized, two other zones can be distinguished. One is the elongation zone, where the cells divide, expand and differenciate. The other zone has differenciated cells and is called specialization zone.

The main parameters that will determine the final root shape are the time of cellular division, the orientation of the division plane, and the cellular expansion. These three processes are deeply related (Aesbacher, R. A., 1994). Examples of root morphogenetic mutants are the *rws1, 2* and *3* conditional mutants, that show radial swelling at restrictive temperature. Mutants with preferential cell expansion in specific cell layers are *cobra, lion's tail, pom-pom* and *sabre* (Schiefelbein and Benfey, 1994).

As in all plant cells and the other plant organs, root growth and development relies on a fully functional cytoskeleton. Microtubules and microfilaments are the main components of the cytoskeleton, along with other elements like motor proteins (Barlow and Baluska, 2002). Profilin is one of those "other" elements. It is an ubiquitous actin-binding protein involved in the cytoskeleton organization of eukaryotes. Interestingly, profilin underexpression provokes seedlings to have shorter hypocotyls and roots than wild type. In contrast, profilin overexpression caused longer roots and root hairs (Ramachandran, S. *et al.*, 2000).

Root hairs

Root hairs are tubular extensions from epidermis cells, the outermost root layer, that are developed in a defined region near the root apex. They facilitate the uptake of water and minerals. The total surface that a root can cover is important in nutrient uptake, and root hairs can increase this area. They can even constitute about 77% of the contact area (Parker, J. S. *et al.*, 2000).

Hair formation requires controlled cell shape changes to occur. Because root hairs are dispensable and plants that lack them are viable, genetic analysis can be easily done. Root hairs are extensions from epidermis cells. However, not all epidermal cells will form hairs. Whether an epidermal cell will or will not form a hair, is due to different mechanisms depending on the plant species. Hairy cells can be defined by asymmetric division or by their relative position with respect to cortex cells.

For example, in Arabidopsis, root hairs are formed only from epidermal cells that are positioned above a transversal wall that separates two cortical adjacent cells. These specialized epidermal cells are named "trichoblasts". Besides this "postional control", the number of hairy and hairless cells seems to be predetermined in Arabidopsis.

Steps in root hair formation are hair initiation, bulge formation, tip growth and growth cessation (Schiefelbein, J. W., 2000). Genes like *CPC (CAPRICE), TTG (TRANSPARENT TESTA GLABRA), GL2 (GLABRA2)* and *WER (WEREWOLF)* control the pattern of root hair formation (Galway, M. E. *et al.*, 1994; Lee and Schiefelbein, 1999; Masucci and Schiefelbein, 1996; Wada, T. *et al.*, 1997).

Root hairs elongate by root tip growth. This kind of tip growth is a sort of polarized cell expansion that also occurs during the growth of pollen tubes, fungi hyphae and other tubular shaped cells. Some genes, like the above mentioned *RDH1, 3, 4* and *TIP1, CAN OF WORMS* and *WAVY* participate in this process (Grierson, C. S. *et al.* 1997; Schiefelbein, J. W., 2000). It has been suggested that these genes can affect tip growth factors. Those factors include Ca^{2+} fluxes, or cytosqueleton components. Actin microfilaments are necessary for vesicle transport, and microtubules have been shown to be required for the normal polar root hair growth. However, microtubules are not necessary for the early bulge outgrowth. Instead, dense F-actin meshworks accumulate at this early stage. The installation of the actin-based tip growth machinery occurs only after expansin-associated bulge formation, and it requires assembly of profilin-supported dynamic F-actin meshworks (Baluska, F. *et al.*, 2000). Indicating the importance of the actin cytoskeleton in root hair growth, ACTIN2 has recently been shown to be essential for bulge site formation and tip growth during root hair development (Ringli, C. *et al.*, 2002).

Interestingly, genes involved in root hair growth are related to hormone or light perception as well. Mutants defective in auxin and/or ethylene responses can also have mutant root hair phenotypes. Based in the study of those mutants, auxin and ethylene are believed to be positive regulators of root hair elongation. Perhaps, they play a role in the extent of root hair tip growth determination (Schiefelbein, J. W., 2000). Additionally, *rdh6* mutants have been shown to have alterations in root hair initiation through an auxin and ethylene associated process (Masucci and Schiefelbein, J.W., 1994). Some examples of hormone related genes that cause root hair alterations when mutated are *CKR1*, resistant to cytokinins, *DWF*, auxin resistant and *AXR2*, auxin, ethylene, and abcisic acid resistant. The ethylene-resistant *etr1*

mutant shows, though not extreme, alterations in the site of root-hair emergence. Light perception related genes are also involved in root hair growth. *HY3* is a phytochrome B gene that produces longer root hairs than normal in the plant when grown in the light, when mutated. *phyB* mutants have also longer roots. Another example is *HY5*, that produces longer root hairs than the wild type when mutated. About twice as long root hairs, they might be caused by delayed cessation of growth (Aesbacher, R. A., 1994; Schiefelbein and Benfey, 1994).

Lateral Roots

Lateral roots in Arabidopsis are derived from pericycle founder cells positioned adjacent to the two protoxylem poles (Blakely, L. M. *et al.*, 1982). Lateral root development proceeds through initiation and emergence phases. Seven developmental stages that precede lateral root emergence have been defined by Malamy and Benfey (Malamy and Benfey, 1997).

Generally, lateral roots do not form near the root tip. An inhibitor has been proposed to explain this lack of lateral root formation near the root tip. The growth of lateral roots means that the root primordium must grow through the cortex and epidermis to emerge to the outside. Microtubules are very important in the control of cellular expansion, this control is lost when microtubules are depolymerized (Aesbacher, R. A., 1994). Once the primary root is growing, the formation of lateral and adventitious root meristems is believed to occur from differentiated cells that must re-differentiate to initiate a new meristem (Malamy and Benfey, 1997). However, the possibility of the lack of previous differentiation has also been suggested (Dubrovsky, J. G. *et al.*, 2000).

Interestingly, mutations resulting in reduced lateral roots numbers seem to affect either synthesis or hormone perception. Lateral root formation and/or emergence are related to auxins, for example. Treatment of developing roots with auxin causes, at high concentrations, development of higher number of lateral roots while NPA, an auxin transport inhibitor, causes the opposite effect. Recently, auxin was shown to mediate cell cycle activation during early

lateral root initiation (Himanen, K. *et al.*, 2002). Mutants with increased levels of endogenous auxin have many lateral roots. Some examples of this are the mutants *alf1, sur1* and *rty (aberrant lateral root formation1, superroot1,* and *rooty* respectively) (Boerjan, W. *et al.*, 1995). On the other hand, defects in auxin transport or sensitivity cause reduced lateral root formation. Auxin resistant mutants, *axr4* and *tir1,* and in the auxin influx carrier, *aux1,* show the latter phenotype (Gray, W. M. *et al.*, 1999; Hobbie and Estelle, 1995; Ruegger, M. *et al.*, 1997).

Mutations in some genes that belong to the Aux/IAA family have been found to cause reduced or lack of lateral roots. Aux/IAA proteins were originally identified as rapidly induced by auxin. Later Aux/IAA family members were found based on sequence similarities to previously discovered members or in yeast two-hybrid assays with these older members (Reed, J. W., 2001). These proteins have four domains and have been shown to interact with ARFs (auxin response factors). Many of these proteins are rapidly degraded *in vivo.* Mutations in domain II render these proteins stable and provoke dominant phenotypes (Rogg, L. E. *et al.*, 2001). Interestingly, Aux/IAA mutants have been shown to be active repressors. Moreover, their activity and stability are modulated by auxin (Tiwari, *et al.*, 2001). Two mutants with reduced numbers or lack of lateral roots belong to this gene family. *solitary root* mutants lack lateral roots and have reduced sensitivity to auxin. The phenotype can not be rescued by applying the hormone. *Solitary root* has a mutation in *IAA14* (Fukaki, H. *et al.*, 2002). On the other hand, *iaa28* mutants have defects in lateral root initiation (Rogg, L. E. *et al.*, 2001). Both mutations are dominant and affect domain II.

Interestingly, Casimiro and colleagues showed that root basipetal auxin transport (toward the root-shoot junction) is required during initiation phase of lateral root development. Conversely, acropetal (to the root apex) auxin transport is required during the emergence phase (Casimiro, I. *et al.*, 2001).

Influence of hormones in root development

Hormones are involved in root hair, and in primary and lateral root growth and development. As indicated in the sections above, many hormone-synthesis or perception

mutants have altered root phenotypes. More examples are provided in this section. Many studies have shown the influence of the plant hormone auxin (Indolacetic acid, IAA) in root development.

Auxin influences not only cell division and differenciation but also cell elongation. It controls many developmental aspects in plants. Auxin resistant mutants (*axr*) show diverse root phenotypes that range from increased root length and reduced root branching (*axr1)*, gravitropism problems (*axr2, axr4, aux1)*, to root proliferation (*axr3*) (Estelle and Klee, 1994).

Sabatini and colleages linked the distribution of auxin to patterning of the root apical region. They used the DR5 - *gus* line, where the DR5 promoter is a synthetic auxin-responsive promoter (Ulmasov, T. *et al.*, 1997). Using mutants (like *pin-formed1,* affected in a putative auxin efflux carrier) and auxin transport inhibitors, they observed changes in auxin distribution that were accompanied by respecification of cell fates and also modification of cell division programs (Sabatini, S. *et al.*, 1999). However, specification of cell fate depended on the relative position of the auxin concentration maximum with respect to the vascular tissue, rather than the absolute auxin concentration.

Auxin transport inhibitors affect also root gravitropism and lateral root initiation, among other processes. Geldner and colleagues showed that inhibitors block the auxin efflux carrier candidate PIN1 cycling and vesicle trafficking (Geldner *et al.*, 2001).

Lateral root development is also influenced by auxin. Proteolysis mediated by ubiquitin plays an important role in auxin-controlled development. Aux/IAA proteins are transcriptional repressors in response to auxin. Their degradation is thought to be promoted by a complex termed SCF (Skp1/Cullin/F-box). Some of the genes that help in the assembly of the active SCF complex are *AXR1* and *TIR.* Downstream of *TIR1*, the transcription activator NAC1 acts to transduce the auxin signal for lateral root development. Xie and collaborators showed that an Arabidopsis homologue of the SINA RING-finger protein from *Drosophila*, termed SINAT5 has ubiquitin protein ligase activity and can ubiquitinate NAC1 (Xie, Q. *et al.*, 2002). However, mutations in the RING motif of SINAT5 result in loss of ubiquinating activity. Interestingly, overexpression of SINAT5 produces reduced lateral root numbers. On the other hand, if a dominant-negative mutant is overexpressed, increased lateral root numbers are observed. Furthermore, these phenotypes correlate with NAC1 *in vivo* expression. If a proteasome inhibitor is used, NAC1 expression is increased. This data indicates that SINAT5

ubiquitinates NAC1 for degradation in order to downregulate auxin signals (Xie, Q. *et al.*, 2002). Auxin-mediated development and transport is also mediated by *Multidrug Resistance*-like genes in Arabidopsis, and auxin transport has been shown to be modulated also by endogenous flavonoids (Noh, B. *et al.*, 2001).

Together with auxin, cytokinin plays also a very important role in many aspects of plant growth and development. Cytokinin appears to act primarily by stimulating cell division.

An indication of the importance of these hormones is the effect they have in cultured cells. The ratio of cytokinin to auxin in the medium can promote shoot development when increased, or root development when decreased (Skoog and Miller, 1957). Both auxin and cytokinin seem to be critical in determining the overall architecture of a plant. Many auxin resistant mutants have also altered sensitivity to cytokinin. A cytokinin-resistant mutant, *ckr1*, has longer roots with shorter root hairs than the wild type, suggesting that endogenous cytokinin inhibits root growth and stimulate root hair elongation (Estelle and Klee, 1994). A screen for root elongation on cytokinin containing media resulted in the finding of the *cyr1* Arabidopsis mutant. This mutant is allelic to the *embryonic flower 2* mutant, and has reduced cytokinin-induced antocyanin accumulation (Deikman and Ulrich, 1995). Another mutant, *stunted plant 1 (stp1*), has roots that elongate more slowly than wild type. Because a cytokinin-treated wild type phenocopied *stp1*, it has been suggested that cytokinin affects root growth via STP1. STP1 mediates effects of cytokinin, but not of auxin, on cell division and expansion in the root of Arabidopsis. The opposite effects of auxin and cytokinin suggest that the balance of these hormones may control the size of the meristem (Beemster and Baskin, 2000).

WOODEN-LEG encodes a two-component His kinase, allelic to *CRE1*, the cytokinin receptor in the two-component circuitry in Arabidopsis cytokinin signal transduction recently described (Hwang and Sheen, 2001, Inoue, T. *et al.*, 2001). It seems to sense extracellular cytokinin on the vascular cell surface, because it is expressed in all cells in the root vascular cylinder and procambium in the embryo. After sensing, it might transmit the signal to the nucleus. Interestingly, *WOODEN-LEG* mutations produce reduced number of cells in the vascular cylinder of roots and hypocotyls (Mahonen, A. P. *et al.*, 2000). Moreover, *CRE1* mutations were shown to impair cytokinin-induced repression of phosphate starvation responses in Arabidopsis (Franco-Zorrilla, J.M. *et al.*, 2002).

However, though cytokinins have been proposed to be involved in root differenciation (in Sheen and Hwang, 2001), this gene seems to control cell divisions but not cell differentiation. The signal has been hypothesized to act to promote vascular cell divisions, allowing differentiation of various cell types (Nakajima and Benfey, 2002).

Interestingly, the cytokinin-resistant *ckr1* mutant is an allele of *ein2*, an ethylene insensitive mutant. Moreover, ethylene-insensitive mutants show also resistance to cytokinin-mediated inhibition of root elongation. It seems that the inhibition of cell elongation in wild type seedlings by cytokinin is mediated by ethylene (Ecker and Theologis, 1994).

Ethylene is a gaseous hormone. It is involved in different plant growth and development aspects, like root initiation. Insensitive mutants that overproduce ethylene (*ein1* and *ein2*), are insensible in hypocotyl and root responses (Guzman and Ecker, 1990). An example of a mutation that affects ethylene sensitivity specifically in the root is the ethylene insensitive root (*eir1)* (Ecker and Theologis, 1994). Ethylene, as well as auxin, is important in root hair initiation. For example *ctr1* mutants show the formation of normally hairless epidermal cells. *CTR1* encodes a negative regulator of the ethylene signal transduction pathway (Kieber, J. J. *et al.*, 1993).

Moreover, ethylene, again like auxin, plays an important role in determining the duration of root hair tip growth. Mutations in genes like *ETR1* and *EIN2*, as well as in *AXR1*, *AUX1*, provoke shorter hairs with normal morphology. In contrast, *eto1* mutants have longer hairs than the wild type, as happens when roots are treated with exogenous auxin or ACC (Schiefelbein, J. W., 2000). Other mutants showing defects in root growth, among others, are the *hydra* mutants. Interestingly, these mutants are defective in sterol profiles and auxin and ethylene signaling, indicating the requirement of sterols for the signaling of these hormones. Sterols are essential components of plant membranes, and are also precursors for brassinosteroid hormones (Souter, M. *et al.*, 2002).

Le and colleagues have suggested that ethylene is a means of fine and fast tuning of root elongation. They point to the fact that slight changes in the concentration of ethylene in the environment modulate the elongation of target cells in the root epidermis of Arabidopsis. This response is immediate, concentration dependent, and reversible on root base, but irreversible on cell base. However, cell differenciation is not affected (Le, J. *et al.*, 2001).

While there are numerous reports about the influence of auxin in root growth and development, and also some about the influence of ethylene and cytokinins in these processes, there are few reports relating the plant hormone gibberellin to root development or growth. However, Ogas and collaborators reported a mutant that is defective in a giberellin signaling pathway that promotes the transition of the primary root from an embryonic to and adult differenciation state (Ogas, J. *et al.*, 1997; 1999). Other hormones, like ABA, have been also reported to have some influence in root growth. In water stressed plants, ABA is required for continued root growth while inhibiting shoot growth. ABA has also been shown to inhibit root hair elongation in wild type seedlings (Schnall and Quatrano, 1992). Moreover, ABA has been reported to play a central role in mediating the regulatory effects of nitrate on root branching in Arabidopsis (Signora, L. *et al.*, 2001).

On the other hand, brassinosteroids seem to be involved in the gravitropic response of primary root of maize (Kim, S.-K. *et al.*, 2000). Moreover, exogenous brassinosteroids have been reported to inhibit root growth. In general, exogenous application of brassinosteroids inhibits primary root extension and lateral root formation, with occasional promotions of elongation or adventitious rooting. There is some evidence for involvement of endogenous brassinosteroids in the control of lateral root initiation. Inhibitory effects, on expansion particularly, seem to be mediated via the induction of ethylene biosynthesis, but not in all cases. Furthermore, brassinosteroids can alter plant cell wall properties and increase the abundance of mRNA transcripts for wall-modifying proteins. These cell wall modifications are necessary for elongation and other morphogenetic processes to occur (reviewed in Clouse and Sasse, 1998).

Like brassinosteroids, jasmonic acid also strongly inhibits root growt. This inhibition is presumed to occur by a mechanism that is not mediated by ethylene. Other regulatory molecules, oligogalacturonides, can inhibit root formation in tobacco thin cell layers or leaves. They also inhibit auxin-stimulated rooting in leaf explants (reviewed in Creelman and Mullet, 1997).

Enviromental influence in root development

Root development is highly influenciated by the environment. Gravity, light, soil structure and obstacles, pathogens, toxic compounds, and nutrients are some of the stimuli that

can affect the development of the root. The incredible root plasticity towards the "outside" conditions means that signals must be perceived, transduced, the genes and/or proteic activity correctly regulated, and that division, expansion and cellular differentiation are modified to cope with the needs. Final root architecture is the result of multiple factors involved, including "inner" factors like hormones and "outer" factors like light, nutrients and so on. The study of the molecular mechanisms needed for signaling and response is hence interesting, and important to understand and possibly enhance desirable plant traits.

There are many reports of gravitropic responses studies. The asymmetric distribution of plant hormone auxin has been thought to mediate growth responses to gravity and light. Interestingly, Friml and collaborators have shown that the lateral relocation of the auxin efflux regulator PIN3 mediates Arabidopsis tropism (Friml, J. *et al.*, 2002).

On the other hand, light influence in seedling development particularly during germination, and in the plant shoot has been well studied. However, the study of light influence in root development has not been so extensive. Perhaps the fact that in nature plant shoots are the part exposed to light is the reason behind this reduced number of studies.

Nevertheless, some studies of light influence in root development of Arabidopsis include experiments where light is given in one side of the plate and the angle of deviation from vertical growth is measured (Okada and Shimura, 1994). Moreover Vitha and colleagues report the use of starchless mutants to study the influence of phototrophic responses in gravitropism. These mutants have reduced gravitropic responses. Phototropism was shown to influence gravitropism measurements (Vitha, S. *et al.*, 2000). In contrast, earlier studies by Okada and Shimura had shown that the phototrophic and gravitrophic strenghts were similar (Okada and Shimura, 1994).Besides a reported blue-light-dependent negative phototrophic response in roots, Ruppel and collaborators identified a red-light-dependent positive phototrophic response in Arabidopsis roots (Ruppel, N. J. *et al.*, 2001). Therefore, Kiss and colleagues suggested the existence of two photosensory systems that elicit phototropic root responses in Arabidopsis. One of these two systems is a blue light receptor system mediated by phototropin. On the other hand, the positive phototropism in roots might be phytochrome-mediated (Kiss, J. Z. *et al.*, 2001). The authors could identify this positive phototropism in mutants with a weakened gravitropic response, indicating that gravitropism can mask the positive phototrophic phenomenon (Kiss, J. Z. *et al.*, 2002).

Besides eliciting the phototrophic responses, light also inhibits the elongation of roots of many species, like pea. It has been shown that the root cap in maize is the light-sensitive part. Based on their experiments with pea seedlings, Eliasson and Bollmark indicated that ethylene might be a mediator of this light-induced inhibition of root growth (Eliasson and Bollmark, 1988). In general, there are few reports about light inhibition of root growth.

Nutrients are another important factor that influence root development. Nutrients like N, P, K and Fe affect developmental processes like root branching, root hair production, root diameter, root growth angle, nodulation and proteoid root formation. Root development can be directly affected by the nutrient concentration in soil or indirectly by the internal nutrient status of the plant (Crawford, N. M., 1994; Forde and Lorenzo, 2001). Interestingly, Forde and collaborators identified an Arabidopsis MADS box gene, *ANR1*, that controls nutrient-induced changes in root architecture (Zhang and Forde, 1998; 2000; Zhang, H. *et al.*, 1999). Since then, the study of the molecular components involved in nutrient-mediated root morphology has increased, but still many studies need to be done to get a better understanding. One of those studies used microarray and RNA gel blot analysis to identify Arabidopsis genes that responded to nitrate at low and high concentrations (Wang, R. *et al.*, 2000). Recently, Kutz and collaborators suggested a role for nitrilase 3, that can produce IAA, in the regulation of root morphology in sulphur-starving Arabidopsis (Kutz, A. *et al.*, 2002).

ROOTS AND PHOSPHORUS UPTAKE

Roots, as the plant organ specialized in nutrient uptake, are responsible for P uptake. Phosphorus is an important plant nutrient, being the second macronutrient that limits plant growth (nitrogen is the first), and it is about 0.2% of the dry weight of a plant (Schachtman, D. P. *et al.*, 1998). As a component of nucleic acids, phospholipids and ATP, phosphorus is vital to plants. It is also involved in the control of enzymatic reactions and in metabolic pathway regulation. However, phosphorus is one of the less available nutrients, due to the interactions of phosphorus with other soil elements like Al, Fe, and Ca, the very low difussion rate of

phosphorus, and its presence as organic compounds where the plant can not use it (Raghothama, K. G., 2000). Phosphorus is assimilated as phosphate, as negatively charged $H_2PO_4^-$ or HPO_4^{-2} ions (Hamburger, D. *et al.*, 2002). From 20 to 80% of the P in the soil is part of organic compounds (mainly phytic acid – hexaphosphate inositol -), and the rest is present as inorganic compounds (with more than 170 minerals). Unavailability, combined with the slow movement of P in the soil (diffusion rate 10^{-12} to 10^{-15} m^2 /s), makes P a limitant nutrient for plants.

P deficiency is a major problem in many poor quality soils, acid or calcareous –basic or alkaline- for example. Southern Mexico has acid soil areas where crops produce very low yields. In acid soils, the very abundant element Al becomes Al^{3+}, that besides inhibiting root growth also prevents P from being absorbed.

A common way to improve soil quality and plant growth is the use of fertilizers. Unfortunately, the recovery of P in a fertilized soil is very low. Approximately 80% of the P becomes immobilized by soil microbes and can not be absorbed by the plants, because it is adsorbed, precipitated or converted to organic forms. Slow diffusion causes that a low-P area forms around the roots (Schachtman, D. P., *et al.*, 1998).

The geometry and morphology of the plant root is then important to achieve maximum P absorbance. A greater root surface helps the plant to get enough nutrients when one or more of them is limiting. With a high surface area to volume rate, a root system can explore a higher soil volume. Mycorrhiza-root relationships are beneficial for plants, because the fungi hyphae increase the volume of soil that a root can explore. This increase in root area can lead to a 3 to 5 times more efficient P absortion (Schachtman, D. P. *et al.*, 1998).

On the other hand, there are plant species that obtain a larger root surface by forming clusters of roots called *proteoid* or *cluster* roots as a response to limiting P. Proteoid roots are groups of fine, specialized roots. These ramified roots develop at certain intervals compact groups of small roots (0.5 to 1 cm long), usually with the shape of an ellipse.
They were described in the Proteaceae family for the first time, hence the name “proteoid”. However, they are present in many other species (some of commercial importance like

Lupinus albus). It has been suggested that they only develop under P or Fe limiting conditions. Contrasting with this idea, although not in very P rich soils, some species can actually develop proteoid roots in normal soils. Also, proteoid roots exudate organic acids (around 23% of net photosynthesis) that quelate metallic ions near the root. By quellating the metallic ions, organic acids allow the mobilization of P and some micronutrients.

In seedlings, these root clusters form in the early roots that develop from the hypocotyl. Each group contains sometimes hundreds of small short roots. And many groups, each one separated by normally ramified regions, can develop in an individual proteoid root –a sort of natural "fractal"-. At the end, the plant gets an increase in the superficial aerea, obtained by a high number of white young tips. This immature surface darkens within few days, and the root clusters and even the complete proteoid root senesce after that.
This kind of roots helps P uptake by exudating high quantities of organic acids, mainly citric acid or citrates. Additionally proteoid roots can aid to reduce some phenolic compounds. Besides P uptake, proteoid roots can help iron uptake and in some species even nitrogen and manganese as well. At night, proteoid roots exudate water, and firmly bind to the soil (Mccully, M. E., 1999).

Other root changes include a general growth increase, structure alterations (like more ramifications), and an increase in number and longitude of root hairs. It has been suggested that in P deficient plants, root hairs can be responsible for around 63% of the total P uptake (Raghothama, K. G., 2000). Calcium seems to play a role in the root response and adaptation of plants to low phosphorus. Being major regulators, plant hormones are also involved in root alterations, root hair elongation and proteoid roots formation

In Arabidopsis, root morphology alterations have been reported in limiting and sufficient P conditions (Williamson, L. C. *et al.*, 2001). Auxin seems to play a role in the root response to low P (Lopez-Bucio, J. *et al.*, 2002). In some ecotypes, roots show a characteristic high or low P phenotype. High P roots are commonly longer and have less numbers of lateral roots than low P roots. Additionally, high P roots have less root hairs, which are also shorter than in low P conditions (Lopez-Bucio, J. *et al.*, 2002).

Not only the root shows changes in low P. There are many other plant responses for the lack of P. For example, low P can cause changes in the respiration rate and phospholipid content in chloroplasts, promote proton secretion (and organic acids as mentioned above) and stimulate P transport. Phosphatase and RNase activities are stimulated as well by the lack of P (Del Pozo, J. C. *et al.*, 1999). Moreover, besides increasing P root absorption, plants redistribute P from "old" leaves to "younger" parts and it uses the reserves that are stored in the vacuoles.

P deficiency induces genes like P transporters, phosphatases and enzymes that are involved in organic acids and anionic channels that help the release of organic acids (Raghothama, K. G., 2000). Organic acids have an important role in P absorption. Improved P absortion was improved by enhanced organic acid exudation in transgenic tobacco and Arabidopsis lines (López-Bucio, J. *et al.*, 2000).

In Indian mustard, Haran and colleagues (Haran S. *et al.*, 2000) studied the expression and secretion of acid phosphatases from root. They found two acid phosphatases that are induced by low P, and they showed that one of them is effectively secreted by the root to the medium. There is a gene homologue of the secreted phosphatase in Arabidopsis. When the promotor of the homologue is fused to the reporter gene *gus* or *GFP* (green fluorescent protein), the reporter gene is expressed in leaves and roots. The expression in the roots has a temporal pattern. First, the reporter gene is expressed in lateral root meristems, and then, there is general expression in the whole root, specifically in low P conditions. Low P conditions also enhance the expression of an acid phosphatase in white lupin. Interestingly, its promoter bears a 50 base pair region with 72% identity to a P-deficiency induced phosphatase promoter from Arabidopsis (Miller, S. S. *et al.*, 2001). Also in tomato P-starvation induced acid phosphatases have been identified (Baldwin, J. C. *et al.*, 2001).

Very important in P absortion are P transporters. Because it involves transport against a concentration gradient, P uptake by the transporters occurs by energy dependent proton/phosphate simport (simport with Na^+ or H^+) (Raghothama, K. G., 2000).
There are two kinds of phosphate uptake systems, as kinetic studies indicate. One system has high affinity to P (3 to 7 μM) and it is induced (or derrepressed) by P deficiency. An example

of a transporter of this kind is the product of the *LePT1* gene from tomato (Muchhal and Raghothama, 1999). The second kind is a low affinity system (50 to 330 μM, but the range varies in different species and tissues). The expression of this low affinity system is constitutive.

In the last few years, genes encoding proton-coupled Pi transporters (H+/H2 PO_4^{-2} symporters) have been isolated from a number of plant species. For example, in Arabidopsis, tomato, potato, tobacco, wheat and *Medicago truncatula* (Muchhal, U. S. *et al.*, 1996) (Leggewie, G. *et al.*, 1997; Liu, C. *et al.*, 1998) (Raghothama, K. G., 2000; Smith, F. W. *et al.*, 2000; Chiou, T.-J. *et al.*, 2001). These transporters have been classiffed into two families, Pht1 and Pht2 (Bucher, M. *et al.*, 2001). Based on the kinetic data that has been obtained for some of its members, the Pht1 family has been believed to contain the high affnity transporters. This family includes 17 out of the18 plant phosphate transporter genes published to date. The remaining gene, from Arabidopsis, shows low affinity kinetics (Mudge, S. R. *et al.*, 2002). This gene is the first member of the Pht2 family that was isolated. It has been shown to be a chloroplast phosphate transporter that influences allocation of phosphate within the plant and phosphate-starvation responses (Versaw and Harrison, 2002).

In Arabidopsis, nine genes code for high affinity P transporters. Schatchtman indicates that one family of transporters is grouped (at least three members) in region of chromosome five (Schachtman, D. P. *et al.*, 1998). The deducted sequence of aminoacids of two of the members of the group is 99% identical, but their promoters are very different, implicating that these genes are differently regulated. Studies of expression of these genes revealed different expression patterns, indicating that their role is not restricted to roots, and is broader than previously thought. Moreover, some of the transporters that were thought to be high affinity, might be low affinity transporters (Mudge, S. R. *et al.*, 2002). Phosphate transporters should not only mediate uptake from the soil, but also permit phosphate movement into other tissues (Chiou, T.-J. *et al.*, 2001).

It has been proposed that P uptake levels are regulated by an increase in the total number of transporters. After induction by P deficiency, the levels of transporters go back to

normality after 30 hrs. This decrease to normal levels is important because it avoids the accumulation of toxic levels of P (Muchhal and Raghothama, 1999).

Until now, the transporters that have been cloned are membrane proteins that have twelve intermembrane regions, separated in two groups of six by a large charged hydrophilic region, typical from transporters. Many P transporters seem to be transcriptionally regulated upon P deficiency (Raghothama, K. G., 2000). It has been suggested that changes in the internal P concentration are the signal that leads to the regulation of these transporters in plants. However, in barley roots this kind of regulation does not seem to be specific: Zn deficiency activates the expression of P transporters even in optimal P conditions. The increase of P transporters in this case gives toxic P concentrations as a result (Huang, C. *et al.*, 2000).

Interestingly, the *PHO1* gene has recently been cloned and it shows no homology with previously studied solute transporters. *pho1* mutants have lower P contents in their leaves, because they are deficient in the transfer of Pi from root epidermal and cortical cells to the xylem. Whereas PHO1 does not resemble any other cloned P transporters, it shares homology with Rcm1, a mammalian receptor for retroviruses and Syg1, involved in the mating pheromone signal transduction pathway from *Saccharomyces cerevisiae* (Hamburger, D. *et al.*, 2002). This mutant was identified in a screen for altered P levels in leaves of soil grown Arabidopsis plants. Besides *pho1*, the *pho2* mutant was also identified during the screen. In contrast to *pho1*, *pho2* mutants have higher P levels in leaves despite having normal P root uptake. This might indicate an alteration in P loading to the phloem vessels of leaves, or in a regulatory protein involved in the control of P level in leaves (Delhaize, E. *et al.*, 1998).

A third mutant, *pho3*, was isolated by screening roots for acid phosphatase activity. Both leaves and roots show reduced P accumulation (Zakhleniuk, O. V. *et al.*, 2001).

Interestingly, *pho1* and *pho2* were used to study the role of inorganic phosphate in the development of freezing tolerance and acclimatization of photosynthesis to low temperature. In this study, *pho1* showed unaltered or improved frost tolerance and accumulation of proline and sugars, while in *pho2* this processes were impaired. Hurry and colleagues concluded from this study that low phosphate plays an important role in triggering cold acclimatization of leaves and in changes in photosynthesis and sucrose biosynthesis enzymes (Hurry, V. *et al.*, 2000).

Besides studies focused on P content, phosphate uptake has also been studied in different Arabidopsis ecotypes. In a study done by Narang and colleagues, it was shown that Arabidopsis ecotypes differ in phosphorus acquisition efficiency when grown using an almost insoluble phosphorus source (Narang, R. A. *et al.*, 2000). Another study was based on the fact that plants can also use DNA or RNA as P sources. To elucidate Pi-starvation signaling pathway(s) in plants, Chen and colleagues developed a screen based on the ability of wild type plants to induce and secrete Pi-mobilizing enzymes in response to Pi limitation. The *psr1* mutant was found in this way and is deficient in the induction of ribonucleases and acid phosphatases involved the scavenging of P form organic sources under P-limiting conditions (Chen, D. L. *et al.*, 2000). Other approaches to study P sensing and uptake have used a phosphate analog, phosphite, that is not metabolized in plants. As a result, the authors of the phosphite study found that phosphite specifically attenuates developmental and molecular responses of Arabidopsis to low P availability (Ticconi, C. *et al.*, 2001). Another alternative is to use mannose, that mimicks P deficiency in some plants (Haran S. *et al.*, 2000; Herold, A.*et al.*, 1987).

In microorganisms, like *Escherichia coli* and *Saccharomyces cerevisiae*, P deficiency activates a multigenic rescue system that allows them to absorb even P traces in the medium. This multigenic rescue system is the *pho* regulon, and has at least 30 genes under the same physiologic and genetic control. Under low P conditions, there is expression of enzymes for P uptake, nucleases, phosphatases, high affinity transporters, proteins that bind P, and sensor protein kinases that monitor the P availability outside the cell (Lenburg and O'Shea, 1996; Torriani, A., 1990). While many scientists support, others are against the idea of the existence of an equivalent system to the *pho* regulon in plants (Goldstein, A.H. *et al.*, 1988).

In the unicellular green algae *Clamydomonas,* the *PSR1* gene (phosphorous starvation response 1) is required for the adaptation to P deficiency conditions. The *PSR1* gene is probably a transcriptional activator and there are homologuous genes in vascular plants. Because of their homology to *PSR 1*, a role in P deficiency adaptation has been proposed for the homologous genes in plants (Wykoff, D. D. *et al.*, 1999).

The study of the morphology and structure of the root as a response of environmental conditions, like nutrient deficiency and others, can help to understand the molecular signaling and response mechanisms involved. P availability induced root morphological changes are a suitable model of study. Though many studies are being performed, little is known still about these signaling and regulatory mechanisms in P sensing and response in plant roots. Succesful results from this kind of studies might be amenable of application of plant growth enhancement in poor soils.

CHAPTER II.

OBJECTIVES / MATERIAL & METHODS

OBJECTIVES

General Objective:

To develop a gain-of-function Arabidopsis mutant population using a transposon based gene activation system suitable for screening for mutants altered in nutrient uptake.

Specific objectives:

1. To design and build a transposon activation tagging (TAT) system in *Arabidopsis thaliana*.

2. To develop a population using the mentioned system.

3. To test and validate the TAT system.

4. As an application example, to screen the resulting population for traits involved in root development and phosphorus uptake, namely:

   -Changes in root arquitechture
   -Enhanced uptake in insoluble P

MATERIALS AND METHODS

I. Development of the Activation Tagging Population

Construction of En-I activation tag vectors

In trans vectors

See **Figure 3**.

I vectors

The *bar*: I (4Enh.) vector was made by ligating 5 fragments in a single step. The fragments were: a *Hind*III/*Sal*I 35S promoter; a *Sal*I/*Xba*I right *I* Terminal Inverted Repeat (TIR); a fragment including the 35S enhancer tetramer and the left *I* TIR; a *Bam*HI/*Eco*RI fragment with a promoterless *bar* gene followed by the *nos* terminator; and a *Hind*III/*Eco*RI pBINplus binary vector (Engelen, F. *et al.*, 1995).

The *GFP: I* (4Enh. + *bar*) construct, named "a" was obtained in a single step ligation with the following fragments: *Hind*III/*Sal*I 35S promoter; *Xho*I/*Xba*I fragment containing the 35S enhancer tetramer and the left *I* TIR (terminal inverted repeat); a *Bam*HI/*Spe*I fragment with the right *I* TIR, fused to the *bar* gene between the *nos* promoter and terminator sequences; a promoterless GPF gene (plasmid PMP2164) as a *Bam*HI/*Eco*RI fragment (Chiu W, *et al.*, 1996); and the pBINplus vector digested with *Hind*III/*Eco*RI (Engelen, F. *et al.*, 1995).

The *GFP: I* (4Enh + mini ORI + *bar*) construct, named OG, was made by ligating, in a single step, the following fragments: an "adaptor" constisting of a piece of a *lox* sequence and the 35S terminator *Hind*III/*Not*I to help the ligation; a *Sst*I/*Not*I fragment containing first a doubly enhanced 35S promoter-AMV leader, followed by the left TIR fused to the 35S enhancer

tetramer and a minimal origin of replication of the plasmid pBR322; a *Sst*I/*Bam*HI (partial digestion) fragment containing the I right TIR and the bar gene (between the *nos* promoter and terminator sequences); a *Bam*HI/*Eco*RI fragment containing the promoterless *GFP* gene, and the *nos* terminator. The last piece was the *Hind*III/*Eco*RI pMOG22 binary vector (containing the *HPT* marker gene).

En vectors

The *su1*-EnTPase construct was already available (Greco, R.), and consisted of a *Bss*HII *En* transposase fragment (without TIRs), between the 35S promoter and terminator. The vector included the *su1* gene as well, between the *SSU* (small Rubisco subunit) promoter plus signal peptide to the chloroplast, and terminator. This construct was built in the binary vector pMOG22.

The EnTPase-4Enh.-GFP, was made by ligating in a single step the following fragments: *Xho*I/*Not*I *En* transposase (between the 35S promoter and terminator); *Not*I/*Hind*III 35S enhancer tetramer; *Hind*III/*Xba*I *GFP* gene between a doubly enhanced 35S promoter plus an AMV leader sequence and the *nos* terminator; and the *Xba*I/*Xho*I pMOG22 binary vector.

In Cis vector

The construct for activation tagging was assembled in a single six-point ligation of multiple fragments excised from specific subclones. The fragments used were: (i) *Not*I-*Hind*III linker, (ii) *Not*I-*Xho*I immobile 35S-En transposase, (iii) left end of the *I* element with terminal inverted repeat (tir), and a *Xho*I-*Xba*I CaMV 35S enhancer tetramer, (iv) a *Bam*HI-*Spe*I fragment containing the right end of the *I* tir fused to the *nos* promoter-*bar*- *nos* terminator, (v) an *Eco*RI-*Bam*HI fragment containing the SSU-SU12 (O'keefe, D. P. *et al.*, 1994) gene, and (vi) a *Hind*III-*Eco*RI digested pBinplus vector –*NPTII*- (Engelen, F. *et al.*, 1995). The transposase fragment was derived from an immobile *En* transposase gene driven by a 35S promoter (Aarts, M. G. M. *et al.*, 1995). The non-autonomous *I* element contains a tetramer of the CaMV 35S enhancer (twice -392 to -90 and -526 to -90) (Odell, J.T. *et al.*

1985) and the *bar* gene (De Block, M. *et al.*, 1987; Thompson, C. J. *et al.*, 1987) and is called AIE (activating *I* element, see **Figure 6**).

Ssu 5': Rubisco small subunit promoter, untranslated region and first 12 aa.
Ssu 3': Rubisco small subunit polyadenylation signal

Arabidopsis transformation

Arabidopsis ecotypes Wassilewskija (Ws-3) and Landsberg *erecta* (L*er*-1), were grown in a climate chamber for 4 weeks under short days (8 hours light, 16ºC) and 2 weeks under long day conditions (16 hours light, 22ºC) at about 70% relative humidity.
The constructs were introduced into the plants using the floral dipping transformation method (Clough and Bent, 1998). The seeds were plated on half strength Murashige and Skoog medium (Murashige and Skoog, 1962) in petri dishes with 1% w/v sucrose, 0.8% w/v purified agar, and 50 mg/L kanamycin or 20 mg/L hygromicin. After a 4ºC treatment for 3 nights they were transferred to a growth chamber (16 hours light, 23ºC) for 9 days. The resistant seedlings were then transferred to soil in the greenhouse at around 22ºC. Primary transformants (T1) were further confirmed by PCR for the presence of the marker genes and the correct position of the main elements.

Parental lines and transposant selection

In cis

High scale selections for parental lines and transposants were done in the greenhouse. For transposant selection, progeny seedlings of first transformants or parental lines were sprayed with 0.7 ml/L Finale (commercial formulation -Aventis- that contains 150g/L glufosinate ammonium) three times, and with 100 µg/L R7402 (DuPont) daily after germination for 8 days. Alternatively, 100 µg/ml pure PPT (Duchefa) was sprayed instead of Finale.

Resistant plants were transferred to new soil 5 to 7 days after the last spray, phenotypes were scored and allowed to set seed. Populations 2, 3 and 4 were transferred to new soil in 96-well trays, with one single plant per well. Population 1 (8 to 20 plants per pot) and population 5 (8 to 10 plants per pot) were transferred to new soil in 14 cm. diameter pots. Phenotypes were pre-scored during plantlet transfer for all sub-populations, and at least once more during the life of the plant for sub-populations 1 and 5. T2/T3, and F1 plants to be used as parental lines, were handled as for transposant selection, but sprayed only twice with 0.7 ml/L Finale, and visually evaluated (dwarf, dark, reduced apical dominance plants were selected). Their seeds were collected and used for transposant selection. For initial media trials, plates supplemented with 10 µg/ml PPT (Duchefa) and/or 1 ng/ml R7402 (Dupont) were used. When only PPT was added, normal ½ MS, 0.8% w/v agar, 1% w/v sucrose medium was used. However, when R7402 was used, sucrose was replaced by 100 µg/ml myoinositol.

In trans

Progeny from the first transformants carrying *I* or *En* constructs were selected in plates with 50 mg/L kanamycin or 20 mg/L hygromicin, and the resistant seedlings transferred to soil. Plates containing kanamycin had 1% w/v sucrose, but the hygromicin plates did not contain any sugar. Crosses were made using *I* lines as female and *En* as male. The F1 seed obtained was selected in either kanamycin/hygromicin (for crosses with *GFP*: *I* (4Enh. + *bar*)) or hygromicin plates (for crosses with *GFP*: *I* (4Enh + mini ORI + *bar*)), and transferred to soil as well. The F2 seed obtained was either collected separately from each plant or as a mix of two plants.

Molecular analysis of excision

In cis

DNA was isolated (Pereira and Aarts, 1998) from 2 leaves or young flower buds, either in Eppendorf tubes or in a 96 tube-rack. Genomic DNA (approximately 20 ng) isolated from the

primary transformants was used in a PCR reaction to reveal empty donor sites. The following primers were employed:

35S-T2: 5'-CCA AAA TCC AGT GGG TAC CGA GC-3'

SSU-301-TF: 5'-GTT GGT TGA GAG TCT TGT GGC CT-3'

The PCR reaction comprised a denaturing step of 95ºC for 3 minutes, followed by 35 cycles of 1 minute at 95ºC, 1 minute at 60ºC and 1 minute at 72ºC, ending with an elongation step of 5 minutes at 72ºC.

In trans

The primers used to analyze excision in *I* constructs were:

35S-for1: 5'-ATC CCA CTA TCC TTC GCA AGA CCC-3'

and **sGFP-R2:** 5'-GCT TGT CGG CCA TGA TAT AGA CG-3'

The combinations of primers 35S-for1 with Int 1 and sGFP-R2 with Int 2 were used to confirm the presence of the transposon in the T-DNA.

Left transposon end: Int 1: 5'-AAG CGT CGG TTT CAT CGG GAC-3'

Right transposon end: Int2: 5'-CAG GGT AGC TTA CTG ATG TGC G-3',

Transposition analysis using Southern hybridization

Approximately 500 ng for individual plants or 1 µg for genomic DNA from pooled plant tissue were digested with *Eco*RI, and electrophoresed in a 0.8 % w/v agarose gel in 1X TAE buffer (40 mM Tris-acetate and 1 mM EDTA) and transferred to Hybond N^+ membranes (Amersham). Digestion with *Eco*RI allows to distinguish between *I* elements in the original full donor site (FDS) and empty donor site (EDS) T-DNAs. To visualize the EDS and FDS, either a ~1.2 kb *Bam*HI/*Pst*I *SU1* fragment or a ~620 bp *Bam*HI/*Pst*I *bar* fragment were used as labeled probes (shown in **Figure 6**). The *bar* probe also allowed visualization of different *I* transpositions in the genome, and was used to estimate the frequency of independent insertions. Transposon insertion display (Tissier, A. *et al.*, 1999) was used to support the

results of Southern hybridization and provide data on the frequency of insertions in the population.

Pollen germination

Pollen was germinated in a solution containing:
-50 mL 10x salt stock solution (1g/L H_3BO_3, 7 g/L $CaNO_3$, 2 g/L $MgSO_4$, 1 g/L KNO_3),
-10 mL buffer pH 6.0 (19.52 g MES (2-[N-morpholino ethane sulfonic acid] in 80 mL H_2O, pH 6.0 adjusted with 5 M KOH, final volume 100 mL –water-),
-10 g sucrose,
-75 g PEG 4000,
All the components were mixed in a final volume of 500 mL –water-. The solution was filter sterilized, divided in several aliquotes and stored at –20ºC. The best visualization was obtained using the "hanging drop" method and the best results were obtained by observing the pollen between 30 to 120 min.

GFP visualization was best obtained in GFP positive plants with the following parameters: GFP3 filter, 470/40 nm exication filter, 495 nm dichromatic beam splitter, and 525/50 nm barrier filter.

II. Characterization of aerial mutants

Genetic Analysis of mutants

The first and fifth sub-populations of stable transposed elements were screened visually for easily distinguishable morphological phenotypes in the greenhouse. The putative mutants were crossed to the wild type ecotype Ws as female or male. A few crosses to other ecotypes, Columbia (Col) or Landsberg *erecta* (L*er*), were also carried out in some cases. The F1 and/or selfed progeny were grown to confirm the heritability and dominance of the mutants.

Southern Analysis

Mutant segregating progeny from self or crosses to wild type (F1) were analyzed by Southern. The procedure was done as described for the analysis of transposants. The only variation was the use of a *bar* probe obtained by PCR instead of by restriction from a plasmid. The primers used to obtain the probe were:
Bar F1: 5'-ACC ATG AGC CCA GAA CGA CGC-3'
Bar R1: 5'-CAG GCT GAA GTC CAG CTG CCA G-3'

Isolation of flanking sequences

To characterize the insertions in different mutants, genomic DNA was used to isolate fragments adjacent to the AIEs by TAIL-PCR (Liu, Y.-G. *et al.*, 1995; Liu and Whittier, 1995; Tsugeki, R. *et al.*, 1996) with the following modifications: The specific nested primers for the *I* element were:
Int2: 5'-CAG GGT AGC TTA CTG ATG TGC G-3',
Irj-201: 5'-CAT AAG AGT GTC GGT TGC TTG TTG-3', and
DSpm1: (Tissier, A. *et al.*, 1999): 5'-CTT ATT TCA GTA AGA GTG TGG GGT TTT GG-3' for the first, second and third TAIL-PCRs, respectively. A fourth primer:
Itir 3: 5'-CTT ACC TTT TTT CTT GTA GTG-3', was used to directly sequence the obtained PCR products with high specificity.
The sequence of the flanking DNA was compared against the Arabidopsis database using BLASTN (Altschul, S. F. *et al.*, 1997) and enabled positioning of the insert in the genome and its context with adjacent annotated genes.

Expression analysis

Total RNA from mutant and wild type whole seedlings, leaves or flowers was isolated as described by (Verwoerd *et al.*, 1989). Approximately 1 µg of total RNA was treated with 1 unit of DNase I as indicated by the supplier (Gibco, Life Technologies). cDNA was obtained

using the M-MuLV Reverse Transcriptase as described by the supplier (Gibco, Life Technologies) in a 20 µl reaction mix that contained 0.1 µg of the DNAse treated RNA, and 1 µM oligo dTprimer $(T)_{23}$-NN among the components (1x M-MuLV RT buffer, 10 µM DTT, 1 mM dNTPs, and DEPC treated water). Two µl of the total RT reaction was used to do the PCR with the gene-specific primers.

For the flavin monooxygenase-like gene (*THREAD)* the primers used were: **Monoox5'**: 5'-TTG GTA CCC ATG GGC ACT TGT AGA GAA-3', **Monoox3'**: 5'-'GTG AGC TCT TAG GAT TTA TTG AAA TGA AGA TGA-3'. The primers used for Actin were 5'-GCG GTT TTC CCC AGT GTT GTT G-3', and 5'-TGC CTG GAC CTG CTT CAT CAT ACT-3'. The reaction conditions for PCR included a denaturing step of 95ºC for 3 minutes, followed by 32 cycles of 1 minute at 95ºC, 1 minute at 54ºC and 2 minutes at 72ºC, ending with an elongation step of 5 minutes at 72ºC. The PCR to analyze the expression of the gene in wild type flowers used 38 cycles (data not shown), and control PCR with actin primers comprised only 30 cycles. The primers for the rest of the mutants were:

THREAD

Monoox 5' (*Kpn* I)

TT- GGTACC – CAT GGG CAC TTG TAG AGA A

Monoox 3' (*Sst* I)

GT - GAGCTC – TTA GGA TTT ATT GAA ATG AAG ATG A

CLOUDY

Mannos 5' (*Kpn* I)

TG - GGTACC – ATG GAT ATA AGG AAG CGG AA

Mannos 3' (*Sst* I)

TC - GAGCTC – GTT TCT AAC TTG CCT CTT TAA

Glucosylt 5' (*Xba* I)

CG - TCTAGA – ATG GCC GTT TCA TCG TCG CA

Glucosylt 3' (*Sal* I)

CC - GTCGAC – CTA AGC ATC AGA ACT TGA CAA C

BOUNTIFUL

DNAbi 5' (*Xba* I)

GG - TCTAGA – GGG CTT TCA TGG CGA ATC

DNAbi 3' (*Sst* I)

CA - GAGCTC – GCA CGT TAA AAT CCT GAC CTA

VARIEGATED

ORF X 5' (*Bam H* I)

TG - GGATCC – GTA GAT ACT ATG GGT CGA C

ORF X 3' (*Xba* I)

TC- TCTAGA – TAT GAG TCA ACC CAT CAT TCG

Overexpression constructs

cDNA of genes: DNAbi (*bountiful,* F7O18.4*),* Glucosyltransferase (*cloudy,* T9L3_160*),* Mannosyltransferase (*cloudy*, T9L3_150*),* and monooxygenase (*thread,* F2I11_210*)* was obtained from the respective mutants by RT-PCR (see **Figures 13** and **17** for loci). Proof-reading *Pfu* DNA polymerase (Stratagene) was used and the products cloned in pGEM-T (Promega). The cloned fragments were sequenced to confirm the identity of the gene. Appropiate restriction sites were added to the primers used for the PCR and allowed cloning between the CaMV 35S promoter and (35S and *nos*) terminator, in a pBI121-based binary vector. The constructs were introduced in the *A. tumefaciens* strain GV3101 by electroporation, and into Arabidopsis by the floral dipping method (Clough and Bent, 1998).

Cloudy hormone treatments

Around 75 segregating mutant plants, from different M2 parents, or at least 25 wild type plants, were grown in the greenhouse.

Treatments began 14 days after transference to the greenhouse. Five treatments were compared: Water, Water with triton X-100 (0.01% v/v), IAA 100 µM, Zeatine 100 µM and Provide, a comercial mix of giberellins A_4A_7, diluted to a conc. of 100 µM giberellin.
The treatments with IAA and Zeatine included Triton x-100 as a surfactant. All treatments were applied twice a week (having 2 or 3 days between each treatment). A hand aspersor was used and same quantities of liquid were used for the same amount of plants when possible (50-100 ml for 1.5 trays).

III. Root and phosphorus nutrition screens

Phosphate screens

The root screens in low P were carried out as described in (Lopez-Bucio, J. *et al.*, 2002). The screens were carried out in square plates (90-100 mm), using 1/10 Murashige & Skoog (MS) medium without phosphate, 0.5 % w/v sucrose and 1% w/v agar for plants (brand). For later comparisons to "high" P, 1 mM NaH_2PO_4 was added to the medium. The seed were sterilized with 70% ethanol for 5 min, followed by a solution of 20% v/v commercial bleach for 7 min. and then rinsed four times with sterile, ultra-distilled water.
Seeds received a 4ºC treatment for 3 nights before placing the plates in the growth chamber. Root measures (length, and lateral root number) were taken after two weeks in the growth chamber. The plates were positioned vertically, with an inclination of around 70 degrees from the horizontal axis. The first subpopulation was screened as a single pool of 3000 seed, derived from non-equal sub-pools. In the first population, one sub-pool was obtained from the mix of the plants grown in one pot (8-20 plants). The total size of the sub-population was of approximately 1100 plants. The second subpopulation was screened using pools of plants grown in two trays (96 plants per tray, 192 plants per pool). This sub-population consisted of 28 trays. 300 seed were used from mixes of trays 1 to 16 and 21 to 28 (both separated in pairs). In total 3600 seed from 2304 plants were used. From trays 17 to 20, 200 seed were used from each tray, accounting for 800 seed from 384 plants.

Growth chamber conditions

The conditions used in the growth chamber for root assays were:
-16 hours light.
- 22ºC (varying from 20-22ºC)
- distance from the shelf to the light source: 60 cm.
- light intensity varied from 100 to 134 µmoles (9 klux per µA).

Light experiments

Square plates (90 mm.) were grown vertically, with an inclination of about 70 degrees from the horizontal axis. The plates were covered with a black paper stripe around them. The stripe covered most of the plate but left approximately 1 cm. open on the upper side. Seeds were sown in the upper part of the plate, about 1 cm. from the top, in a way that the green parts of the seedling could receive light. In contrast, the roots receive less light than the upper part.

X-Gluc staining (assay for GUS activity)

Seedlings were harvested after different times, and immersed in a X-Gluc solution. The solution was prepared with 50 mM phosphate buffer (1M Na_2HPO_4 and 1 M NaH_2PO_4, pH 7); 10 mM EDTA; 0.1% Triton X-100; 2 mM Potassium ferricianide; 2 mM Potassium ferrocianide; 100 µg/ml chloranfenicol; 1 mg/ml X-Gluc (Gallaher, S. R., 1992).
The seedlings were left in the X-Gluc staining solution at 37 ºC for from 1 to 4 nights (depending on the line) in the dark. After the treatment at 37 ºC, the X-gluc solution was replaced by 70% ethanol, and left for 1 night at 4 degrees. The ethanol solution was replaced afterwards by a clearing solution (20% lactic acid and 20% glycerol).

RT-PCR mutant 17

The RT-PCR procedure was done as described before. The primers used include restriction sites that could be used for further cloning in overexpression vectors. These primers were designed according to the predicted ORFs.

For the unknown gene, the used primers were:

Desc17 5' (*Kpn* I)

AT-GGTACC-TCG ATA ATG ATC CAT CAA GCT

Desc17 3' (*Sac* I)

TT-GAGCTC-GTT GAA ATG TTT TAC ATG ACG GT

For the putative kinase, the primers used were:

Cin 17 5' (*Xba* I)

TA-TCTAGA-ATG GAA TGG ACT AGA GGA AGA

Cin 17 3' (*Sac* I)

AA-GAGCTC-AAT TTA CAA TTC CCC CAC CA

$Ca_5HO_{13}P_3$ screen

$Ca_5HO_{13}P_3$ was used as an insoluble source of phosphorus in this screen. To avoid soluble phosphorus traces, it was washed several times using deionized water. It was washed at least 4 times before sterilization and at least 1 time more after it.

The composition of the medium was 1/10 MS without phosphorus sources, 1% w/v agar for plants, 0.5% w/v sucrose, and 5 mM $Ca_5HO_{13}P_3$. pH was adjusted to 8 using NaOH or KOH. A 100 mM $Ca_5HO_{13}P_3$ stock was sterilized separately from the rest of the components, to allow more washing. It was added to the medium just before pouring, when medium temperature was low enough to pour, to avoid quick precipitation. Plate pouring was preceded by thorough medium mixing for each plate. Seed were sterilized as indicated as indicated for the phosphate screen. It was sown using 0.1% agarose in deionized water. The pH of the agarose solution was adjusted to 8 - 8.5.

Seed were stratified for 2 nights in the tube after sterilization and immediately sown in plates. Plates were transferred to a growth chamber with 16 hours light, at 24ºC for 21 days.

CHAPTER III.

RESULTS (Part I)

PRODUCTION OF A TRANSPOSON ACTIVATION TAGGING POPULATION

Activation Tagging Transposon System Design

An activation-tagging system was designed and developed in the model plant *Arabidopsis thaliana* using the two-component *En-I* maize transposon system. The *En-I* system was used to construct vectors suitable for developing collections of stable *I* transposon insertions that could serve as activation tags in plant genomes.

The elements used for this activation-tagging system have been modified to ease their use. One modification is the lack of terminal inverted repeats in the En autonomous element. Because of this lack of terminal inverted repeats, the *En* element is incapable of transposing. However, the transposase that it codes for is active, placed between the CaMV 35S promoter and terminator, and can cause the transposition of the *I* element (Aarts, M. G. M. *et al.*, 1995). On the other hand, only short sequences of the *I* non-autonomous element that include the terminal inverted repeats are used. The enhancer is placed between those short terminal sequences. To make the transposon system easier to use, marker genes to select for excision, transposition or to segregate the transposase source out of the genome were used in different occasions.

Having two elements, there were different possibilities to use them. Therefore, two strategies were followed initially as an attempt to find the most efficient strategy to generate a collection of stable insertions. The two elements (autonomous and non-autonomous) of the transposon system were placed either in a single vector "*in cis*", or in separate vectors "*in*

trans" (see **Figure 2**). In both cases it is desirable to segregate the transposase source out to ease the study of interesting mutants. For this purpose, a negative selection marker (*su1*, which confers sensibility to sulfonylurea) was placed in the transposase bearing constructs.
For sake of clarity, each strategy will be described separately.

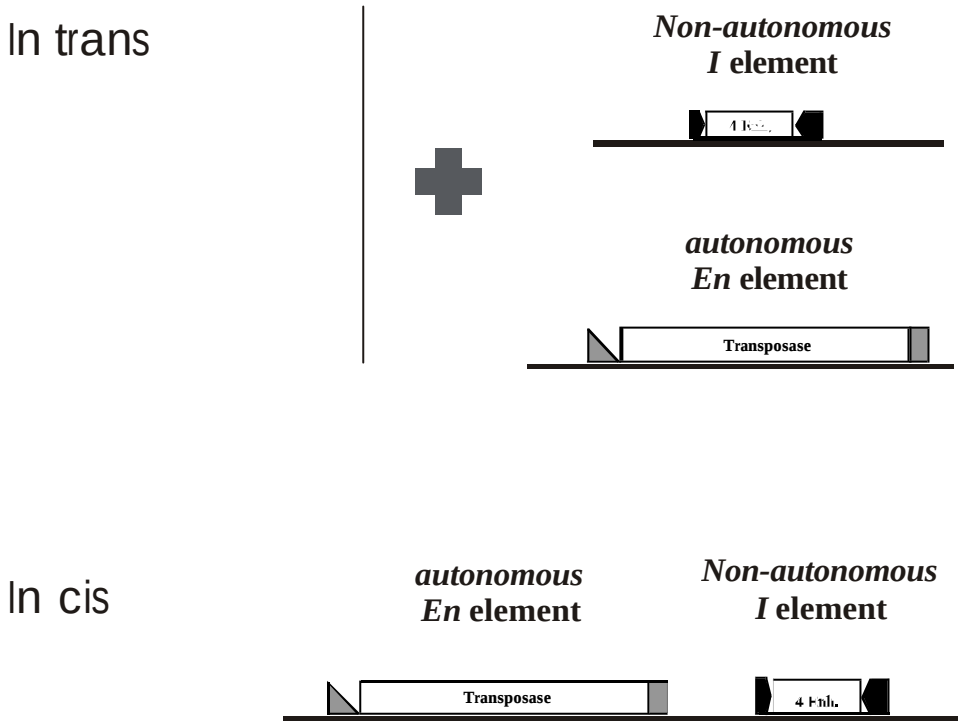


Figure 2. Schematic representation of the *in trans* and *in cis* strategies.

1. *In trans* strategy.

In the *in trans* strategy, the autonomous *(En)* and the non-autonomous *(I)* elements were placed in separate vectors. A schematic representation of each construct is shown in **Figure 3**.

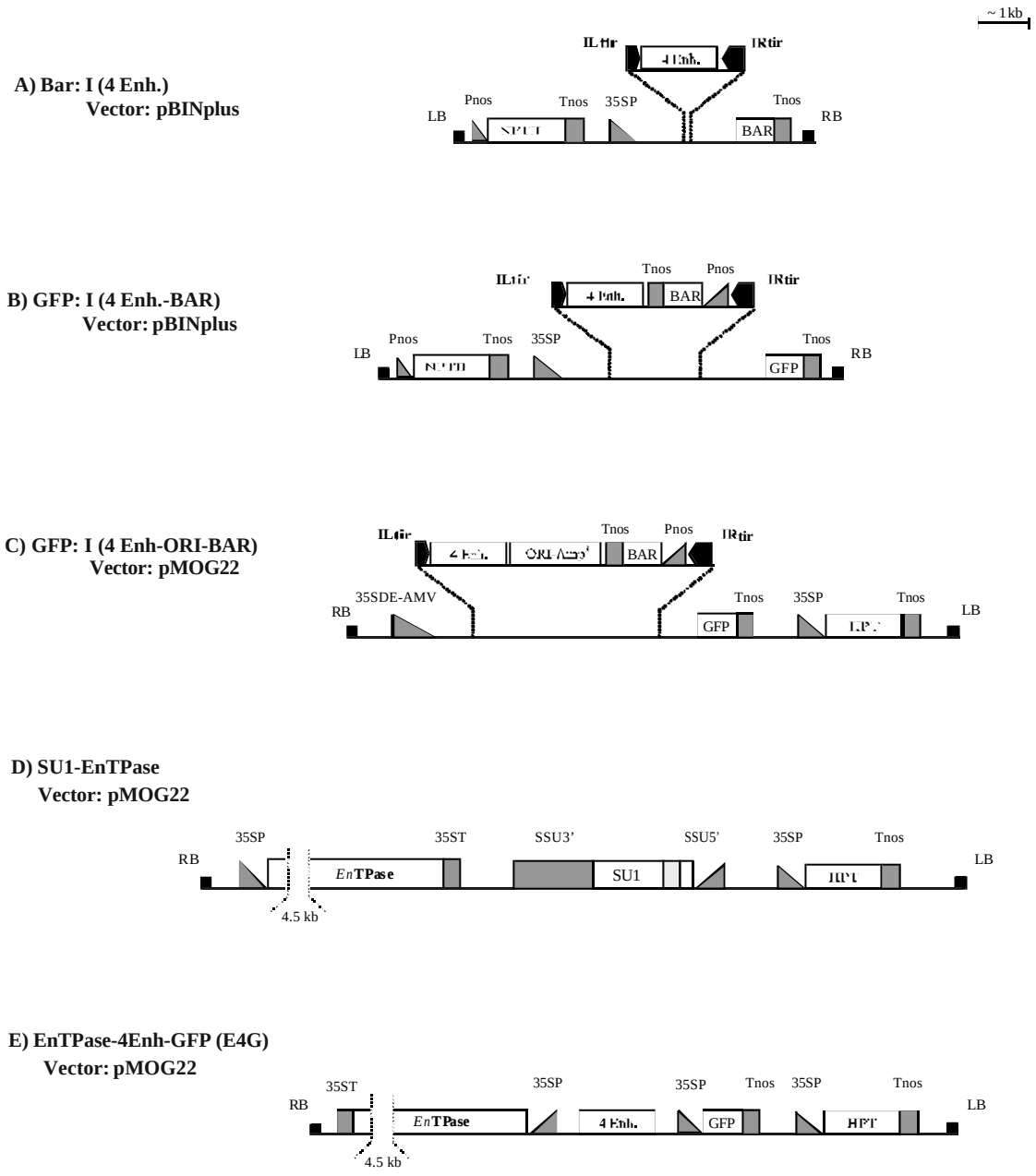


Figure 3. Schematic representation of the constructs designed and built for the *in trans* strategy. LB, Left border; RB, Right border; 35SP, 35ST, CaMV 35S promoter and terminator, respectively; EnTPase, En immobile transposase source; ILtir, IRtir, I element left and right terminal inverted repeat, respectively; 4 Enh., tetramer of the CaMV35S enhancer; Pnos, Tnos, promoter and terminator sequences from the nopaline synthase gene, respectively; SSU5', SSU3', promoter and transit signal peptide to the chloroplast, and terminator of the small subunit of RUBISCO gene, respectively. ORI-AmpR, origin of replication and ampicillin resistance gene from pBR322; GFP, green fluorescent protein; HPT, hygromicin phosphotransferase gene; 35SDE-AMV, double enhancer 35S promoter, fused to the AMV leader.

Three *I*, and one *En* vectors were tested:

i. Constructs with the non-autonomous *I* element:

a) *bar*: *I* (4Enh)

Vector: pBINplus (Kan)

b) *GFP*: *I* (4Enh- *bar*).

Vector: pBINplus (Kan)

c) *GFP*: *I* (4 Enh-ORI- *bar*)

Vector: pMOG22 (Hyg)

ii. *En* Transposase construct:

EnTPase + *su1*

Vector: pMOG22 (Hyg)

I vectors contain only the transposon, which has the 35S enhancer tetramer inside, while *En* vectors contain only the transposase. Both *I* and *En* vectors contain also marker genes. For example, the *En* vector contains also the *su1* gene, a negatively selectable marker. On the other hand, in the case of *I* vectors, construct "a" has the *bar* gene as an excision marker, while constructs "b" and "c" use the *GFP* gene for the same purpose. As excision markers, the expression of *bar* or *GFP* is "blocked" by the *I* element (containing the enhancer sequences). The expression of the marker gene is supposed to be restored upon *I* element excision. The *bar* gene inside the transposon in constructs "b" and "c" acts as a transposition marker. Additionaly, construct "c" contains the origin of replication and ampicillin resistance gene of the pBR322 plasmid inside the *I* element. This origin of replication -and resistance gene- would allow the recovery of the gene of interest using plasmid rescue.

Transgenic lines containing each of the *I* and *En* constructs were obtained for the Ws and Ler ecotypes. The number of transformed lines for each *I* transposon construct is indicated in **table IV.**

All transformants were tested for the presence of their respective components by PCR. For the *En* transposase construct, three transformants were found that had all the components (the transposase and the *su1* gene).

I element construct	4Enh.	ORI	Transp. marker	Excision marker	# plants
bar: I (4 Enh.)	✓			*bar*	5
GFP:I (4Enh-*bar*)	✓		*bar*	GFP	42
GFP:I (4Enh-ORI-*bar*)	✓	✓	*bar*	GFP	29

Table IV. Components and number of plants with *I* constructs. Shaded columns indicate the components present inside the *I* element.

As mentioned above, in the first construct *bar*:*I* (4 Enh), the *bar* gene is used as an excision marker. Interestingly, the first transformants with this construct were already resistant to the herbicide Basta, without the excision of the transposon. A possible explanation for this resistance is that the transposon end could act as a cryptic promoter. This construct was made as a first attempt to build an activating transposon. However, it lacks a transposition marker (a marker inside the transposon that can help to select for insertions) and it was not used in further experiments.

In the following steps, only constructs *GFP*:*I* (4Enh-*bar*) and *GFP*:*I* (4Enh-ORI-*bar*) were used to build a population of activation insertions.

In order to saturate the genome, a number of *in trans* lines with T-DNA insertions carrying the transposon were obtained. At least 10 insertions per chromosome were expected, so that local transposition events could lead to tagging genes in neighboring regions. By crossing these lines to lines carrying the transposase source (*En*-SU1), a number of "active" F1 lines were generated.

A low scale preliminary experiment was done to test for excision of the *I* element. *I* excision was assayed by PCR, using primers on both sides outside the TIRs. The excision frequency can be affected by the position of the original T-DNA insert containing the *I* element in the genome and the position of the insert containing the transposase. One same transposase line was used to avoid the position effect in this experiment. On the other hand, more than one line of each transposon construct was used.

For construct *GFP*:*I* (4Enh-*bar*), where the crosses of two *I* lines were studied, this excision test showed an excision frequency by PCR of 6.7% (1 plant of 15 showed a band). For the construct with the origin of replication, GFP:I (4Enh-ORI-*bar*), the excision frequency

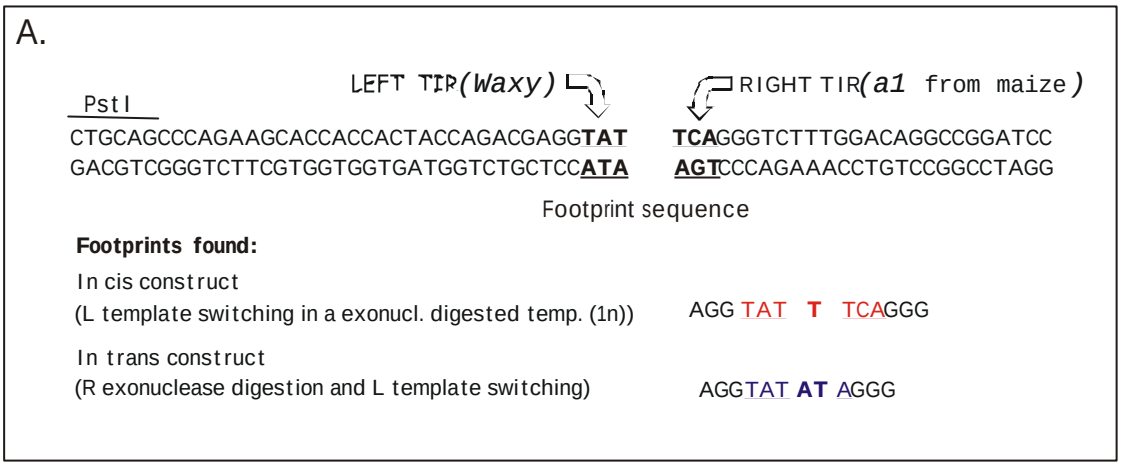
was 15% (3 plants of 20 showed a band), using the crosses of three *I* lines. The bands obtained for the last construct were very faint. Faint bands are indicators of somatic excision. They are faint because excision occured only in a small sector of the tissue from where DNA was isolated.

The fragment obtained by PCR in the *GFP:I* (4Enh- *bar*) line was sequenced, showing the footprint that the transposon left upon mobilization (**Figure 4**). Interestingly, since the two *I* transposon terminal inverted repeats present in AIE were isolated from different inserts in the maize genome, the left junction from the *waxy* (Pereira, A. *et al.*, 1985) and the right junction from the *a1* (Schwarz-Sommer, Z. *et al.*, 1987) mutant loci, they contained different flanking sites that do not form a target site duplication (**Figure 4**). Since excision of AIE was found to be normal, these results indicate that the presence of target site duplication is not necessary for excision.

For one excision band from the construct *GFP:I* (4Enh-ORI- *bar*), the sequenced fragment did not show a clear footprint, but rather a sequence that belonged to other parts of the vector. The obtention of such a sequence could be due to two possibilities. First, it could be that the transposon indeed excised, but a rearrangement occurred when that happened. And second, this fragment could be obtained for a line where a rearrangement of the construct occurred during transformation, allowing a PCR band to be obtained without excision. However, when the original *I* parental plant was tested with the same primer combination no band was obtained, indicating that a rearrangement during transformation was not the cause of the unexpected sequence obtained. The number of tested plants from the line that did not show the footprint was not enough to conclude if this was a general trend in the line (obtain excision bands without clear footprints), but because most of the plants did not show any band it seemed not to be the case.

A small sample of plants was observed under the microscope to detect restoration of GFP expression due to *I* excision. Unfortunately, no GPF fluorescence could be observed when compared to control plants (containing a 35S - *GFP* construct). The trials for GFP detection included visualization of germinated pollen, seeds, and seedlings in F1 and some F2 plants. Whether the absence of GFP fluorescence was due to the fact that the plants were

assayed too early (and transposition would have occurred in sectors that were too small to be seen) or to problems of restoration of gene expression after excision was not further tested.



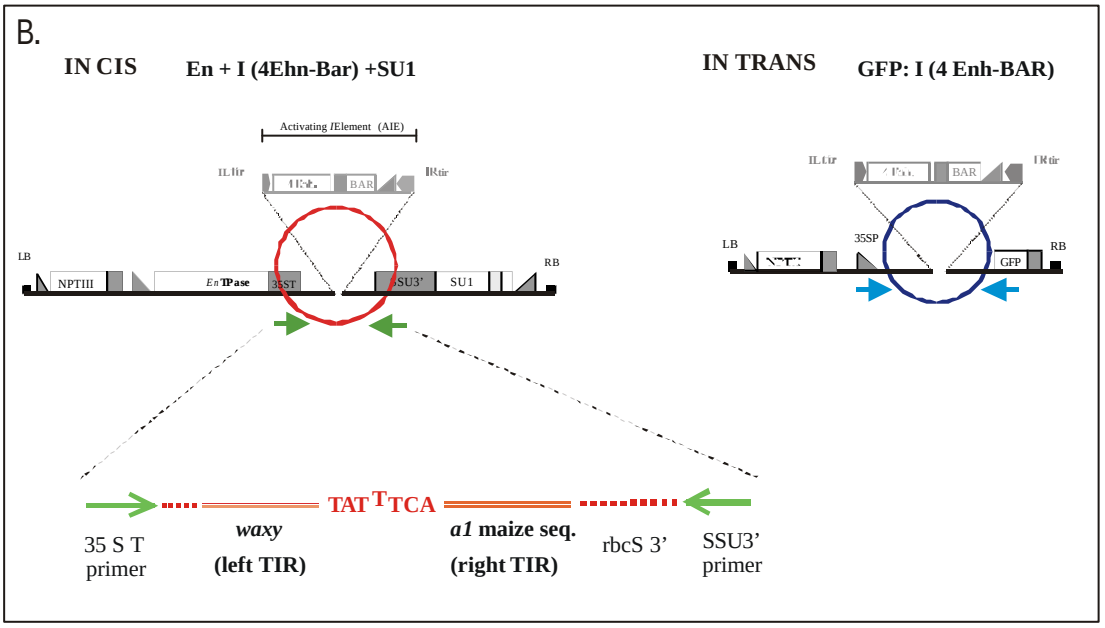


Figure 4. Transposon footprints in the T-DNA vectors. **A**, top: Expected footprint. Bottom: footprints found in En-I (in cis) and I (in trans) vectors. Footprints for *in cis* vectors are shown in red and for the *in trans* construct in blue. The left TIR was cloned from the waxy and the right TIR from the a1 maize loci. The Pst I site indicates where the left piece was cloned. The footprints are not perfect repeats. A possible explanation for this is given in the text. **B**, schematic representation of the PCR to obtain the footprints. Arrows represent the primers used. The gray parts depicted in the constructs are those corresponding to the mobile I element, not present in the T-DNA after excision occurred. TIR, terminal inverted repeats of the transposon; rbsS 3', Rubisco small subunit 3'; 35S P and T, 35S promoter and terminator, respectively; GFP, Green Fluorescent Protein.

In order to generate a population of transposon insertions and at the same time to test the parental lines, approximately 60 transposon lines were crossed to the 3 transposase lines in two rounds as shown in **Table V**. The three available En-*su1* lines were used to compare their activity.

Construct *GFP:I* (4Enh-*bar*)

	2	3	4		8	10	11	13	14	15	16	17	18	…	44
1	O	O	O	OX	O	O	O	O	O	O	O	OX			
4				X				O		O		OX			
5	X			X			X	O		O		OX	X		

Construct *GFP:I* (4Enh-ORI-*bar*)

	1	2	3		5	6	7	8	10	11	12	13	14	…	29
1	OX	O	O	OX	O	O	O			O	X	O	OX		
4	X			X		O				O	OX	O	X		
5	X	X	X	X	X	OX	X	X	X	X	OX	OX	X		

Table V. Examples of *En vs. I* crosses
O first round of crosses X= second round of crosses
Vertical numbers, transposase lines. Horizontal numbers, transposon lines.

Homozygous *En* plants were preferably used, though it was not possible in all cases. F1 seed were sown directly on plates with kanamycin/hygromycin or just hygromicin (for the plants containing the construct with origin of replication) depending on the resistance gene included in the T-DNA vector in the parental lines. From the two crossing rounds, a total number of around 1700 selected F1 plants were transferred to soil. Approximately 1200 were expected to be functional for transposition. Plants showing mutant phenotypes were observed among them. In many cases, the phenotypes were displayed in sectors of the plants (**Figure 5**), as expected for active transposase-containing plants. The presence of mutant phenotypes in this collection suggested active transposition. However, because the F1 plants were carrying non-stable insertions, many of the observed mutant phenotypes could not be recovered when the progeny seed of the putative mutants was sown, which complicated the study of the system. Phenotype evaluation was hindered in the first stages of development by the fact that the plants were selected first in plates with antibiotics, in the sense that some young plantlets

had deformations that could be due to insertions but could also be due to the antibiotic (as had been seen in other cases).

Though these *I* constructs showed excision by PCR and a variety of aerial mutant phenotypes were observed, they were not suitable to obtain large numbers of stable insertion plants when compared to the other strategy (*in cis*).

On the other hand, the size of the *in cis* construct was a disadvantage initially because only a few transformants were obtained. However, the transformation of higher numbers of plants was very useful to overcome the size problem. Finally, the *in cis* strategy showed to be easier to work with, particularly because the mutants obtained were directly stable after selection. The stability of the insertions made easier the study of the mutants, and was used then further to prove the system. Therefore, the lines generated with the *in trans* were not followed anymore.

However, after the comparisons between both systems, another *En* transposase construct was built. The EnTPase+4Enh+*GFP* was made because from the experiments comparing the *in cis* and *in trans* strategies, the *in cis* construct seemed to have a higher transposase activity. This could be attributed to the proximity of the 35S promoter of the transposase and the 35S enhancer tetramer (see **Figure 6**). A new transposase construct was then designed where the 35S enhancer was placed next to the transposase, and could increase its activity. A visible marker (*GFP*) was also added (see **Figure 3**). This marker does not affect so grossly the phenotype of the plant that contains it, as *su1* does. This construct can be used either for the *in trans* strategy with a variety of engineered *I* vectors, or to remobilize insertions in stable mutants (also the ones obtained by the *in cis* strategy).

2. In cis strategy

Development of an *En/Spm-I/dSpm* transposon *in cis* system

The *En-I* system was used to build a vector where both elements were present and could be used to obtain stable activation *I* insertions. The activation tag construct shown in

Figure 6 consists of three main components: 1) The *En/Spm* transposase coding sequence under control of the CaMV 35S promoter and terminator sequences; 2) A mobile, non-autonomous *I/dSpm* component harboring a tetramer of the CaMV 35S enhancer and the *bar* gene between the terminal inverted repeats, denominated Activating *I* Element (AIE), and 3) The negatively selectable marker *su1*, adjacent to the transposon components within the T-DNA (Tissier, A. *et al.*, 1999).

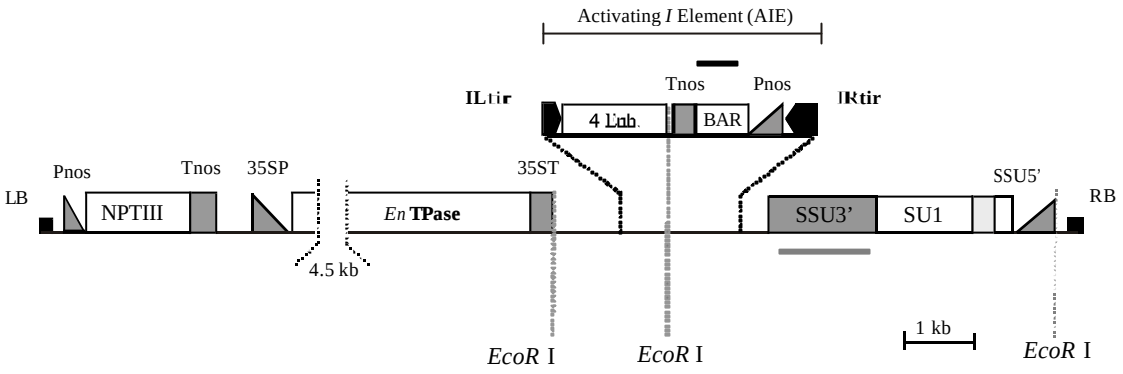


Figure 6. Schematic representation of the construct used for plant transformation for the strategy *in cis*. Relevant *Eco*R I sites used for the molecular analysis are indicated. LB, Left border; RB, Right border; 35SP, 35ST, CaMV 35S promoter and terminator, respectively; EnTPase, En immobile transposase source; ILtir, IRtir, I element left and right terminal inverted repeat, respectively; 4 Enh., tetramer of the CaMV35S enhancer; Pnos, Tnos, promoter and terminator sequences from the nopaline synthase gene, respectively; SSU5', SSU3', promoter and transit signal peptide to the chloroplast, and terminator of the small subunit of *RUBISCO* gene, respectively. The gene specific probes (*bar* and SSU3') used for blot hybridization are indicated as bars above or below the figure.

The transposon construct was introduced by Agrobacterium-mediated transformation into the Arabidopsis ecotypes Wassilewskija (Ws) and Landsberg *erecta* (L*er*). Twenty- six primary transformants termed WAT (Ws) and LAT (L*er*) were generated from the transformation experiments. Southern blot and segregation analysis of a selection of primary transformants revealed that they contained from 1 to 7 T-DNA copies at one or more loci (data not shown).

PCR was performed to analyze excision of AIE from the original construct in the primary transformants. Sequencing of PCR products confirmed the presence of an empty site

in the donor T-DNA (**Figure 4**). As for the *"in trans"* strategy, excision of the AIE element showed a hybrid footprint, confirming that the presence of the target site duplication is not necessary of excision. Over 90% of the first batch of primary transformants showed an empty donor site (EDS) by PCR, due to excision. Individual transformants displayed varying intensities of EDS fragments suggesting that there was enough transposase activity to produce excisions in the majority of transformants, and also indicating variation among them.

Preliminary analysis of the efficiency of independent AIE transposition was done by characterizing the kanamycin resistant progeny (containing the donor T-DNA) of one of the first transformants showing a high intensity EDS fragment (line WAT2). Among 50 kanamycin resistant plants analyzed, 17 different insertion bands could be seen in a Southern blot suggesting a high frequency of independent transposition (data not shown).

Selection and evaluation of active transposing lines

To select for stable transposed insertion events (transposants), selfed T2 seeds (progeny seed of primary transformants) were sown at high density in the greenhouse and sprayed with Basta (formulation Finale) and R7402 (DuPont) after germination. Surviving doubly resistant seedlings were counted to estimate the stable transposition frequency (STF, ratio of surviving plants to seeds sown for each family). Genomic DNA from these plants was subjected to Southern analysis to determine different AIE insertions and estimate the independent transposition frequency (ITF) (Aarts, M. G. M. *et al*., 1995). ITF is the ratio of the number of different insertion bands observed (by Southern, in this case) to the total number of plants analyzed from a family. This measure gives an indication of the frequency of independent transposition events and can be used to select families useful for generating new and independent insertions.

Pilot experiments were performed to determine the relationship between the STF and the ITF. For this, seeds from 4 lines were sown in soil and selected by spraying with Basta and R7402. Doubly resistant plants from each line were individually analyzed by Southern blot to detect different insertions. The results obtained are summarized in **Table VI**[a]. The primary

transformants displayed an inverse correlation between the STF and the ITF of each line, as observed previously (Tissier, A. *et al.*, 1999).

Single plant analysis [a]					
Line	**# plants**	**# plants analyzed**	**# Different insertions**	**STF%** [c]	**ITF%** [d]
WAT1	23	23	2	13.33	8.9
WAT2	18	18	9	0.23	50
WAT3	11	11	2	3.8	18.2
WAT3	18	18	4	4.82	25
WAT5	18	18	6	2.83	31.5
Pooled plants [b]					
Line	**# plants**	**# plants analyzed**	**# Different insertions**	**STF%** [c]	**ITF %**
WAT3	19	6	1	4.52	16.67%
WAT8	31	7	8	1.9	114%
WAT10	25	8	5	1.7	62.5%
WAT14	29	6	5	1.86	83.3%
WAT15	8	8	1	0.8	12.5%
WAT18	29	10	1	2.45	10.0%
WAT20	8	8	1	0.69	12.5%
WAT21	9	9	6	1.18	66.6%
WAT23	10	10	3	0.82	30.0%
WAT24	32	7	2	1.31	28 %
WAT29	3	3	3	0.92	100.0%
WAT30	3	3	2	0.68	66.7%
WAT32	7	7	3	0.39	42.8%
LAT2	6	7	4	0.83	57.1%
LAT3	37	13	2	8.2	15.4%
LAT26	6	6	1	1.43	16.7%

Table VI. Stable and independent transposition frequencies of sprayed *in cis* progeny.

[a] Data obtained with single plant Southern analysis.

[b] Data obtained using pooled samples for the Southern analysis.

[c] The stable transposition frequency (STF) was calculated as the ratio between the number of double resistant plants and the number of double-sprayed seeds.

[d] The independent transposition frequency (ITF) was estimated as the ratio of the number of different fragments observed to the total number of plants analyzed from a family.

The inverse correlation between IFT and STF can be attributed to the timing of excision: early excisions produce large clonal sectors in the plant, giving rise to a high number of stable progeny but with a low diversity of AIE insertions. Late excision events give rise to small clonal sectors that are represented as a few resistant progeny seedlings with a high diversity of independent insertions. On the basis of STFs and ITFs, lines WAT2 and WAT5 were identified as potentially suitable parents to generate a population of independent stable insertions.

In order to create a large population, additional parental plants useful for insert generation were needed. With this aim, T2 seeds from the remaining primary transformants were used in double selection experiments on a bigger scale, and the STFs per line calculated. Surviving seedlings after the double selection with Basta and R7402 were transferred to new soil and DNA was extracted from inflorescences in pools of 8-20 plants from the same transformed line. The DNA from the pools was analyzed by Southern blot analysis and transposon insertion display (TID) (Tissier, A. *et al.*, 1999; Yepremov and Saedler, 2000) was carried out to identify independent insertions. Southern analysis proved to be sensitive enough to estimate single AIE inserts in the pool based on reconstruction experiments (data not shown). The autoradiogram shown in **Figure 7A** displays the variation in insert number from the pooled DNA from stable insertion lines derived from different transformants.

The ITF ranges from less than 5 to 114% (where at least one of the sampled plants had more than one independent AIE) among the different lines tested. For example, 8 different bands can be seen in the **Figure 7A** in the pool of plants derived from WAT8, which included 7 plants. In contrast, only 1 band is visible in a pool of ten plants from line WAT18. The small sample of plants used in this analysis did not allow a precise comparison with the STFs but supported the selection of optimal lines. **Table VI[b]** shows the ITF and STF for most primary transformants. This larger double selection experiment resulted in the generation of an initial population of 1300 doubly resistant plants (denominated population 1). The total number of independent inserts in population 1 was estimated to range between 350 and 500.

Preliminary analysis of individual stable plants revealed that the number of AIE insertions was low, ranging from 1 to 4 per plant. In a random sample of 17 doubly resistant plants from population 1 obtained from different primary transformants, 76.4% contained one AIE, 17.6% three and 5.8% four AIE inserts (**Figure 7B**). In two additional independent

samples of plants derived from the same primary transformant (WAT2), the majority of the plants contained one insert (60% and 73% in each group), and the rest had two (13 and 33%) or three insertions (13 and 8% in each group). Transposon insertion display (Tissier, A. *et al.*, 1999), using representative pools of plants provided data on the distribution of insertions in population 1. **Table VII** shows the chromosomal distribution of sixty-nine insertions obtained from DNA of plant pools derived from different lines.

Line	Chr. I	Chr. II	Chr. III	Chr. IV	Chr. V	Total insertions[a]
WAT2	1	2	7	6	2	18
WAT3		1			1	2
WAT8	2		5	4	1	12
WAT10	2	2	3			7
WAT13	1	2	1	2		6
WAT14	1	4			1	6
WAT17	2	1	1	1		5
WAT19		2	1	2	1	6
WAT21	2		1			3
WAT24			1	1		2
WAT30					1	1
WAT32				1		1
total	11	14	20	17	7	69

Table VII. Number of AIE inserts in each chromosome.

[a] Inserts in representative plants from population 1.

Figure 8 shows the approximate position of inserts found in progeny of two lines, WAT2 and WAT8, in each chromosome. These results show that insertions in most Arabidopsis chromosomes can be obtained in the progeny from a single line and that the progeny of different lines produces an even distribution of insertions in the Arabidopsis genome (**Table VII**). This suggests that the diversity of insertions in populations generated from these lines will be large and most probably should be able to saturate the genome when an adequate number of plants is used.

Lines showing the highest ITF (more than 30-40%) as well as an acceptable STF (less than 3%, like in lines WAT 2, 5, 8, 10, 14, 21, 23, 29, 30, 32, LAT2 and 26) were chosen for further double selections to increase the insert collection (populations 2 to 5). The unselected

T2 and T3 plants from the chosen lines were used to generate more stable insertions with the double spray selection method. To check the ITF of the following generation, plants resistant to the double selection were analyzed by Southern blot. Line WAT8 still showed an ITF of 50% with mainly single inserts, while line WAT10 had an ITF of less than 30% independent insertions indicating that the calculated frequencies were adequate enough to make predictions for the population (data not shown).

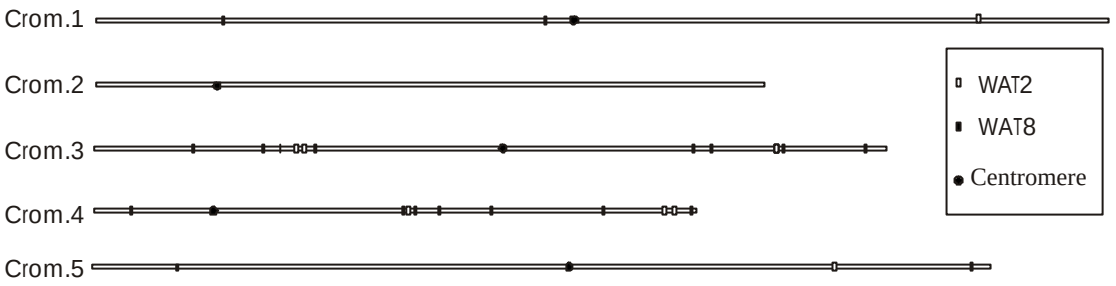


Figure 8. Schematic representation of AIE inserts deriving from two different transformants, WAT2 and WAT8, in the genome of Arabidopsis.

The AIE number per plant remained consistent in different generations for the analyzed lines (WAT8, WAT10 and WAT14), being 4 the highest number of inserts found per individual. For example, from 20 T3 WAT8 plants, 14 have one (70%), five have two (25%), and one has three inserts (5%). Progeny derived from outcrossing T3 WAT8 plants with the wild type still bear a low number of insertions, as can be observed in **Figure 7C**.

Generation of an En-I Activation Tag population

Around 1% of the plants bearing stable insertions in population 1 were visible morphological dominant mutants. This frequency suggested that the mutations were due to activation and not just to knock-outs, because many plants were expected to be heterozygous for the insertions. Moreover, the frequency of visible morphological dominant mutants seemed to be higher than the reported with activating T-DNA (Weigel D. *et al.*, 2000). Because of these encouraging results, the experiment was scaled up to obtain more stable transposants.

Five populations were obtained in slightly different ways. The transformed genotypes that revealed optimal frequencies of stable transposants and ITFs on the basis of molecular genetic analysis, were selected to generate a larger population of inserts. Initially the selfed T2 and T3 progeny were used as shown in **Figure 9** for construction of populations 1 to 3. These lines often gave high numbers of *su1* homozygous plants and could harbor fixed insertions that occurred early in the transformed lines. To avoid these potential problems, the T2/T3 progeny of the primary transformants was crossed to wild type and the F2 progeny used for selection of stable inserts to obtain populations 4 and 5 (**Figure 9**).

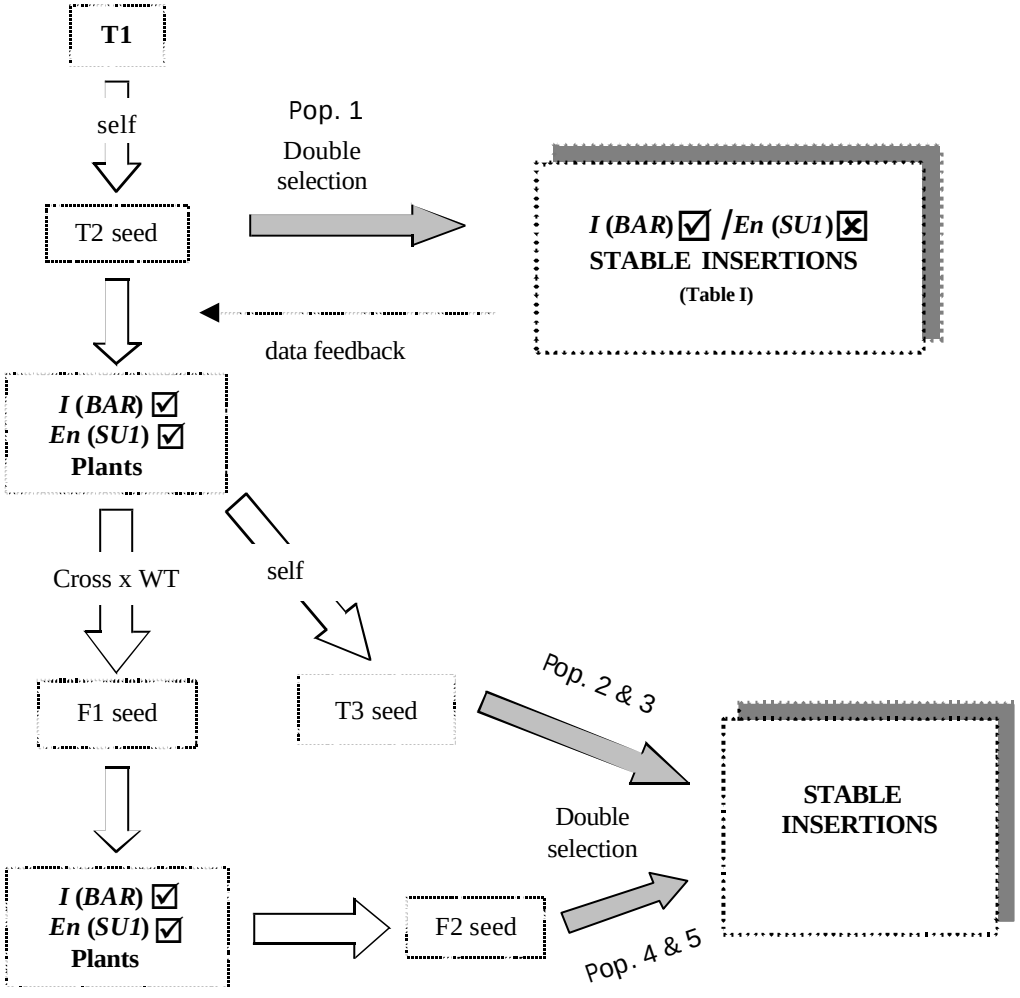


Figure 9. Steps followed to generate the population of stable insertions. The process starts from the first transformants (T1) containing the construct shown in **Figure 6**. Several rounds of stable insertion selections were carried out using either self or F2 seed as parental lines. The resulting population number reported in **table VIII** is indicated for each case.

Molecular analysis of sets of plants from selected populations using the TID technique and Southern blots revealed the ITFs of different lines and allowed an estimate of the frequency of stable insertions. Lines with very high numbers (more than 20 stable plants out of 1000 tested seeds) were regarded as producing redundant or non-independent inserts. On the other hand, lines that produced a few inserts were considered as mostly independent. Based on this data, low frequency of stable transposition (under 20 plants per 1000 seeds) was used as an indicator for selection of lines for generating new transposant populations.

Because population 5 was grown under optimal conditions for mutant identification, this population was therefore ideal to estimate the dominant mutant frequency. Some mutants could not produce seed due to sterility or weakness, and thus were not used further in the analysis. An example of a sterile, miniature, bushy mutant is shown in **Figure 10.**

Among 26 putative mutants from population 5 for which the progeny could be analyzed, 25 showed a dominant or semi-dominant inheritance of the phenotype. These 25 mutants from 2500 estimated stable activation tags show that the frequency of observed dominant mutants is about 1%. This result is consistent with the data generated obtained in the population 1.

The mutants selected from population 5 showed diverse phenotypes with alterations in a wide range of characteristics such as fertility, height, flowering time, branching, flower structure, plant architecture etc. An example of a developmental mutant with a serrated leaf structure is shown in **Figure 10**, indicating that developmental mutants can also be obtained using activation tagging with this transposon system. In the resulting total population (consisting of 5 sub-populations) of about 13000 plants containing an estimated of 8300 inserts, a variety of aerial morphological mutants have been identified as shown in **Table VIII**.

Population number	# of parental genotypes[b]	# of selected Transposants[c]	# of Insertions Estimated[d]	# Mutants (dominant)[e]
1[a]	54	1346	400	8 (4)
2	188	2688	800	1 n.d.
3	246	1248	600	4 (3)
4	1250	4960	4000	8 (8)
5[a]	726	3000	2500	35 (25)
Total		**13242**	**8300**	**56 (40)**

Table VIII. Number of stable transposants and estimated insertions in different populations.
[a] Plants screened in pots at low density.
[b] Number of segregating T2/T3 or F2 genotypes used.
[c] Plants with stable insertions.
[d] Estimated on basis of molecular analysis and stable insertion frequency.
[e] Number of confirmed dominant mutants based on segregation.
n.d. not determined.

CHAPTER IV.
RESULTS (Part II)

AERIAL MORPHOLOGICAL MUTANTS FOUND IN THE POPULATION

Mutant identification and characterization

Many visible morphological mutants, most of them Ws, were observed during the construction of the activation tagging population, identified during the transplantation to trays or pots, and for some populations, during later visual screens. To determine the dominant nature of some of these mutants and the type of genes affected, the mutants identified from population 1 were characterized in detail.

Characterized Mutants (population 1)

Population 1 was selected from amongst the original (T2) progeny of the transformants and was expected to have mainly heterozygous insertions. Phenotypic screens for obvious morphological mutants in the greenhouse revealed some new as well as previously observed phenotypes within this population. Inheritance tests were done using selfed or backcrossed seed. Though some mutant phenotypes did not reappear in the progeny, 8 phenotypes were shown to be heritable. After segregation analysis two mutants were found to be recessive, four dominant, and the inheritance of the remaining two remained unclear, but were apparently dominant. Inheritance studies were done by crossing the mutants to wild type plants, as male as female when possible. Always Ws, the parental ecotype, was used. Sometimes different ecotypes like L*er* and Col were also used.

The recessive mutants included the well-described mutant *fiddlehead (fdh)* with fused inflorescences that originally did not display a leaf fusion phenotype, although the progeny showed a phenotype similar to previously isolated *fdh* mutant alleles (Yephremov, A. *et al.*,

1999, Pruitt R. *et al.*, 2000). Variegated leaves and highly variable flower phenotypes characterized the second recessive phenotype (**Figure 11 A and B**).

The dominant mutants observed included *thread, empty siliques, cloudy,* and *bountiful,* and they are further described later in the text (*bountiful, thread,* and *cloudy*, phenotypes are shown in **Figure 11 D, H** and **I**, respectively). Inheritance studies of the other two mutants have not been conclusive, but strongly point to dominance. For example, one phenotype could not be recovered when Col wild type plants were used as female parents for the cross (data not shown), but was recovered when Ws plants were used. One of these two mutants was found to be extremely small in size, with a profusion of small, relatively serrated and wide leaves, reduced shoots and very little seed set **(Figure 11)**. In the second one (ecotype L*er*), two different phenotypes were segregating in its progeny: one sterile with trichomes and the other fertile without trichomes. Both plant types also had very short, thick, crooked siliques and curved leaves (**Figure 11**).

To further characterize the dominant mutants selected from population 1, first, Southern analyses were performed in the progeny of mutant vs. wild type crosses to determine whether an insertion cosegregated with the mutant phenotype. When this was confirmed, molecular characterization of the insertion was sought. Most mutants showed only a single insertion. To characterize the nature of the insertions conferring the different mutant phenotypes, DNA flanking the inserts was sequenced and used to position the insert in the genome and determine its context with respect to adjacent annotated genes. The *fiddlehead* mutant had the most recognizable phenotype and isolation of flanking DNA confirmed the mutant to be a knockout in the *FDH* gene (Yephremov, A. *et al*., 1999). For the dominant mutants, however, the AIE was not found to be present inside a predicted or experimentally defined coding region. The predicted/annotated genes in the region around the insertion were then identified, and primers were designed to clone them and analyze their expression. Genes closer to the enhancer of the AIE were regarded as primary candidates to be overexpressed and to account for the observed phenotype. The expression level of the candidate mutant genes was first examined by semi-quantitative RT-PCR analysis in mutant and non-mutant siblings (wild type control) among the progeny of the original mutant. In all cases, increased levels of RT-PCR products from a gene near the insertion site were obtained from the plants showing the phenotype as compared to the wild type.

In general, in the dominant mutants the insert was placed upstream of the overexpressed gene. The enhancer tetramer is present next to the left border of the mobile element. However, as the activating *I* element is small, the enhancer sequences can act on genes that are on either side of it. The phenotypes and details of the molecular characterization of each dominant mutant of population 1 is described below. All these mutants have a Ws background.

empty siliques

The *empty siliques* mutant owes its name to its developed but seedless siliques (**Figure 12**). This mutant has a slower development than wild type, and flowering is delayed. Crosses to the wild type in both (male and female) directions resulted in the formation of viable seed. Fertility is lower than in the wild type in both cases, with the mutant being even less fertile as a male. When pollinated by wild type pollen, the mutant silique grows further, and finally becomes larger than unpollinated mutant siliques and wider than wild type ones. The stem grows in an undulating fashion and the F1 seeds (produced by the female mutant) are larger than the wild type seeds while ovule and embryo sac development are normal (Chun-Ming Liu, personal communication).

The AIE in the mutant *empty siliques* (**Figure 12**) is inserted around 500 bp upstream of a cytochrome P450 gene homologue classified as *CYP78A9*, as shown in **Figure 13.** *CYP78A9* was previously identified by T-DNA activation tagging (Ito and Meyerowitz, 2000).

thread

The name *thread* was given to this mutant because it shows a pronounced apical dominance. Due to this enhanced apical dominance, only a single stem is present during a long period of the life cycle of this plant. This stem is not erect, but rather "loose", laying on a side and giving the impression of a thread. In the last fourth of the life of this mutant, more stems emerge, both from the rosette and from the main stem to form secondary branches.

This is an early flowering mutant. In contrast to the wild type that in long days flowers when the rosette has 8 leaves, thread flowers when the rosette has only 5 leaves.

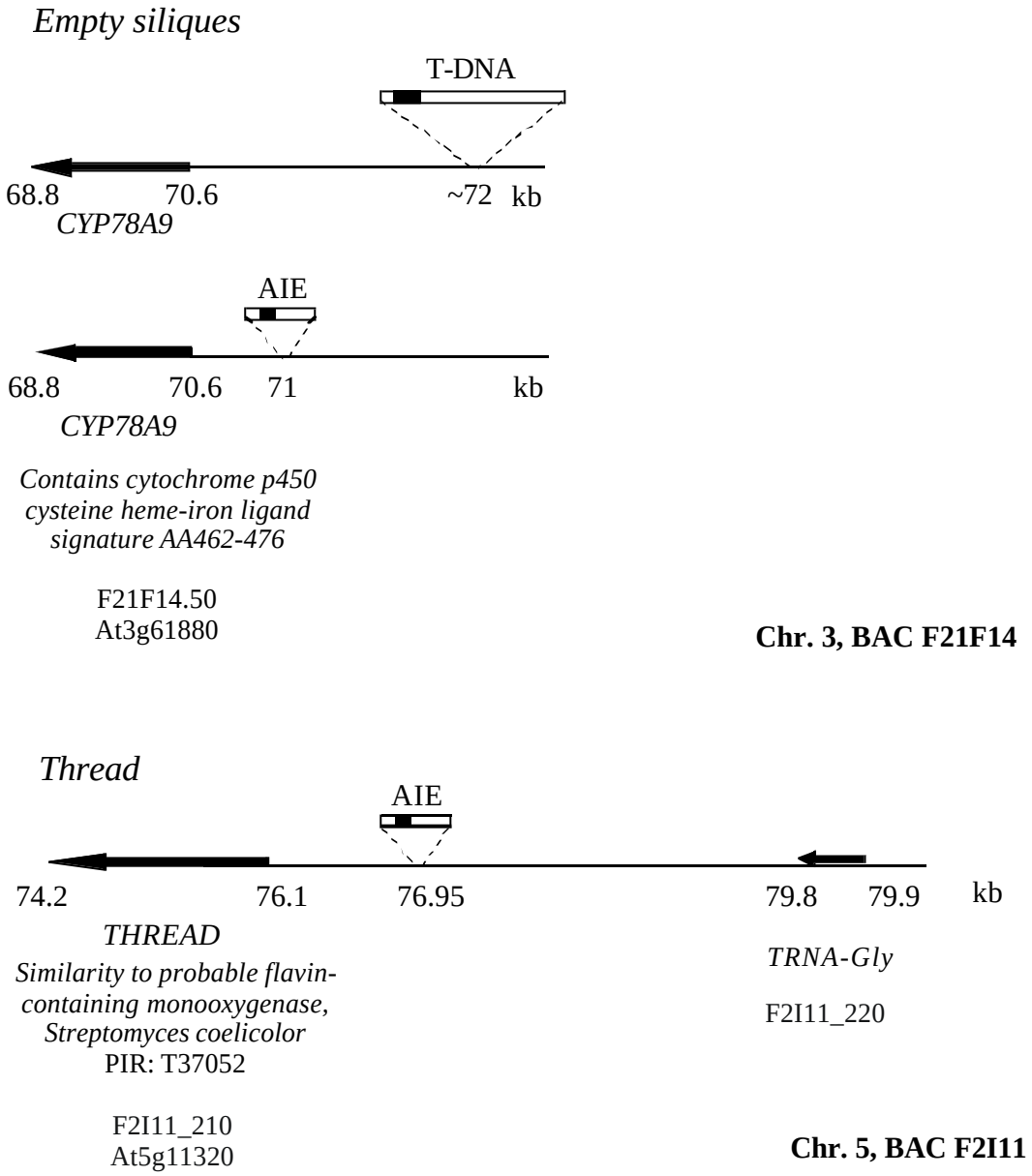


Figure 13. Position of the Activating I Element (AIE) and T-DNA insertions in the mutants *empty siliques* and *thread*. A, AIE insertion in the thread mutant. B, AIE and T-DNA (Ito et al. 2000) insertions affecting the CYP78A9 gene (empty siliques phenotype). The dark box inside the "insertions" represents the enhancer tetramer.

Very reduced, these leaves show an epinastic growth habit: their "tip" curves downwards. Their size is about 5 to 10 times smaller than a wild type leaf, and the leaf petiole seems longer than its wild type counterparts. These plants also show very elongated hypocotyls upon germination.

Additionally, *thread* flowers are very reduced and sterile. They do not open normally. Crosses using wild type pollen allow the recovery of few seeds per silique (from one to ten seed). However, seeds were not obtained when using *thread* pollen to fertilize wild type emasculated flowers. Silique shape is also changed in this mutant. Instead of a rounded silique tip, *thread* siliques have a pointed tip, and they never reach the size of a fully mature wild type silique. Like leaf petioles, siliques seem to have a longer petiole. When they mature, is common to observe siliques where the top is almost white, the middle is yellow/brown and the bottom is still green, as the petiole and the stem.

This mutant has an AIE –Activating *I* element- insertion 828 bp upstream of the predicted translation start codon of the *THREAD* gene (F2I11_210) (**Figure 13**). A clear PCR product is visible only in the RT-PCR samples of segregating seedlings showing the mutant phenotype, but not in wild type plants, suggesting overexpression of the gene in the mutant and its possible role in causing the phenotype (**Figure 14**). No PCR product could be visually detected following 30 amplification cycles for wild type flowers, but after 38 cycles a fragment of low intensity could be observed (data not shown).

To confirm that the observed phenotype was due to gene activation, a gene construct was made in which the *THREAD* coding sequence (from Ws) was placed under control of the CaMV 35S promoter and transformed to wild type ecotypes Col, L*er*, and Ws (the original ecotype) plants. **Figure 12** shows the overexpression phenotype obtained by transforming the *THREAD* gene as compared to the original *thread* mutant phenotype (**Figure 12).** The phenotype was recovered in the three ecotypes transformed, although with some differences. From 7 Ws transgenic lines, all had epinastic leaves and short siliques, showing a very similar phenotype to the original mutant except one that had elongated siliques. These results confirm that the phenotype observed in the activation mutant is due to the enhanced transcription of the *THREAD* gene. From 13 Col transformants, 11 showed the epinastic phenotype in the leaves, with varying degrees of severity. The original mutant in the Ws ecotype had short siliques, but in some of the Col transformants the siliques were elongated, even though the epinastic leaf phenotype was evident. From 10 L*er* transformants, all displayed the leaf phenotype but the siliques, with one exception, were elongated. This illustrates the variation in phenotype not only among transgenic lines, but also among ecotypes.

The *THREAD* gene has been annotated and predicted to encode a flavin monooxygenase-like enzyme although no phenotype or function has been experimentally

determined previously. However, the predicted THREAD protein shares 66% amino acid identity with YUCCA, encoded by a cytochrome P450 gene that has recently been characterized by T-DNA activation tagging (Zhao, Y. *et al*., 2001). The *YUCCA* gene family has 9 members, and the overexpression of some of them leads to the overproduction of auxin, which results in the *yucca* phenotype, similar to the *thread* overexpression phenotype (Zhao, Y. *et al.*, 2001). The *THREAD* gene described here is thus a new member of the family that displays a similar phenotype, suggesting similar functions.To confirm whether the *thread* phenotype was related to auxin overproduction, *thread* mutant plants were crossed to the marker line DR5 - *gus,* and the F1 progeny analyzed (**Figure 15**). This line uses a synthetic promoter, DR5, which consists of 7 tandem repeats of an auxin-responsive TGTCTC element and a minimal 35S CaMV promoter. This synthetic promoter is fused to the *gus* marker gene, and it helps to monitor auxin in the plant (Ulmasov, T. *et al.*, 1997).

Interestingly, *thread* seedlings showed an intense blue X-gluc staining in the tissues with the characteristic mutations. In contrast, wild type seedlings showed a remarkably less intense staining pattern (**Figure 15**).

Some of the other dominant mutants were also crossed to DR5 - *gus* but no significant changes in *gus* expression were observed. Maybe closer observations might reveal subtle but interesting changes in those mutants. In any case, *thread* showed the greatest contrast to the original DR5 - *gus* line *gus* expression. This increased staining pattern may suggest increased auxin content in the mutant, as would be expected from the similarity of the *thread* to the *yucca* phenotypes and genes.

bountiful

bountiful mutants are characterized by wrinkled leaves of increased size, hence the name *bountiful.* These plants are also slow in general development and show a late flowering time phenotype. Additionally, their siliques are vertically oriented, giving them a particular aspect in comparison to wild type plants (**Figure 16**). Interestingly, these mutants display differences when homozygous or heterozygous. Homozygous plants are plants of reduced size with more severe mutant features. For example, remarkably bigger leaves and almost vertical

siliques. Southern analysis revealed of a single insertion showing cosegregation with the *bountiful* phenotype.

bountiful had an AIE insertion in chromosome three, BAC F7O18 (see **Figure 17**)**.** Gene At3g04560 (F7O18.3) lays to the right side and codes for an unknown protein, but a cDNA clone is available. The start codon is around 1800 n away from the insertion, and around 3800 n from the enhancer.

To the left side, and hence nearest to the enhancer, an intronless gene is present. The start codon begins just 154 n after the insertion, and around 350 after the enhancer. Interestingly, this gene At3g04570 (F7O18.4) is a putative transcription factor. It is predicted to have an AT-hook DNA binding site. Moreover, a full length cDNA is available (Ceres:15781). A very similar gene named *ESCAROLA* was reported by Weigel *et al.*, and might belong to the same family. Like *bountiful*, *escarola* mutants have wrinkled leaves, that make them look like a lettuce. However, further description of *esc* mutants has not been made publicly available yet (Weigel, D., *et al.*, 2000).

Because of the proximity of the promoter of the gene to the enhancer inside the AIE and its predicted function, F7O18.4 was thought to be causing the *bountiful* phenotype. Molecular analysis confirmed this idea. Non-quantitative RT-PCR experiments clearly showed the overexpression of the gene in leaves (**Figure 14**). Rosette and cauline leaves from wild type and mutant siblings were assayed. Also wild type seedlings and flowers were included in the experiment. Wild type-like siblings did not show a visible band in rosette and cauline leaves, whereas siblings displaying the *bountiful* phenotype did. Interestingly, wild type seedlings showed the presence of a band, though less intense than those observed from *bountiful* leaves. Moreover, transformation of wild type plants with a F7O18.4 cDNA after a CaMV 35S promoter caused a severe phenotype in the first transformants, which was attenuated and more similar to the mutants in following generations.

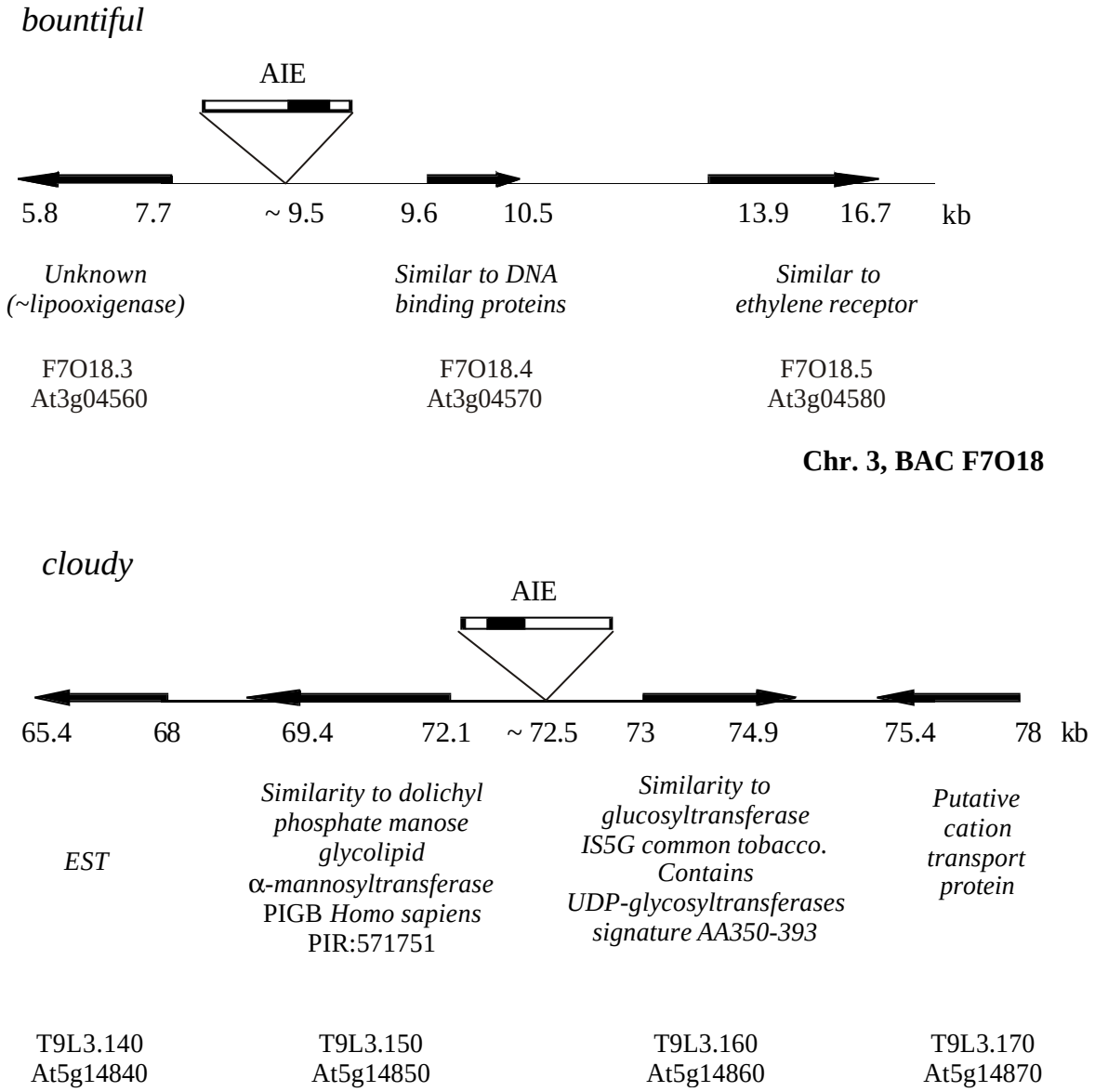


Figure 17. Transposon insertions in *bountiful* and *cloudy* mutants identified in population 1. Position numbers (kb) are rounded. The dark rectangle depicted inside the AIE represents the 35S enhancer tetramer.

cloudy

The *cloudy* mutant shows a wild type phenotype up to 14 days after germination. Young rosette leaves seem normal as they emerge, but as leaves mature, they start to show

upwardly curved growth. The ends of older leaves appear longitudinally rolled. Flowering occurs slightly earlier than WS wild type plants in long days (6-7 vs. 7-9 leaves). Initial flowering shoots seem also normal. However, as it develops, the stem begins to show a wavy growth fashion. This occurs for the main and the secondary stems as well.

Flowers have also a special phenotype, with thinner petals that do not form a completely closed flower and seem to have gaps between them. At later stages the mutant develops a higher number of lateral and secondary shoots than wild type plants. These stems are generally thinner than wild type and also show a wavy growth fashion (**Figure 18**). This increased number of fine stems is why the mutant was named *cloudy*. It has very reduced fertility at the beginning and middle of its reproductive phase.

The siliques are very short (less than half WT and have very few seed, if they have). The stems at this moment are shorter than the control, being the total height of the plant about a half of the WT. Surprisingly, at later stages some stems start to elongate more, almost at normal height, and start to set seed. *cloudy* lives longer than the WT. About the time that the control plants are at the end of their life, the mutant starts to form small, axilary stems that are able to flower. Some shoot tips show double siliques at this stage.

Interestingly, when the plants are under stress, like when exposed to numerous flies, the mutant phenotype (like the characteristic rolled leaves) is very mild, almost inexistent. The *cloudy* phenotype appears to be environmentally conditioned in some cases.

Five plants showing a similar phenotype were identified in population 1, and could be traced back to the same original parental line. Crosses versus Ws wild type plants were performed to analyze inheritance. In two lines the phenotypes of the progeny were not clear enough to give a clean score. In the other three lines, the inheritance indicated that the mutation was dominant. Though no further analysis was done, the results obtained with the original crosses suggested that the phenotype was not well transmited as male (see **Appendix I**). There seems to be a male fertility reduction. No phenotype was found in the progeny of crosses to wild type plants when the mutant was used as a male. Moreover, analysis of those plants by Southern revealed no insertion band.

Southern blot analysis showed cosegregation of a band with the *cloudy* phenotype. One of the original mutants with clear mutant progeny had a single band while the other two had an extra band. All the lines showing a clear mutant phenotype shared the same band. For the mutants with mild phenotype progenies, the same band correlated with the mild phenotype in F1 progeny (mutant vs. Ws).

A TAIL-PCR was performed in order to find the position of the transposon insertion in the genome. It was located in chromosome 5, in the BAC clone T9L3 (accesion AL391149), around nucleotide 72,482 (see **Figure 17**). In that area the closest predicted genes were T9L3_150 (At5g14850) and T9L3_160 (At5g14860). T9L3_150 was annotated as highly similar to dolichyl phosphate mannose glycolipid a-mannosyltransferase PIGB from *Homo sapiens* (PIR:571751).

On the other hand, T9L3_160 was annotated as highly similar to glucosyltransferase IS5G from common tobacco, containing the UDP-glycosyltransferases signature AA350-393. The start codon of the T9L3_150 gene was circa 400 bp far from the left terminal inverted repeat of the transposon, whereas the translation start of T9L3_160 was placed around 500 bp from the right end of the transposon. The 35S enhancer tetramer is positioned next to the left of the transposon, thus, 2 kb extra (representing the *bar* gene, including a *nos* promoter and terminator) must be considered to calculate the distance from the enhancer to the beginning of the gene to the right side of the insertion.

A non-quantitative RT-PCR was performed to know preliminarly if one of these genes, or both, were overexpressed (**Figure 14**). Cauline leaves, whole seedlings and flower tissues were used to compare expression. Additionally, samples of segregating plants showing and not showing the mutant phenotype (and with or without the related insert band in the Southern, hence) were used for the experiment.

Using the mannosyltransferase (PIGB) primers, a faint band was observed in the flowers from plants showing the mutant phenotype. A smear was present in wild type flowers. However, when the glucosyltransferase primers were employed, clear bands of the expected size (around 1461 bp cDNA) were present in the seedling, leaf and flower samples from the mutant plants. Segregant (without phenotype and the related insertion) and wild type samples did not show the 1.4 kb PCR band. The intensity of the band was higher in flowers, decreased

in the leaf, and was the lowest in the seedling samples. Based on those results, the glucosyltransferase cDNA from the mutant was used to build an overexpression construct. The construct was introduced to wild type Wassilewskija, Landsberg *erecta* and Columbia Arabidopsis ecotypes. However, this 35S-promoter overexpression construct of the glucosyltransferase-like gene did not result in the *cloudy* phenotype in wild type plants.

cloudy hormone treatments

Initially, in order to investigate if the phenotype was related to hormone availability (which could happen if the glucosyltransferase overexpression could result in IAA glycosilation), the following experiment was done. Mutant and wild type plants were sprayed with auxin (IAA), giberellin (mix of GA4 and GA7) and cytokinin (zeatin), and their phenotypes observed. Water and water with Triton X-100 were used as a control.

Besides the expected effects of each hormone in the plants, like higher size with giberellins, or shorter with cytokinins, the mutants seemed to show reduced leaf rolling after spraying with IAA (when the shoot was around 9 or 10 cm. long in the water treated plants). On the other hand, cytokinins seemed to cause a more severe rolling. Leaf rolling was not induced in wild type plants by these hormone treatments (**Figure 18**). In later stages, after flowering, the effect of the different hormones in reverting the mutant phenotype was not so evident as the effect of IAA in the leaves, maybe because flowers were only treated twice a week instead of daily. However, though not carefully measured, it seemed that the IAA treatment had a slight influence in seed set.

Other effects included the higher frequency and earlier appearance of features like the double siliques in the shoot tip for the plants treated with zeatin, and the appearance of small axilary shoots in the plants treated with gibberellins (**Figure 18**).

Interestingly, these four dominant mutants seemed to live longer than wild type plants, which complete their life cycle in about 4-6 weeks. At later life stages, many of them developed aberrant organs at the top of stems. Moreover, the mutant phenotypes seemed attenuated in late development.

Mutants in characterization (populations 2-5)

Population 2, 3 and 4 were grown in 96-well trays, which made difficult the visualization of mutants. However, some interesting mutants were observed. Some insertions in the mutants obtained in the following populations were also analyzed, but many have not been proved to cause the mutant phenotypes yet.

In population 2, a visible morphological mutant was *surco* (**Figure 19**). This mutant has round, slightly wrinkled leaves with a deep furrow, therefore the name (in spanish, "surco" means "furrow"). This is a "half sized" plant. The main infloresence is only around 15 cm. long, contrasting with the at least 35 cm. of a wild type plant grown in the same conditions. It does not have many secondary stems, as occurs with some other dwarf plants. Also in contrast with other dwarfs, *surco* is not dark green. *surco* is a semidominant mutant: F1 plants did not show the clear leaf phenotype but the stems showed an intermediate size between the parental mutant and the wild type. This mutant has a unique AIE insertion in chromosome three, P1 clone MRP15. As in other dominant or semidominant mutants, the insertion is not inside a gene. This mutant has a Ws background. In population 3, at least four mutants with interesting aerial phenotypes were identified in the Ws background:

- ***picos*** was a plant of reduced size, which reached a maximum diameter of 3 cm and a height of also 3 cm. It showed extremely thick, dark green leaves. Interestingly, the leaves were markedly narrow, transversally curved, and their borders had several very pronounced serrations that looked like sharp points[3]. The plant had a longer life when compared to the wild type and its siblings, and it did not show any normal infloresence stem or flowers (**Figure 20**). However, it had a stem-like structure that was flat and curved, covered with hair-like structures, growing in between the leaves. In time, *picos* developed a high number of leaves. Because no flowers were present at all, no seed was obtained from this mutant. Fortunately, leaf tissue was collected and DNA was obtained from it to make Southern analysis and TAIL-PCR. No other *picos*-like plant was found among the progeny of the parental line, indicating the possibility of being a new insertion not present before in the parent.

[3] "Pico" means "sharp point" in Spanish.

- ***bolita***. This mutant had also reduced rosette and stem size. Its leaves were dark, thick, and longitudinally downwards curled, giving the impression of being longer than wild type rosette leaves. As a special feature, the leaves seemed to elongate spirally. Likewise, the stem grew in a spiral fashion, resulting in a very compact stem that did not grow straight. This compact stem was never taller than the rosette itself, also very compact. Because of this lack of stem elongation, the flowers were all compacted in a short space (**Figure 20**). Flowers had also a mutant phenotype but they were fertile, giving rise to many seed after autofertilization. The total plant height was around 3 cm. It looked like a small "ball", hence the name "bolita" (small ball in Spanish). Segregation analysis showed this mutation to be dominant.

- The mutant ***arrugas*** was named after its round and slightly corrugated or "wrinkled" leaves (wrinkle is "arruga" in Spanish). The corrugation was mainly in the central part but not in leaf borders. This mutant has a higher number of shoots and flower buds. Interestingly, some flowers seemed completely normal and produced seed after fertilization while others had shorter pistils and did not produce seed (**Figure 20**). Therefore, adult mutant plants had stems with a mix of elongated wild type siliques and extremely short unfertilized pistils. Segregation analysis strongly point to dominance. Unfortunately, molecular analyses were not yet conclusive.

- A fourth visible mutant was ***largo*** ("long" in spanish). Initially, it develops normally. When the shoots appear they seem also normal. However, as the stems grow, a "bald" shoot tip can be seen. There are no flower buds at the top but rather a stem-like structure with hairy protuberances that look like unfertilized pistil hairs. The flowers are female sterile but male fertile. The pistil is extremely thin and never fills to give seed (**Figure 20**). This causes siliqueless stems. This lack of full siliques gives the stem the impression to be longer than wild type stems. This mutation was inherited in a dominant fashion. The name of this mutant was later changed to ***needle***.

Population 4 was grown in a greenhouse where the temperature increased to more than 30 degrees more than once. Therefore, phenotype scoring was more difficult. Many mutant plants did not survive these conditions. A few mutants could be moved to better conditions after some weeks. Unfortunately, not all these mutants were in conditions to give viable seed

and some were lost. Nevertheless, the phenotypes that were seen were different from those observed before, and indicated the diversity of novel insertions present in this population.
A few examples of the observed mutants in Ws background are:

- *down siliques*, seemed normal, but the siliques pointed downwards and were sometimes arranged in bunches.
- *loops* had loop-like leaves, and a lot of very small shoots.
- *messy* had dissorganized leaves growing in different directions. Never had a shoot and died without giving seed, as *loops*. It had a normal color but was very reduced in size.
- *nice*, which had reduced, oval, and very hairy leaves (like peach skin). It had also many tiny shoots. It produced very little seed (only 12 seed were obtained from it).
- *serrate*-like had slightly serrated leaves that were very contrasting to wild type when it was a young seedling, but it seemed normal for the rest. Seed was obtained from this mutant and later proved to be dominant. A leaf phenotype caused by the overexpression of a gene, *ATHB13*, nearby the insertion was already reported, and might be the one that is overexpressed in the mutant as well (R. Greco, personal communication, (Hanson, J. *et al.*, 2001)).
- *trishoot* received its name because three main shoots appeared when the plant started to flower.

Also some interesting mutants with a L*er* background were obtained. But they also grew in poor conditions. One of them produced only 4 seed. Population 5 yielded also a number of mutants, as shown in table VII. Fortunately, these mutants were grown in better conditions than mutants found in populations 2 to 4 and they could be better analyzed. Though some were sterile, many of them produced viable seed. A few pictures of mutants found in population 4 and 5 are shown in **Figure 10**.

The finding of many novel and previously mutants, identified by T-DNA activation tagging, indicated that this Transposon activation tagging population could be used for gene isolation. As a trial to assess the potential of the transposon activation tagging system to be applied to study postembryonic root development, the roots of some mutants that were identified in population 1 were studied. The results are described in the next chapter.

CHAPTER V.

RESULTS (Part III)

POSTEMBRYONIC ROOT DEVELOPMENT AND PHOSPHORUS NUTRITION STUDIES

Root studies of aerial mutants (activation tagging population 1)

To test the potential of the transposon activation tagging system for root studies, some mutants that were identified in the first activation tagging subpopulation were analyzed to find out if they also showed altered root development. For this initial test, seed were sown in ½ MS, 1% w/v sucrose, 1% w/v purified agar, and their roots were measured after 4.5 and 14.5 days of growth.

In the conditions used for this experiment seedlings grew very fast. The last root measurement was done after two weeks of growth but the roots were already touching the plate walls and the roots of other plants. The results are shown in **Table IX**.

The plants with the most striking root phenotype were the *thread* mutant and the transformant with the *THREAD* gene (encoding a flavin-monooxygenase) under the control of the CaMV 35S promoter (**Figure 21**). Roots of only one Ws overexpression line (35S – *THREAD*) out of 7 Ws lines were measured. Nor Col or L*er* overexpressing lines were studied.

In both cases the main root was shorter than the wild type controls. Moreover, an increased number of lateral roots, covered by numerous root hairs, gave a fuzzy appearance to

the root. However, the phenotype displayed by the original mutant and the overexpressor transformant was not exactly the same. The main difference between the original *thread* mutant and the 35S thread transformant was the severity of the phenotype in different tissues.

Plant	**4.5 days**	**n**	**14.5 days**	**n**
Cloudy	1,04 (0,36)	10	12,66 (1,65)	5
Cloudy x DR5	1,43 (0,29)	10		
WT Ws	1,25 (0,20)	10	7,72 (2,19)	6
VAR	0,71 (0,35)	10	7,27 (1,12)	9
VAR x DR5	1,44 (0,83)	10		
WT Ws	1,33 (0,16	10	7,23 (1,62)	8
Thread	0,30 (0,30)	11	3,75 (2,08)	11
35S – *MONOOX*	0,57 (0,67)	18	4,73 (4,29)	18
-minus longest*	0,12 (0,07)	12	2,46 (1,9)	14
-grown in Kan.	0,1 (0)	5		
WT Ws	1,17 (0,14)	19	8,04 (2,08)	19
Thread x DR5	1,46 (0,65)	9		
WT Ws	1,17 (0,13)	9		

Table IX. Root length of aerial mutants found in population 1. The average length and standard deviation (in parenthesis) are shown.

* The progeny of the first transformant was segregating. Therefore, the values of the longest roots were removed to show the average length of those plants displaying the mutant phenotype.

** After 14 days roots grew extensively in a disordered way (mixing with others and touching the plants below them and the plate walls), which can make these measurements not reliable.

The overexpressor line shows a very marked typical auxin-induced root phenotype. It has shorter, brush-like hairy roots, while the original mutant has a lighter root phenotype (still different from the wild type, though). In contrast, the original mutant had a more severe phenotype in the aerial part than the transformant with the overexpression construct. The aerial part of the original mutant developed a kind of stem from where very small, downward folded rosette leaves grew, but the transformant with the overexpression construct had an almost normal rosette (**Figure 21**).

The mutant *cloudy* showed shorter roots than the wild type at 4.5 days, but seemed to have longer roots than the control after two weeks. A mutant displaying variegation in leaves had shorter roots than the wild type. The F1 seedlings of crosses of the mutants vs. the marker

DR5 - *gus* were in general longer than the mutants, and than wild type Ws itself. The roots of F1 plants of crosses of each mutant vs. a DR5 - *gus* line were stained after 12 days in the growth chamber. The mutant *thread* showed very intense blue staining in the whole seedling, in contrast to the DR5 - *gus* (**Figure 15**).

Root architecture screen under P deficiency

Populations 1 and 2 were screened under P deficiency conditions, under which root mutants had been found in an EMS mutagenized population (López-Bucio, personal communication). The first screen was done in P deficiency conditions, and the aim was to find plants with altered root phenotypes. Altered root phenotypes in this case could mean general mutations in root development or specific response alterations to P deficiency.

During the first screen, using seed of population 1, 28 candidate mutants were observed, showing different root phenotypes. The group of mutants obtained was analyzed once more in both low and high P conditions. From this re-scoring of phenotypes, six putative mutants were chosen. The main putative mutant phenotypes observed were shorter root and/or less number of lateral roots. Data of the average primary root length, lateral root number and root density (number of lateral roots per centimeter of primary root) are shown in **Table X**.

Plant line	0 P			1 mM P		
	Primary root length (cm)	Lateral root #	Lateral root density (# LR/cm)	Primary root length (cm)	Lateral root #	Lateral root density (# LR/cm)
Ws WT	**2.1 (0.18)**	**14.6 (0.81)**	**7.1 (1.1)**	**5.5 (0.20)**	**8.4 (2.1)**	**1.5 (0.3)**
Q	4.2 (0.27)	4.2 (0.75)	0.9 (0.14)	4.2 (0.26)	1.5 (0.9)	0.3 (0.2)
N	1.1 (0.24)	3.4 (1.0)	3.4 (1.0)	3.0 (0.12)	1.0 (0.30)	0.3 (0.09)
LL	2.0 (0.35)	1.4 (0.51)	0.6 (0.25)	3.7 (0.3)	0.8 (0.4)	0.2 (0.1)
D	4.3 (0.03)	5.2 (0.4)	1.2 (0.08)	4.0 (0.1)	2.8 (0.4)	0.7 (0.09)
M	1.9 (0.6)	2.0 (0.6)	1.0 (0.16)	2.0 (0.36)	0.7 (0.60)	0.2 (0.2)
H	4.0 (0.3)	5.4 (1.5)	1.4 (0.28)	4.9 (0.2)	3.4 (0.24)	0.7 (0.06)
G3	1.6 (0.25)	12.6 (1.6)	8.2 (1.2)	3.0 (0.16)	2.5 (0.6)	0.8 (0.15)
*3	4.2 (0.23)	2.8 (0.25)	0.7 (0.08)	5.5 (0.15)	3.4 (0.5)	0.6 (0.09)

Table X. Selected putative root mutants obtained in the root architecture screen of population 1. Plants were grown in 1/10 MS medium for 16 days. NaH_2PO_4 was used as the P source. The average length of the root of 5 seedlings is shown, and also the standard deviation (between parenthesis). Data provided by López-Bucio, unpublished.

During the screen of population 2, around 90 putative mutants were found, from which 17 were chosen, and **Appendix II** provides more information about their features. To re-evaluate them, 5 seed from each mutant were used (López-Bucio, unpublished). All, except one (#41) were recovered. They were assayed in low and high P showing in both conditions a reduced number of lateral roots.

Some candidate mutants had reduced fertility or were not fertile. Infertile mutants were not further studied. Moreover, some mutants displayed also modified aerial phenotypes (see **Appendix III**). For example, mutant D showed half the size of a mature wild type plant, G3 showed a variegation, and LL, N and Q a dwarf phenotype.

Mutants # 22, 26, 29, 33, 37, 38, 39, and 41 displayed a very similar aerial phenotype (see **Figure 19**)**.** Round leaves, with a deep furrow, characterize these mutants, and give the impression of having a short petiole. The size of the mutants was approximately 15 cm. A similar phenotype had been observed before in mutant *surco,* described in chapter 4, found also in population 2.

Molecular Analysis of putative mutants

Seed from a group of fertile candidate root mutants were selected in PPT plates (phosphinotricin, the active compound of the herbicide Basta), to ensure the presence of the *I* element. Alternatively, plants were grown directly in soil and one leaf was "painted" with concentrated finale, and the effect scored next day. The concentration of Finale was high, causing the death of sensitive plants even if only one leaf was painted. Resistant plants showed damage in the painted leaf, but the rest of the plant remained alive.

DNA was isolated from the Basta resistant plants, and used for Southern analysis to visualize AIE insertions in the plants (see **Figure 22**). The analysis was carried out as for the aerial mutants found when building the whole TAT population, described in the part "Activation tagging population". *bar* and *su1* probes were used to hybridize the blots (see **Figure 6**). The first one shows the different transposon insertions, and the second one reveals the presence of the original T-DNA –and hence the transposase-.

From two to five Basta resistant progeny plants per candidate root mutant were used for the Southern analysis, which revealed that most of the plants had one single insertion (see **Figure 22**)**.** Mutants # 11 and 19 had one transposon insertion band that seemed to have the same size. Similarly, mutants 26, 29, 33, 37, 38, 39 and 41 had one very similar-sized band. Mutant 22 seemed to belong to that group as well, and mutant 38 had also a second band of different size. DNA from G3 plants did not give a clear band, but two or three faint bands were distinguishable (data not shown). The Southern blots were then stripped and hybridized then with the *su1* probe to visualize T-DNA insertions. The DNA of most of the mutants did not hybridize with the *su1* probe. However, mutants LL, N, and Q did show hybridization with the *su1* probe and while mutant Q showed a single band, mutants LL and N showed both another band of the same size, higher than the expected. The higher band could be due to T-DNA rearrangements. Moreover, because only one band was seen with the *bar* probe, that corresponded to the size of the band observed with the *su1* probe, it seems that the transposon is still inside the T-DNA. The isolation of the flanking sequence in mutant LL confirms the position of the transposon inside the T-DNA.

DNA from candidate root mutants (D, G3, H, LL, N, Q, 11, 19, 22, 26, 29, 33, 37, 38, 39, 41, 52, 68 and *surco* to compare) was used to do TAIL-PCR to find the flanking sequences to their AIE insertions (see **Figure 23**). For this, DNA from plants having only one insertion was used. Not all the mutants were analyzed. From a group of mutants sharing a similar-sized band, one or two were selected. The sequences obtained were compared to Arabidopsis databases using the BLASTN tool. Some interesting insertions are shown in **table XI.** Interestingly, TIGR, NCBI and TAIR databases gave different blast results in some cases. An example is shown in Figure 23 for the insertion Mutant 22. Near the insertion, some genes are predicted in the NCBI database while they are absent in the database from TIGR. Moreover, there are changes in the length of the predicted gene, or the number assigned for some genes in the clone between both databases. Moreover, in at least two other cases, comparisons against the TAIR database did not give any result, while the NCBI database did. NCBI information is mainly reported here. Interesting candidate genes seemed to be near some of the insertions in the candidate root mutants. Unfortunately, efforts to analyze further the phenotypes of some of these and other mutants did not give clear results. Mainly, the wild type plants in these assays showed very variable phenotypes, which hampered the study of the mutants.

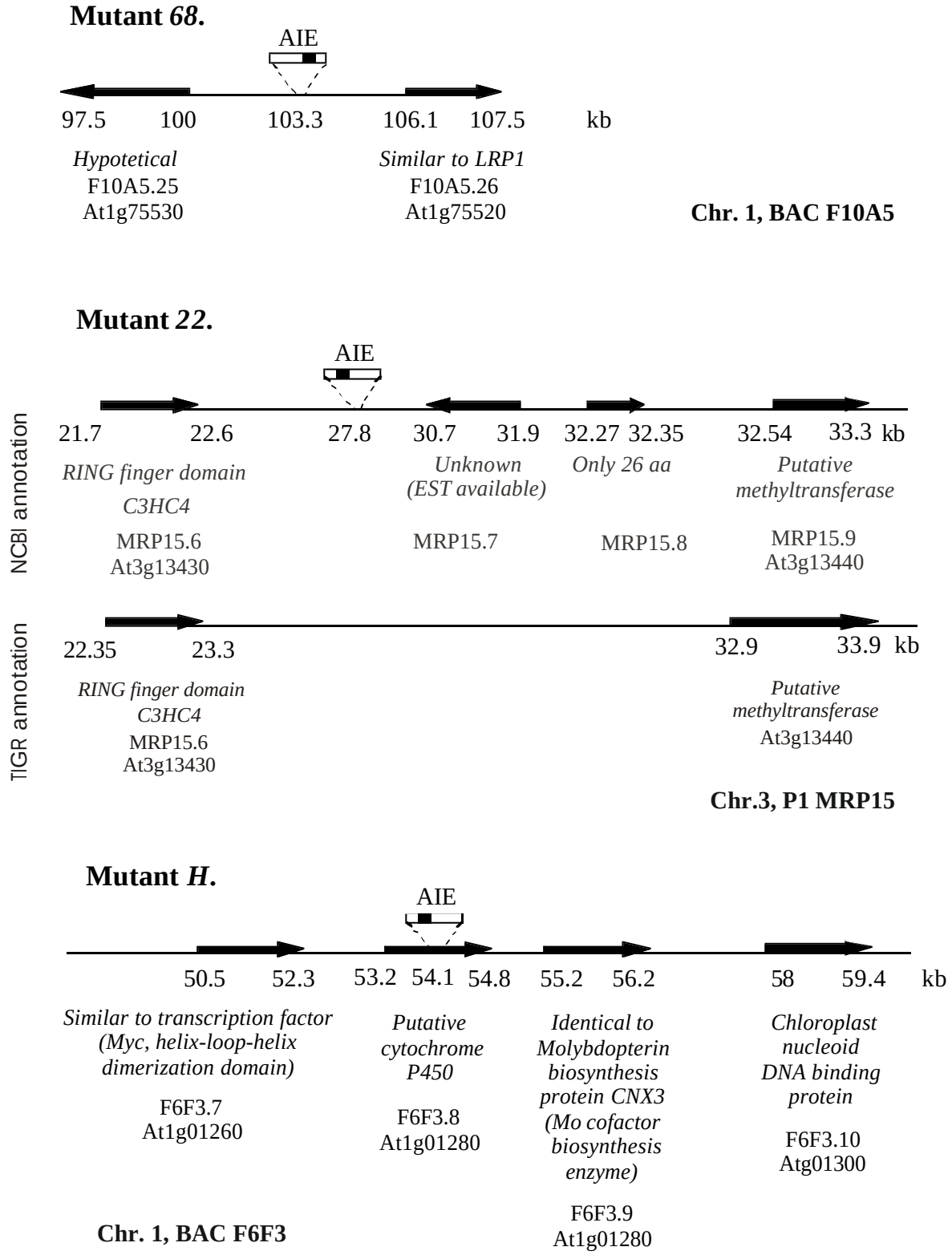


Figure 23. AIE insertions in some putative root mutants.

Mut	Chr.	clone	position	Genes to the sides (accession)
D	3	F4F15	~ 5000	Inside a putative transm. Protein G1p, putative nuclear DNA-binding protein G2p, m1 protein, putative chlorophyll synthetase, putative transm. Prot G5p, putative acyl-CoA dehydrogenase (AF049236), or a gene for sulfate transporter (clone A5T12)
	3	Comp. Seq.	~19133000	Kinesin heavy chain
H		F6F3	~ 53,180	Inside a putative cytochrome p450 (F6F3.8). Left: similar to Myc-type transcription factor –helix-loop-helix dimerization domain (F6F3.7). Right: Molybdopterin biosynthesis protein protein CNX3, Molyb. Cofactor biosynthesis enzyme (F6F3.9).
LL				Cloning vector pSLJ8313 (confirmation of transposon still inside the T-DNA insertion).
11 or 19	2	Sector 182 of 255	~ 20,237	
17	2	T26B15		Left: hypothetical protein (no homology with known domains) (AAC25932.1). Right: Putative protein kinase (high homology to MAPK kinases) (AAC25933.1)
22*	3	P1 MRP15	~27,821	Left: RING –Zn finger-, and right: unknown gene, right (MRP15.6 and MRP15.7). EST for the unknown gene from flower buds (AV533483). Same insertion as in the mutant *surco*.
52	4	F19H22	~41,676	Insertion inside a gene that has many ESTs, similar to more than 20 put. Prots. (fructose-bisphosphate aldolases). Left: similar to kinesin, Right: similar to salt-tolerance protein, putative Zn Finger (F19H22.170).
	4	Contig. Frag. 90	~79,686	
68	1	F10A5 or F1B16		First 32,040 of F1B16 overlap with bases 97,508 to 129,547 of F10A5. Left: similar to LRP1 (F10A5.26, AAF87119.1). Right: hypothetical protein (F10A5.25, AAF87125.1)

Table XI. Some insertions in the putative root mutants from populations 1 and 2. This information is based on the data available at present at the NCBI database, and it might change in the future.

Mutant 17

Mutant 17 was isolated from population 2 during the root screen in P deficiency conditions, and showed an interesting phenotype. Its main root characteristics are a reduced number of lateral roots and a short primary root (see **Figure 24**). Moreover, it also appears to have less hair roots in low P conditions, when compared to wild type seedlings. **Table XII**

shows the average length of the root of seedlings in medium with or without P. The composition of the medium was 1/10 MS, 0.5% sucrose, 1% agar. Square 9 cm petri dishes were used, and sealed with "egapack", a thin plastic film used to pack food. The light source was at least 53 cm. from the top of the plates, and had an intensity of 100-134 µmoles.

	0P				1 mM P			
	Root length (mm)		**# visible lateral roots**		**Root length (mm)**		**# visible lateral roots**	
	Average (St.dev.)	n	Average (St.dev.)	n	Average (St.dev.)	n	Average (St.dev.)	n
WT Ws	20.6 (6.9)	8	8.4 (1.2)	8	>40	6	3.2 (1.5)	6
Mut. 17	11.9 (3.1)	8	0	8	18.4 (3.2)	8	0.1 (0.4)	8

Table XII. Root length and number of visible lateral roots of wild type and mutant 17 seedlings. The seedlings were grown in 1/10 MS, 0.5% sucrose plates with 1mM or without P. The measurements were taken after 26 days in the growth chamber.

Another experiment where mutant 17 was grown in different conditions was done. A slightly different medium composition was used (½ MS, 1% sucrose) and other differences with the original experiment were the size of the plates (14 cm) and the material used to seal them, parafilm in this case. The most significative difference was the space between the top of the plates and the light source, less than 15 cm in this experiment vs. at least 53 cm in the original experiment.

In this experiment with altered conditions, Mutant 17 showed again reduced primary root length in comparison to wild type seedlings. **Table XIII** shows the average length of the seedling root at 4.5 days, compared to wild type. However, root length after 2 weeks was extremely variable under these conditions. An interesting feature of these older roots was that a few of them showed a light-green color in the main root. Moreover, after this period, many lateral roots were observed in the mutant, in contrast to the earlier experiment where no lateral roots were observed at this time.

Plant	Root length (cm) at 4.5 days Average (standard deviation)	n
17	0,51 (0,37)	14
WT Ws	1,23 (0,12)	9

Table XIII. Root length of mutant 17 and wild type seedlings. The seedlings were grown in ½ MS, 1% sucrose, 1% agar plates for 4.5 days. The average and standard deviation (in parenthesis) are shown.

Mutant 17 also seems to display a slightly altered aerial phenotype when moved from plates to soil: it developed slightly pointed leaves, in a higher number. It flowered slightly earlier than the wild type, and finally has a higher number of stems. When grown for a long time in plates with 1% w/v sucrose, the leaves show a wavy border. It remains to be seen if this aerial phenotype occurs when the seed is directly planted in soil, and is not the consequence of the plate-grown period.

Mutant 17 was crossed to the reporter line *IAA28* promoter – *gus*. The *IAA28* gene belongs to Aux/IAA genes, and a gain-of-function mutation leads to the lack of lateral roots (Rogg, L. E. *et al.*, 2001). Interestingly, F1 progeny plants showed three segregating phenotypes: wild type (high numbers of subramified lateral roots and root hairs), intermediate, and mutant (very low numbers or lack of lateral roots, with almost no ramifications and reduced root hairs). The presence of a phenotype between the mutant and the wild type, suggested that the nature of the mutation is semidominant. Interestingly, the seedlings that displayed the severe mutant phenotypes showed reduced expression of the *IAA28* promoter - *gus* gene fusion **(Figure 25)**.

Furthermore, in preliminary experiments where different concentrations of exogenous auxin were supplemented to the medium, root length was always shorter than the wild type. The auxin used was 2,4-D and the concentrations were 1×10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} M (López-Bucio, data not shown). In experiments where the seedlings were moved from medium without to medium supplemented to NPA 10 µM, and then to medium with different concentrations of 2,4-D, the mutant seemed to be less responsive. At 1 and 5×10^{-8} M 2,4-D, the mutant did not have any visible lateral roots while the wild type controls did (data not shown).

The progeny of plants of the first and third generations of the original mutant was analyzed by Southern. All the progeny of the mutant's first generation showed a single, same insertion. However, in the progeny of the third generation, two plants of 11 showed also a second insertion **(see Figure 26)**. Interestingly, the progeny of one of the plants with two bands did not display the mutant phenotype, but rather a mix between the wild type and mutant phenotype. A TAIL-PCR was done using DNA from a plant that showed a single insertion by Southern analysis **(Figure 26)**. The insertion of the transposon appeared to be in chromosome 2, clone T26B15 **(Figure 27)**. The position of the insertion in the BAC clone is near nucleotide 18,716. The orientation of the transposon is left to right with respect to the BAC sequence, with the enhancer near the left end. Near the insertion are two putative candidate genes that could be responsible for the observed phenotype. One gene is situated upstream the insertion (left, complement from positions 15,732 to 17,481). This gene encodes a hypothetical protein, with nucleotide sequence similarity to only one Arabidopsis gene of unknown function (T26B15.6, accession number AAD24844). When translated, its aminoacid sequence has only low homology with a gene product from *Halobacterium sp.* (28% identities and 38% positives), but there is nothing else from Arabidopsis or other organisms with similarity in the current NCBI database.

The second putative gene (T26B15.7, At2g32510, complement from positions 19,182 to 20,300), possesses a Serine/Threonine protein kinase active-site signature and protein kinases ATP-binding region signature. This gene shows high homology to a long list of genes whose products are very similar, are related, or have been identified as MAPK or MAPKK proteins, and has been classified as MAPKKK17 (Jonak, C., *et al.*, 2002).

In a first attempt to study gene expression, primers were designed for both genes. Using those primers, a RT-PCR experiment was done. Unfortunately, no product was detected for either gene in the tested conditions that included seedlings, roots, flowers and mature leaves. However, PCR products were obtained from all samples when assayed with actin primers.

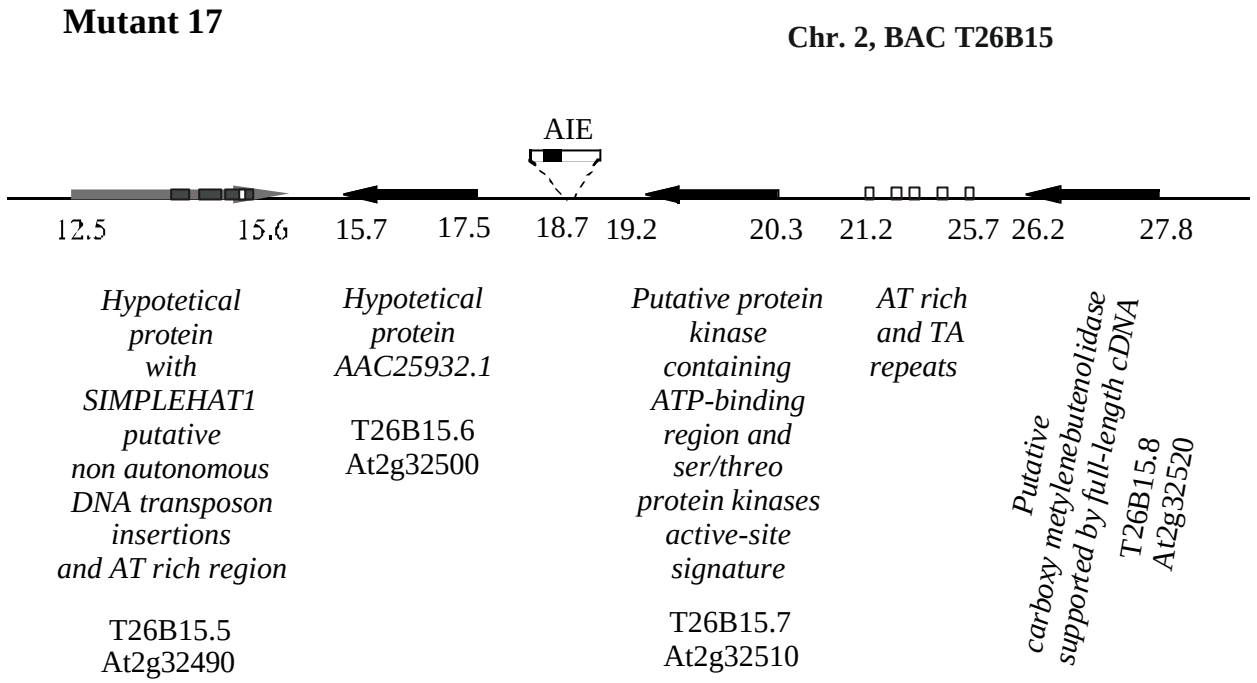


Figure 27. Schematic representation of the position of the AIE insert in the genome of mutant 17. The representation is not at scale.

Shadowed roots

It was not easy to analyze the phenotypes of many mutants with insertions near interesting genes. Possibly, this difficulty was caused by environmental variations in the growth conditions. To try to improve the reproducibility of the results by reducing variation in one condition, light, an experiment where the roots were shadowed was done. However, there can still be some variations in root responses depending on the angle in which the plates are placed, which affects the angle in which the light enters to the shadowed area. Nevertheless, in this experiment the roots were covered from the light, while the aerial part of the plant received it normally (see **Figure 28**). Early observations did not show a marked difference between light and shadow-grown roots. However, at 14 days differences could start to be observed. First, the wild type roots have a different response to light and shadow. Covered roots elongate more than when illuminated. Second, normally the shoots of plants grown

without P seem to be stressed when their roots are illuminated. The aerial parts of plants with illuminated roots show delayed growth and are purple-colored due to antocyanin accumulation. Interestingly, when the roots were covered, the aerial part of the plants did not seem to show the same degree of stress after more than 15 days. The shoots had a green color, without visual signs of antocyanins, and they were developing like the illuminated plants grown with P **(Figure 28)**.

Another interesting observation relates to the results obtained with *Ler* plants. One of the problems in the early attempts to re-study the root of the mutants, was that mutants with a L*er* background were difficult to score. L*er* roots are very similar in low and high P conditions when grown in the light. They have almost the same length and show lateral roots in both conditions. In contrast, L*er* wild type plants seemed to show clearer differences in low and high P when their roots were covered. Both elongated, but more lateral roots could be observed in low than in high P conditions. Covering the roots can help to study L*er* mutants and hence, to score adequately phenotypes of crosses to L*er* that are used to map interesting mutations.

Along with the differential response of the wild type plants, interesting phenotypes were observed in some of the candidate root mutants. Some mutants displayed a different behavior of root growth in light or darkness, when compared to the wild type. The most visible mutant phenotypes observed in the first trial experiment, were:
G3; most of them had a light-grown phenotype in the shadow.
22; some showed the light-grown phenotype in the shadow, though not all.
68; all seedlings had roots that were about 1/4 shorter than the wild type in the shadow.
Q; showed roots that were longer and less ramified than the wild type (in shadow and in light).
N / LL; remain shorter than the wild type (light-grown like) and more ramified in the shadow.

Others, like 85, had a subtle phenotype, with slightly less elongated roots in the shadow when compared to the wild type.In the original trials to analyze the root phenotypes, the phenotypes of roots of the plants that still had the T-DNA insertion were thought to be due to the presence of the *su1* gene (mutants LL, N, and Q). However, the root response was

different among mutants that still contained *su1*, suggesting that a mutation caused by the T-DNA insertion could influence the root phenotype, rather than the marker gene. On the other hand, it could still be possibleis that the presence of the SU1 gene in different positions of the genome changes the phenotype. Accordingly, mutants N and LL showed a similar behavior, opposite to Q. These three mutants were shown to contain the *su1* gene when a Southern analysis was performed, and N and LL had the same double-band pattern (**Figure 22**). Complementation studies were not done in these mutants, but these results might indicate they have the same mutation. Mutants with defects in light receptors (*hy1, 3, 4* & *8*) were also tested with the shadowed roots system to get more information about the pathways involved in the root-growth inhibition induced by light. **Table XIV** shows the mutants used, their light deficiencies and defects.

Mutant	Light deficiency	Defect
hy1	Red, far-red	Phytochrome chromophore biosynthesis
hy3	Red	Phytochrome B
hy4	Blue	Chryptochrome (blue-light photoreceptor)
hy8, fre1, fhy2	Far red	Phytochrome A

Table XIV. Reduced light response mutants tested in the shadowed roots experiment. Taken from (Chory and Susek, 1994).

Most of the light perception mutants had a wild type root response in the conditions here evaluated, but *hy8* showed a different response. It seemed to grow in the shadow as if it was in the light (less root elongation).

In this experiment root hairs were not carefully analyzed (mutations in these light receptors affect root hair growth in some cases). However, *hy8* seemed to have an altered response to light and shadow in this preliminary experiment. It had a light grown-like root phenotype in the shadow. These mutants are characterized by insensitivity to inhibition of hypocotyls elongation by continuous far-red light (Chory and Susek, 1994). They have been reported to appear normal under continuous white or red light. Additionally, PHYA has been suggested to play a specialized role in allowing seedlings to germinate in extreme shaded environments that are enriched in far-red light (Chory and Susek, 1994). If this is confirmed, it is interesting how the lack of a photoreceptor could cause, in the shadow, the effect of

growing like when roots are exposed to light. Maybe it does not sense far-red light and it allows root inhibition.

The experiments here described were initial trials to reduce the variability in the root study. Results obtained with wild type plants were comparable in three different experiments, but experiments with the putative mutants were done only once. In this "very preliminary" study, the influence of light and nutrients in root development was conjunctly studied, with interesting results. The shadowed-roots system helped to obtain more uniform results that in earlier trials, but there are still variations found. These experiments should be repeated to give more solid conclusions and to further refine the system.

Nevertheless, a low scale screen was performed then, using a pool of seed of a part of population 4. Two different mixes of seed were used, comprising around 50 plants in total. The conditions of the screen included 0 P or 1 mM P in light or with shadowed roots. A number of putative mutants were observed in the different combinations of conditions.

After identifying interesting candidates, they were transferred to soil and sprayed three times with Basta to eliminate those plants that did not contain any transposon insertion. The surviving plants were measured at the end of their lives and their "probable" ecotype determined visually. The ecotype determination is important because L*er* controls showed variation. For example, in some experiments, there were single L*er* plants in the control group that were small, with small leaves and short root (2 to 3.8 cm). Taking this in account, some putative mutants might just be L*er* seedlings that developed like the ones in the controls.

The candidate mutants obtained in this low scale screen are shown in **Appendix IV**. Some mutants were chosen not only because of their root length or number of lateral roots. Other criteria were the architecture of their root and growth in contaminated plates. The root architecture varies between ecotypes. For example, Ws seedlings show more lateral roots in the root tip in low P, whereas L*er* seedlings have more developed lateral roots in the upper part of the root (see **Figure 29**). Seedlings showing a root architecture that was different from the observed for Ws were chosen, and afterwards their ecotype determined visually.

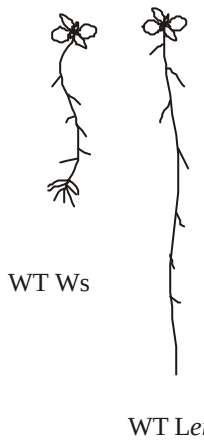


Figure 29. Typical root architecture of wild type Ws and L*er* plants grown for two weeks in phosphorus deficiency conditions.

On the other hand, plants growing in contaminated plates (fungi or bacteria) generally have very enhanced shoot and root growth. Their roots are longer, have an increased number or lateral roots and have very long root hairs. Some candidate mutants were found that were smaller than the rest in these conditions, and were taken as well. The seed of these plants was collected for further characterization.

Insoluble phosphate uptake screen

In some poor soils P is present but is insoluble, and is not used by many plants. In order to look for genes that could improve P uptake when activated, all populations from the transposon activation tagging collection were screened using an insoluble source of phosphorus. The insoluble source used was $Ca_5HO_{13}P_3$. To minimize the presence of soluble phosphorus, $Ca_5HO_{13}P_3$ was washed several times with deonized water before adding to the medium. **Table XV** shows the amount of seed used per population. Besides the mutant collection obtained using the *in cis* vector, a mix of seed of a few plants obtained using *in trans* vectors were included (25 mg in total). Seed were sown in plates containing $Ca_5HO_{13}P_3$ as the only phosphorus source. After 21 days, seedlings were visually inspected to search for putative mutants. Two criteria were used to evaluate candidate mutants: leaf size and color in

comparison to the rest of the seedlings in the plate. As reference, wild type Ws and L*er* seed, and transgenic citrate synthase overexpressors (Col background) were used (De La Fuente J.M. *et al.*, 1997).

Population	~ Population size	# Pools	Pool size	Seed (mg) per pool	~ # seed per pool	Seed density ~ #seed/plate
1	1100 plants	1	1100 plants	164	8200	~ 500
2	28 trays of 96 plants	7	384* plants	~70	~3500	~ 500
3	13 trays of 96 plants	26	48 plants	~ 10	~500	~500
4	53 trays of 96 plants	53	96 plants	~20	~1000	~500
5	465 pots of 4-10 plants	21	20 pots**	~ 7-11	~ 350 - 550	~ 250

TABLE XV. Activation tagging collection used for $Ca_5HO_{13}P_3$ utilization screen.
* One pool had 576 and another 182 plants.
** Two pools had 40 pots.

A number of putative mutants were observed. Some displayed a very clear phenotype, with increased leaf area and darker green color (**Figure 30**) while the rest of the seedlings in the plate were pale and small. However, there were some seedlings with increased size but with dead leaves. Putative mutants were sorted according to their phenotype. Clear, medium and slight phenotypes were separated and transferred to soil. Recovery of the plants before adding water with 1/10 MS was considered to choose plants for molecular analysis. Controls that showed poor growth in the medium with insoluble phosphate recovered only after watering with 1/10 MS.

Furthermore, some altered aerial phenotypes were also observed when looking for plants with enhanced growth in the medium supplemented with insoluble phosphate, like pale or extremely compacted seedlings **(Figure 30)**. Some displayed the altered aerial

Southern analysis of 12 clear enhanced growth phenotype, 21 medium and 4 plants with altered aerial phenotypes showed that most of them contained a single AIE insertion (data not shown), as observed for the morphological mutants characterized before.

CHAPTER VI.

DISCUSSION

I. ACTIVATION TAGGING POPULATION AND IDENTIFIED MUTANTS

Strategies in cis vs. in trans

The *En/I (Spm-dSpm)* maize transposon system was used for Activation tagging in Arabidopsis.The system has two components, an autonomous and a non-autonomous element. Initially, these two components were introduced in Arabidopsis either separately (in different vectors and plant, *in trans*) or together in a single vector *in cis*. Both approaches were tested to find the most suitable to generate a collection of stable activating insertions. Originally, the strategy designs were developed having in mind that they would have different advantages and drawbacks. For example, having the elements *in cis* would avoid the need to make crosses in order to mobilize the transposon, while using them *in trans* would directly give rise to a stable library of transposon-containing T-DNA inserts. These stable inserts or "starting lines" could then be used for directed tagging or to cross to different sources of transposase.

I constructs used for the system *"in trans"* showed excision by PCR when a transposase source was present. Moreover, a variety of aerial mutant phenotypes were observed in a population generated using this strategy. However, the constructs used for this approach did not seem to be suitable to obtain large numbers of stable insertion plants when compared to the other strategy (*in cis*), for the following reasons:

First, many phenotypes were displayed in sectors, indicating they were caused by somatic transposition, which difficulted the study of putative mutants and hence, made it difficult to test the system. In contrast, in the *in cis* system, stable insertions could be directly obtained, making easier the study of candidate mutants and the system itself.

Moreover, the fact that in the *in cis* system crosses between plants containing the *I* transposon and the *En* transposase to get plants with active *I* transposition were not required to start a population was already a time-saving advantage over the *in trans* system.

On the other hand, while convenient selection against the *En* transposase source in following generations is possible with the *in trans* system, and can be done as for the *in cis* system, by spraying or selecting in plates with the R7402 pro-herbicide, a major drawback of the *in trans* vs. the *in cis* system is that the T-DNA constructs that contain the *I* transposon do not carry any negatively selectable markers. In any case, there would be ways to select plants where excision from the original T-DNA occurred in the *I* element containing T-DNA. For example, if there is excision, *GFP* expression would be restored, which can serve to select plants where excision occurred. Then, to select for *En* T-DNA-free plants where transposition occurred (from the *I* containing T-DNA), besides spraying with Basta and the pro-herbicide, one should also look for GFP-positive plants, which would have the T-DNA from where the *I* element excised.

Unfortunately, the selection for *En* T-DNA and *I* T-DNA-free plants where transposition occurred (from the *I* containing T-DNA) would be complicated. To select for plants with only the *I* element, but none of the T-DNAs (originally containing *I* or *En*), one could look for Basta and R7402 resistant plants that are sensitive to kanamycin or hygromicin (depending on the T-DNA). Still the difficulties of scaling up a kanamycin sensitivity test for a large number of plants and the possibility of silencing of the NPTII gene in the T-DNA are disadvantages of the *in trans* strategy when compared to the *in cis* strategy. Another possibility would be to develop PCR assays combined with the herbicide and proherbicide treatments to find plants with stable insertions, devoid of T-DNAs for the *in trans* system, but again, this is far less practical than the herbicide and proherbicide applications that the *in cis* system requires. Therefore, the *in cis* system was found to be the most suitable to create large numbers of plants with stable insertions and was used to generate a population.

Development of a population of stable insertions

A large population was then built using the construct shown in **Figure 6**, where the immobilized autonomous and the non-autonomous elements are present.

In order to position the 35S enhancer tetramer in the *I* element close to adjacent genes, fragments of 200 and 400 bp of the *I*-element terminal inverted repeats were used. These fragments are much smaller than those described previously (Tissier, A. *et al.*, 1999). Excisions were observed in most transformants that contained a complete T-DNA, indicating that these short transposon terminal inverted repeats are still functional for efficient transposition. The total size of the Activating *I* element (see **Figure 6**) is 3.7 kb and the enhancer tetramer is only 200 bp away from the left end and around 2 kb from the right end of this modified transposable element.

It has been previously reported (Weigel, D. *et al.*, 2000) that genes further than 3 kb from the enhancer can be activated to give a mutant phenotype. In one case, we found that the 35S enhancers could activate genes on both sides of the AIE (data not shown) requiring confirmation of the gene responsible for the observed phenotype. To visualize the different stable insertions in the double resistant progeny of the primary transformants, Southern blot analysis was done on individual and pooled plants. An inverse correlation was observed between the frequency of stable insertions and the ITFs in progenies from different transformants. Analysis of these parameters in different families of transformants allowed the selection of suitable parents with high ITFs that were useful for making a large population of insertions, as described in Results. Either selfed T2/T3 or F2 seed from crosses with the wild type were sown and then sprayed with both R7402 and Basta to select new stable insertions. Selfed seed has the disadvantage that a number of parents homozygous for the T-DNA or containing multiple T-DNA inserts will not be able to produce stable transposants. Moreover, insertions that are "fixed" as early transpositions will eventually be obtained in the next generation at a high frequency. In view of this, segregating F2 seed was used for the generation of the latter batches of the population. The parental F1s were obtained by crossing

T2 or T3 plants with a wild type Ws plant as the female parent to facilitate identification of F1 progeny as compared to self-pollinated escapes, lacking the *su1* phenotype.

The variety of independent inserts can be increased in this way over several rounds of selection as outlined in **Figure 9**. The progeny of the selected lines can behave differently than the original progenitors or their siblings (data not shown). Because it was not possible to analyze molecularly each new stable transposant seedling to decide whether to include it in the collection, the inverse correlation between the STF and ITF provided a parameter to aid selection. To avoid the selection of stable insertions originating from plants with very low ITF and early clonal sectors that give a high percentage of stable transposants, the seeds from crosses of different progenitors were handled separately. In this way, seeds that give rise to a very high number of double resistant seedlings (and suspected to have a low ITF) can easily be identified and only one or a few seedlings are then included in the population. Thus individual families having a low to moderate frequency of stable transposants were used to select plants and build up the population of independent inserts. This strategy is different from that used previously (Tissier, A. *et al.*, 1999) where the best lines were bulked and used for transposant generation.

The selection of parental line whose seed would give rise to stable insertions, was done by spraying them with Basta to ensure the presence of the *I* element. Most conveniently, the *su1* gene serves as a visual marker as it confers a dwarf and dark green phenotype that allows the selection of transposase-containing plants for use as parents for the double spraying experiments. Each step of stable transposant selection resulted in batches of 1000 to nearly 5000 individuals (as shown in **Table VIII**). The total number of plants obtained to date is around 13000, with 8300 estimated independent inserts (**Table VIII**)**.**

One advantage of using populations with low number of insertions per plant is that they allow the rapid identification of genes that are responsible for a phenotype of interest. Analysis of individual stable plants from different sub-populations, derived either from single or from a number of starting lines showed that they contain one (the majority) or just few transposon inserts. Additionally, the inserts seem to be well distributed throughout the genome, as observed in a random sample of stable plants (**Table VII**) suggesting that genome

saturation of insertions within these genotypes is possible. In addition, the seeds of each transposant plant were collected separately in all sub-populations, enabling the screening of families and the correlation of phenotypes within a line. This can be particularly useful in cases where screenings for sensitive mutants to certain compound or environmental condition are used. It also avoids the problem of loosing phenotypes that have been identified (as occurs when pooled seed is used). However, to facilitate large screens, pools of seed can also be made and seed from the original individual can be traced back once an interesting phenotype is identified.

Characterization of transposon Activation Tagged Mutants

In the pilot experiment on population generation, a number of dominant mutants were identified. Characterization of these mutants revealed that two were novel, and two were affected in previously reported genes or gene families, isolated by means of T-DNA activation tagging. The recovery of activation tagged genes similar to those obtained with T-DNA insertions indicates that the mechanisms involved in activation are similar for the transposon system, and validates the use of this system for gene activation. One of the genes identified in our screen, belonging to a gene family identified by T-DNA activation tagging is the *THREAD* gene encoding a flavin monooxygenase-like enzyme, which belongs to the family of the *YUCCA* gene (Zhao, Y. *et al.*, 2001). YUCCA was shown to be an enzyme involved in the Trp-dependent auxin biosynthesis pathway. This family has 9 members, and the overexpression of some of them leads to the overproduction of auxin that results in the *yucca* phenotype (Zhao, Y. *et al.*, 2001). THREAD shares a 66% aminoacid sequence identity with YUCCA. The overexpression phenotype that THREAD produces is remarkably similar to the one described for the *yucca* mutant: long hypocotyl, enhanced apical dominance, narrow and epinastic leaves, and sterility. The auxin content in the *thread* mutant was not directly quantified. However, its phenotype suggests the effects of elevated levels of this hormone.

Moreover, F1 seedlings from a *thread* vs. DR5 - *gus* cross showed enhanced *gus* expression in the tissues showing an altered phenotype, i.e. leaves. In the DR5 - *gus* wild type lines, *gus* is expressed in response to auxin (Ulmasov, T. *et al.*, 1997). Therefore, the higher

GUS activity produced by the DR5 - *gus* construct in the *thread* background may indicate enhanced auxin levels or enhanced levels of responsivity to auxin. Due to the previously reported activity of the YUCCA protein, highly similar to THREAD, the higher level of expression of DR5 - *gus* seems to reflect higher auxin levels indeed.

RT-PCR experiments reveal expression, though low, of *THREAD* transcript in wild type flowers. This is supported by the presence of an EST corresponding to the *THREAD* gene in a flower bud cDNA library (Asamizu, E. *et al.*, 2000). It is unclear whether other family members are expressed similarly or differ in their regulation, but no ESTs from some other members were found in the same library.

Interestingly, the transformants obtained with the overexpression construct display some differences between ecotypes. This might be due to the different genetic backgrounds which give rise to different gene interactions in each ecotype.

Another morphological mutant identified in population 1 is *empty siliques*, in which overexpression of a cytochrome P450 (CYP78A9) gene causes a seedless phenotype. The *CYP78A9* mutant was originally identified in a T-DNA activation tagged population by screening for suppressors of the *apetala2-1* phenotype. The insertion in the T-DNA mutant line was located around 2 kb away from the *CYP78A9* gene (**Figure 13**) (Ito and Meyerowitz, 2000), and produced plants with siliques without seeds. In the transposon mutant line described here, the seedless phenotype (shown in **Figure 12**) was also observed but in a wild-type Ws background. Other phenotypic features besides the lack of seed, such as silique length and increased width when pollinated, were also similar. Some observations suggest that the lack of seed formation is caused by failure of fertilization with no zygotic and endosperm development in the ovules and could be due to a signal missing from the ovule or at other stages in the fertilization process (Chun-Ming Liu, personal communication).

It is intriguing to know whether the occurrence of these similar mutants in different activation tagged populations (Ito and Meyerowitz, 2000; Zhao, Y. *et al.*, 2001) is just due to the fact that only a few genes produce morphological alterations when overexpressed or whether the likelihood to overexpress particular genes or members of certain families is higher. Interestingly, there are slight differences in the enhancer tetramer sequences used in

the T-DNA and the transposon activation tagging populations, described later, that do not seem to produce remarkable differences in the observed phenotypes, at least in these mutants.

Interestingly, the analysis of a number of tagged genes reveals that in some cases there is an ectopic overexpression of the tagged gene, although the presence of a low level of expression of the wild-type gene in certain tissues cannot be ruled out.

The mutants showing novel phenotypes in population 1 included *bountiful* and *cloudy*. Both showed clear PCR products in the RT-PCR in tissues where a product was not detected in the wild type. *bountiful* could be phenocopied by introducing the gene fused to the 35S promoter, and an exact copy was obtained by using the endogenous promoter fused to the 35S enhancer tetramer (R. Greco, personal communication). Interestingly, *BOUNTIFUL* shows high similarity to *ESCAROLA*, an AT-hook DNA binding gene. A mutant in which *ESCAROLA* is overexpressed was briefly described by Weigel and colleagues in a report of their T-DNA activation tagging population, and had also altered leaves (Weigel D. *et al.*, 2000), but no other reports about the function of these genes are available.

On the other hand, the AIE in *cloudy* was placed between two predicted genes, both reading to opposite directions away from the AIE: a glycosyltransferase-like and a mannosyltransferase-like gene. The *cloudy* phenotype seems not to be caused by the overexpression of the glycosyltransferase gene, as originally thought. The glycosyltransferase gene seemed to be the best candidate to be causing the phenotype due to overexpression. This gene was clasified as having homology to IAA-Glycosyltransferases in the MIPS database. Moreover, when hormones were applied to the mutant, auxins seemed to decrease the mutant phenotype in leaves, while cytokinins seemed to enhance it. Conjugation of auxin to glucose is a step in auxin regulation. Recently, an Arabidopsis glucosyltransferase that glucosylates IAA has been reported. Its overexpression produces an altered leaf and root phenotype (Jackson, R.G. *et al*., 2002). Auxin glycosylation seemed to be a possible explanation for the observed phenotype in *cloudy*. Additionally, RT-PCR experiments showed it to be overexpressed in a higher degree than the gene on the other side of the insertion.

Nevertheless, the mutant phenotype could not be obtained by transforming wild type plants with a construct where the expression of the glycosyltransferase-like gene was driven by a 35S promoter. Moreover, preliminary observations of F1 seedlings from a DR5 – *gus* vs. *cloudy* cross did not show remarkable changes in GUS expression, indicating that there are not

major changes in auxin activity. However, because the mutant phenotype is not visible in the first 2 weeks after germination, GUS expression should be studied afterwards before concluding that auxin is not involved in the observed phenotype.

Still, the phenotype might be caused by the gene on the other side of the insertion, a mannosyltransferase-like gene, or by the overexpression of both genes. Interestingly, the mannosyltransferase-like gene has homology to the human gene PIG-B. Many eukaryotic cell surface proteins are bound to the membrane via the glycosylphosphatidylinositol (GPI) anchor that is covalently linked to their carboxy-terminus. The GPI anchor precursor is synthesized in the endoplasmic reticulum (ER) and post-translationally linked to protein. The PIG-B human gene (phosphatidylinositol-glycan of complementation class B) is involved in transferring the third mannose. PIG-B encodes a 554 amino acid, ER transmembrane protein with an amino-terminal portion of approximately 60 amino acids on the cytoplasmic side and a large carboxy-terminal portion of 470 amino acids within the ER lumen (Takahashi, M. *et al.*, 1996). However, overexpression transformants with the mannosyltransferase-like gene using the 35S promoter could not be obtained to date (R. Greco, personal communication). Perhaps the 35S promoter is too strong and might cause lethality. Nevertheless, attempts to obtain double transformants are now under way.

Interestingly, this mutant shows different degrees of severity according with environmental conditions, like the presence of a high number of fly larvae in the soil, eating the plants. This environmental regulation might indicate that the overexpression is not necessarily constitutive. Therefore, other possibility that should be considered is the use of the endogenous promoters of these genes with a tetramer of the 35S enhancer, in order to mimick the original mutant. Moreover, the genes here used were obtained by PCR with primers designed according to the predicted sequence in the databases. However, it might be possible that the prediction is not accurate and that the introduced genes are incomplete. This possibility should be also studied further.

An interesting feature of the four mutants described above was that they seemed to live longer than the wild types in general, maybe because they flowered later or were sterile or semisterile. Moreover, in some cases the phenotype was less severe in late life stages, as if enhancer effects were lower in late development, or endogenous ways to reduce the effects of the overexpression were present at that stage. Moreover, some of the candidate mutants

developed aberrant organs at the tip of their stems at late life times. This could be also caused by their longer lifetime.

In other populations (2-5) a number of mutants were identified. Most of them were not observed in population 1, and some were also not described before and seem to be new. The characterization of these mutants was continued by Raffaella Greco, Antonio Chalfun-Junior and Asaph Aharoni. Interestingly, in *picos* and *bolita,* overexpressed genes adjacent to the insertion have been identified and overexpression constructs could cause picos and bolita phenocopies when transformed in wild type plants (Raffaella Greco, personal communication). Also overexpressed genes were found in *largo* (now *needle*) and in another mutant named *twisted*, with spirally curled siliques (shown in **Figure 10 I,** Antonio Chalfun-Junior, personal communication). The finding of overexpressed genes in most of the analyzed mutants indicates the efficiency of the Transposon activation tagging system here reported.

Activation tagging –some considerations-

This approach uses enhancer sequences that can promote the overexpression of genes. The main advantage of this strategy is that it generates dominant mutations that are distinguishable in the transgenic plants, in contrast with recessive mutations. The availability of the Arabidopsis genomic sequence is very helpful because it allows the position of the insertions to be identified and gives information about the genomic context (The Arabidopsis Genome Initiative, 2000).

There are some considerations that should be taken into account when working with this strategy. For example, if transposons were used, reversion would probably not lead to derivative alleles, like in the classical knock-out strategies, as explained later in the discussion. On the other hand, a possible limitation of activation tagging might be the presence of insulators in the plant genome (Bell, A. C. *et al.*, 2001). Insulators have not been well studied in plants, but they play an important role in gene regulation, and might decrease or block the effect of an enhancer in a tag. Post-transcriptional regulation could also decrease the phenotypic effects of overexpressing a gene. Translation efficiency or regulation, as well as

protein modifications, might modulate the level of an active protein and therefore could interfere with the effects of gene activation by the enhancer.

Moreover, an important drawback of activation tagging as here described (using a constitutive strong enhancer) might be that dominant mutations that cause total sterility can not be mantained as recessive ones. Recessive mutations can be normally maintained in heterozygous plants that do not show mutant phenotypes and give homozygous progeny that display the mutant phenotype. A way to analyze the genes involved in such dominant mutations would be to obtain DNA from the original mutant and find the position of the insertion in the genome. Then, candidate genes can be introduced in overexpression vectors into a wild type plant, and the phenotype can be evaluated.

However, for the case of dominant mutations that cause lethal or extremely weak phenotypes, it would be very difficult to recover the mutation, unless another mutation that supresses it is present and can segregate in following generations. Some options to minimize the problem would be to use specific, or better, inducible activators. Another option would be the use of unstable transposons, as described later in the text. Nevertheless, activation tagging has been shown to be a useful tool in isolating previously unknown gene functions.

Transposons as activation tags

The identification of at least four gain-of-function activation mutants in population 1, with approximately 400 activation tagged inserts and 25 in an independent sub-population containing about 2500 inserts (population 5), indicates a high frequency of activation tagging using the *En-I* transposon-based activation tagging system. Using the T-DNA activation tag system one mutant per thousand individuals was reported (Weigel D. *et al.*, 2000).

Since it is likely that in the reported T-DNA populations there are several T-DNA inserts per individual, the frequency of activation mutants identified in this transposon tag collection is considerably higher. A higher frequency was also observed in a transposon tag population produced using a Ds element containing an intact 35S promoter (Wilson, K. *et al.*,

1996). This would support the notion that activation tagging is more effective using transposons instead of T-DNA.

The vector used for the T-DNA population reported by Weigel and colleagues was derived from a vector reported before by Walden and collaborators (Walden, R. *et al.*, 1994). It has a tetramer of the –90 to –420 transcriptional enhancer sequence of the 35 S CaMV promoter. On the other hand, the enhancer sequence used in the system reported here is shown in **Figure 31**. The differences in the enhancer are slight and were not considered to be the cause of the difference in the frequency of aerial morphological mutants in each population.

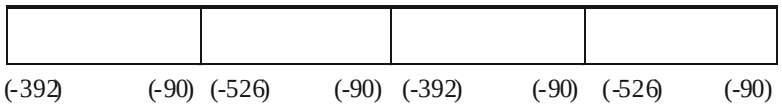


Figure 31. CaMV 35S enhancer tetramer used in the AIE. The parts of the CaMV 35S promoter used for the activation tagging system are depicted. The numbers indicate the original position of nucleotides in the CaMV 35S promoter.

Most likely, the lower frequency of T-DNA activation mutants could be caused by a high incidence of complex and repeated loci in the T-DNA tagging population. Such complex loci often occur in T-DNA integration and are associated with gene silencing (Matzke, M. A. *et al.*, 2000). Complex T-DNA insertions may interfere with enhancer action by inactivating the enhancer through RNA silencing-like mechanisms and/or methylation (Mette, M. F. *et al.*, 2000; Sijen, T. *et al.*, 2001).

Analysis of some lines from the population generated with the Activation Tagging T-DNA, revealed enhancer methylation. Moreover, crosses of the lines where the enhancer was methylated to transgenic lines carrying a 35S - *gus* construct, showed reduction in GUS activity when compared to crosses to wild type controls (Chalfun-Junior *et al., in preparation*). Enhancer methylation and reduced GUS activity from a 35S - *gus* transgene in some T-DNA activation tagging lines might indicate that enhancer silencing, the explanation suggested here for T-DNA lower efficiency for activation tagging, might be indeed happening.

On the other hand, in complex loci, the enhancer could also become positioned too far away to activate plant genes efficiently.

The double selection system used here to obtain stable transposon insertions assures in most cases the elimination of the original T-DNA from where the transposon excised. Therefore, in contrast to complex T-DNA insertions that are often generated during plant transformation, the non-autonomous enhancer-carrying transposons produce insertions containing a single copy of the intact element that could therefore be less susceptible to silencing phenomena.

Another possibility that could explain the activation efficiency would be a greater bias of the *En-I* transposon for insertion near transcriptionally active sites. However, recent analyzes of junctions of 1000 T-DNA insertions done by Szabados and colleagues, indicate that "the majority of T-DNA insertions landed in chromosomal domains of high gene density, 4.7% of insertions were in interspersed, centromeric, telomeric and rDNA repeats, and 0.6% of sequenced tags identified chromosomally integrated segments of organellar DNAs". Furthermore, the authors of the T-DNA study indicate that "35.4% of T-DNAs were positioned in intervals flanked by ATG and stop codons of predicted genes (62.2% in exons and 37.8% in introns)". Moreover, the authors noted that there is an increased frequency of T-DNA insertions in 3'- and 5'-regulatory regions of genes (Szabados, L., *et al.*, 2002). Therefore, again, the formation of complex structures that directly or indirectly affect the enhancer activity seems to be the best explanation for the higher frequency of activation mutants observed with transposons to date.

Besides producing higher frequency of mutants, the use of a transposon as the carrier of the enhancer offers further advantages. Once an activated gene has been identified, the transposon could be re-mobilized to activate related genes or copies near the original, if present, as well as to produce knock out insertion phenotypes (Wilson, K. *et al.*, 1996). Unstable transposons could also be helpful, particularly in recovering insertions that if germinal would confer embryo lethal or very weak activation phenotypes. Somatic insertions would instead display aberrant phenotypes only in specific sectors of the plant, from where tissue can be obtained to culture or to isolate DNA and identify the genes involved.

As indicated in the introduction, one of the advantages of transposons over T-DNA as a tag in knock-out strategies, is that reversion (the excision of the transposon from the original site) can be used to analyze whether an insertion is the cause of a specific phenotype. In some cases, the footprint that the transposon leaves when it excises can also cause a mutant phenotype, creating a derivative mutant allele. For activation tagging, reversion can also be used. However, in this strategy, a phenotype is normally not only caused by gene knockout, and the insert is not necessarily inside. Therefore, reversion would relate the phenotype to the presence of the insert, but would not give specific information about the affected gene. Moreover, the footprint that the transposon leaves would probably not create a dominant derivative allele, because the modified sequence is not directly the one of the affected gene in many cases. An exception could be a case where the insertion is in the promoter of the overexpressed gene and the footprint would generate changes in the gene regulation, creating then recessive or dominant mutations.

For the case of the *Ac-Ds* maize transposon system, the presence of "hot spots" for *Ds* insertion in the genome of Arabidopsis have been reported (Ito, T. *et al.*, 2002). Two of these "hot spots" are adjacent to the to nucleolus organizer region, at the top of chromosomes 2 and 4. This region consists of ribosomal RNA gene clusters that are tandemly repeated and form a loosened chromatin structure, due to the high transcription of ribosomal RNA. Interestingly, these hot spots were reported in an earlier *Ac-Ds* population, but not in *En/Spm-I/dSpm* ones (Ito, T., *et al.*, 2002).

Though the presence of specific "hot spots" for the *En/Spm-I/dSpm* system in Arabidopsis have not been reported, the GC content has been proposed to be relevant for transposon insertion. Most *I/dSpm* insertions are found in AT rich sites (A. Pereira, personnal communication). Transcribed regions are in many ocassions enriched for AT content, and this might suggest that *En- I* insertions in transcribed regions are favored. In any case, if specific "hot spots" exist for different transposon families, it might be interesting to try insertional mutagenesis systems with different transposon families.

Interestingly, the first transformants with the "*in trans*" trial construct *bar*: I (4 Enh.), which uses the *bar* gene as an excision marker, were already resistant to the herbicide Basta without the excision of the transposon. This resistance could be explained by the presence of a

cryptic promoter in the transposon end. Because the CaMV 35S enhancer tetramer is positioned next to the transposon end, it can strengthen the putative cryptic promoter so the adjacent *bar* gene is then transcribed. This construct was not used in further experiments. However, if indeed transcription can be initiated by a cryptic promoter in the transposon end, it would then be possible to find mutants caused by gene silencing. This could occur if a gene downstream the *I* end, in an inverted position with respect to it, would be inversely transcribed producing complementary mRNA. This could lead to double stranded RNA molecules that could then trigger silencing mechanisms (Mette, M. F. *et al.*, 2000). If this really happens, it would be interesting to see then if only that single gene or a number of genes are silenced.

The silencing phenomenon was not observed among the mutants that have been more extensively studied. Nevertheless, the possibility of this mutation mechanism remains open.

In conclusion, an alternative strategy to efficiently produce populations of stable transposon-based activation tags in the Arabidopsis genome is described here. The *En/I* activation tag system is validated by describing some of the mutants obtained. Moreover, the principles developed here are applicable to many plants that do not have efficient transformation systems (only a small number of primary transgenic lines is required to generate the activation population) or where somaclonal variation is high, a disturbing feature in forward mutant screens. The efficiency of the transposon activation tags, suggests that this system could be employed to saturate the Arabidopsis and other more complex genomes with inserts.

II. ROOT & PHOSPHORUS UPTAKE SCREENS

Root studies of aerial mutants (population 1)

The roots of the dominant mutants with alterations in the aerial part were studied. The most remarkable root phenotypes (short roots with a fuzzy appearance) were shown by *thread* and a 35S - *THREAD* overexpressor. However, it is interesting that the original mutant and the monooxygenase-overexpressing line show differences in shoot and root phenotypes. The *thread* mutant has a more severe phenotype in the aerial part and a subtler phenotype in the root. In contrast, the overexpressing line used here has a softer shoot phenotype and a more severe root phenotype. A possible explanation for this difference, besides the involvement of the surrounding DNA in the position of the T-DNA containing the 35S - *THREAD* fusion, is given below.

The CaMV 35S promoter is constitutive and highly active in most organs of transgenic plants (Odell, J. T. *et al.*, 1985). In its enhancer, two domains have been defined. Downstream, the A domain includes nucleotides –90 to +8, and includes the TATA box at –31 to –25. On the other hand, domain B is upstream and includes nucleotides –90 to –343. These two domains confer different developmental and tissue-specific expression patterns, though there is synergism between both domains (Benfey, P. N. *et al.*, 1989; 1990a). Domain A is responsible for expression in non-vascular root tissue in seedlings. Additionally, domain A causes strong expression in lateral roots and the pericycle, from which lateral roots develop (Benfey, P. N. *et al.*, 1989; Katagiri, F. *et al.*, 1989). In contrast, while domain B gives the strongest expression in the aerial part of the seedling, it gives lower expression levels in the non-vascular tissue of the root in early developmental stages. However, domain B gives expression in the vascular tissue and the root tip. Moreover, when a part of the B domain is used as tetramer, it can enhance expression in the root. In mature plants, different B subdomains are active in the root cap, hairs and phloem (Benfey, P. N. *et al.*, 1989; 1990a; 1990b).

The original mutant line contained the activating transposon insertion, which carries four copies of the CaMV 35S enhancer B domain. A schematic representation of the enhancer

tetramer is shown in **Figure 31**. On the other hand, the *THREAD* overexpressing lines have the whole 35S promoter that also includes domain A. Besides the influence of the position of the transgene in the transformant, the presence of domain A can explain the more severe root phenotype observed in the overexpressing transgenic line when compared to the original mutant.

The lack of domain A in the enhancer tetramer used in the AIE might be a drawback to use this sytem to obtain very severe root mutants. However, this first test indicated that it is indeed possible to obtain altered root phenotypes.

From the other aerial mutants identified before, *cloudy* mutants seemed to have slightly longer longer roots than the WT. The recessive *var* mutants, on the other hand, displayed shorter roots than the Wt. Interestingly, F1 plants that contained the DR5 - *gus* construct showed longer roots than the Ws wild type control as well. This might be caused by ecotype differences because DR5 - *gus* plants have a Col background (Ulmasov, T. *et al.*, 1997).

Root architecture screen under P deficiency

While root evaluation of the group of mutants described before did not include the influence of nutrient status in root morphology, another experiment was done with a subset of the activation collection. This experiment included nutrient stress, specifically phosphorus deficiency, as a special condition to study postembryonic root development.
Unfortunately, some of the aerial mutants obtained when building the TAT population were handled separately from the rest of the collection. Therefore, they were not included in the tested populations in the root and P nutrition screens.

Unequal pools were used in this screen, and important candidates with reduced seed might have been missed. Nevertheless, a number of putative mutants were obtained from populations 1 and 2. Many of these putative mutants were assayed in medium without phosphate and in medium supplemented with 1 mM phosphate, showing in both conditions a reduced number of lateral roots. The lack of lateral roots in both conditions implies that the affected genes are probably directly involved in lateral root formation (López-Bucio, personal communication).

Some mutants showed aerial phenotypes, and some of them coincided with previously observed phenotypes, such as variegation or reduced size with round, deep-furrow leaves. On the other hand, N, LL and Q showed a characteristic dwarf and dark green aerial phenotype and were suspected to have still the *su1* gene, which causes such a phenotype.

Molecular analysis of putative root mutants

Southern analysis of some fertile candidate mutants, done using Basta resistant plants, showed that most of the plants had one insertion. The dwarf mutants N, LL and Q had indeed the *su1* gene. Mutants with a very similar aerial phenotype showed a band of the same size, suggesting they were carrying the same insertion. Among them, one had two bands. Unfortunately, this mutant was not studied further, because the common insertion with the other plants was thought to cause the observed phenotype, but should be studied to assess if the second insertion can influence root development.

Plants with single AIE insertions were chosen, and TAIL-PCR was used to obtain flanking sequences to their insertions. Some of the plants that showed altered aerial phenotypes that were seen before in the population, showed to have inserts in the same positions as the ones identified earlier. For example, plants from the G3 group, showing a variegated phenotype, had an insertion in the same position of the insert in the earlier variegated mutant. Other examples are the plants with the "furrowed leaf" phenotype, with the same insertion as the mutant *surco*, previously identified, with the same AIE insertion in chromosome 3. It is important to note that the genome sequence that is available in the databases was obtained from the Col ecotype, while the ecotype of most plants in the TAT population is Ws and a few L*er*, and this difference can complicate the positioning of the AIE in the genome in some cases.

Nevertheless, some plants seemed to have insertions near genes with interesting features. One of these genes was annotated as similar to *LRP1 (lateral root primordim 1)*. *LRP1* was identified using a transposon gene trap, and it showed a lateral and adventitious root primordium-specific expression. This gene is activated during the early stages of root

primordium development, but is not expressed prior to the emergence of lateral roots from the parent root. However, the insertion of the transposon gene trap inside the gene does not cause a phenotype in homozygous plants (Smith and Fedoroff, 1995). The authors of this study report that *LRP1* codes for a glycine and serine-rich novel protein. They report aminoacid similarities with the zinc binding sites of the activation domain s of the protein kinase C family of proteins (Smith and Fedoroff, 1995).

Other interesting genes include a gene that has a domain that is similar to Myc-type transcription factor –helix-loop-helix dimerization domain. Myc genes are retroviral oncogenic genes, derived from a single Myc proto-oncogenic gene (Lewin, B., 2000). A molybdenum cofactor biosynthesis enzyme, a RING finger-like protein, a salt tolerant-like Zn finger protein, and kinesin among others might also be interesting if they are indeed overexpressed in these mutants.

Unfortunately, the phenotypic analysis of these and other mutants was hindered by large variation among the mutants and the wild type controls. This variation was probably due to other environmental factors other than just the P deficiency. For example, light, humidity, variations in the composition of ingredients of the medium used, like agar, and changes in temperature among others could account for these lack of uniformity.

Some interesting phenomena were observed while trying to study the roots of these mutants and the wild type, though. One of them was the effect of fungal or bacterial contaminations in the plate in root and plant growth in general. When fungi were growing in one side of the plate, without touching the plants, the seedlings on the other side of the plate seemed to grow stronger and larger than seedlings in uncontaminated plates, and their roots had many lateral roots and hairs. When moved to soil, the plants coming from contaminated plates were visibly more fit than those from clean plates. This effect of fungi in the plant is similar to the reported auxin-mediated effect of *Pseudomonas thivervalensis* colonization in the root of Arabidopsis (Persello-Cartieaux, F. *et al.*, 2001).

Additionally, roots growing under or above the agar displayed also different morphologies. Roots growing under the medium, touching the inside of the bottom part of the plate, had numerous short lateral roots in P deficiency conditions when compared to the roots

that had grown above. In contrast, roots growing under the medium supplemented with P had many long lateral roots.

Nevertheless, some mutants showed root phenotypes that were reproducible under the conditions assayed in these experiments. These mutants included Mutant 17 and G3 (with aerial variegated phenotype). Mutant 17 showed an almost normal aerial phenotype, and was further studied.

Mutant 17

Mutant 17 had a reduced number of lateral roots, some plants lacking them completely under conditions where the wild type controls had many. It also seemed to have less root hairs. Moreover, the primary root was shorter than wild type roots (**Figure 24**). It also appeared to have slight aerial alterations (**Figure 19**).

Interestingly, under conditions where the light source was closer, more nutrients, and more headspace in the plate, the altered root phenotype of this mutant was not visible after two weeks. Leaves displayed then a different phenotype, serrated, while they seemed more pointed in earlier conditions (less nutrients, farther light source and less headspace in the plate). Moreover, roots became greenish after two weeks growing in these conditions.

Crosses of mutant 17 vs. the marker line *IAA28* promoter - *gus* gave interesting results when assayed for GUS activity. First, they showed the semidominant nature of the mutation. Second, stained F2 progeny showed decreased expression of the reporter gene in plants with the mutant phenotype. The *iaa28* mutant is known for having no lateral roots, and the mutated gene belongs to the Aux/IAA genes, that are involved in auxin signaling (Rogg, L. E. *et al.*, 2001).

Because changes in regulation of the affected gene in mutant 17 give reduced lateral root numbers as well, the possibility of direct or indirect interactions between 17 and IAA28 should be considered. These results seem to confirm this idea, and point to transcriptional regulation of *IAA28* by 17.

Interestingly, in the fourth progeny of the original mutant 17, the insertions in a Southern analysis were not segregating as expected if they were stable (progeny of plant 17-2, see **Figure 26**). These plants are not supposed to contain the transposase anymore, though PCR confirmations were not done. If the heterologous transposase is indeed absent, is there then an endogenous transposase activity that can act in the *I-dSpm* exogenous element? If this is true, it was not observed in any other case before, indicating that this is a rare case.

Moreover, because progeny of the plants showing two inserts in the Southern did not show the original mutant phenotype, it might mean that the original mutant was homozygous, and in these plants showing two bands one of the AIE insertions was re-mobilized, rendering the plant heterozygous for the mutation. Moreover, if this is true, it suggests that the mutation was indeed caused by the AIE insertion in the original mutant.

The insertion in mutant 17 was found to be in chromosome 2, BAC clone T26B15 (**Figure 27**). In the position of the insert, two genes are located to the sides. While one gene codes for an unknown product, with similarity to only another gene in Arabidopsis and low similarity to one of *Halobacterium sp.*, the other one has a protein kinase signature. The start codon of the first one is closer to the enhancer than the second one (around 1.1 kb vs 1.6 plus 2 kb from the transposon).

The putative kinase gene is interesting because when blasted shared features with MAPK-like and MAPKK genes from different organisms. Mitogen-activated protein kinases are a specific class of serine/threonine protein kinases. They control many cellular functions in all eukaryotes. Three protein kinases are the components of MAPK cascades that activate successively by phosphorylation. The final targets of these cascades can be transcription factors, protein kinases or cytoskeletal proteins (Whitmarsh and Davis, 1998). In plants, they link stimuli that are activated by external sensors to cellular responses and are involved in stress signaling, plant-pathogen interactions, cell division, and hormone action (Jonak, C. *et al.*, 2002; MAPK group –Ichimura K., *et al.*- 2002).

In yeast and animals, they have been shown to play a role in actin cytoskeleton organization and cell growth polarization Recently, a MAPK from alfalfa, SIMK was shown to play a crucial role in root hair tip growth. The authors of this report show its relation with

the actin cytoskeleton. When overexpressed, SIMK caused increased root formation (Samaj, J. *et al.*, 2002).

Interestingly, the putative kinase near the AIE insertion in mutant 17 has been classified, coincidently, as MAPKKK17. MAPKKKs have been classified in three families: MEKK, SIK, and Raf families, and MAPKKK17 belongs to the first of them (classification from the site: www2.rhul.ac.uk/~ujba110/mapk/mapkkk_genes.htm indicated in Jonak, C., *et al.*, 2002)

Unfortunately, a first attempt to study the expression of these genes by RT-PCR gave negative results. It is important to note that this initial trial was done using the seedlings and plants grown in the conditions where the mutant did not display the clear altered root phenotype. Neff and collaborators, who used T-DNA activation tagging, studied a suppressor gene of the *phyB* phenotype. Interestingly, they observed that the gene, *BAS1*, was not constitutively overexpressed as a result of the neighbouring activating T-DNA. Instead, its expression was just increased, but it retained its normal light regulated expression pattern (Neff, M. *et al.*, 1999). Experiments to study expression of these genes in conditions where the mutant phenotype is observed should be done to address whether this is the case. Other possibilities include lack of overexpression, with the mutation being not related to it. Moreover, there are examples of gene miss-annotation in the databases. In that case, the primers used for this analysis would not be useful. For example, if they were inside an intron instead of the predicted exon, and other primers should be used.

Shadowed roots and insoluble phosphate screens

By shadowing the roots interesting observations could be made. Initially, root observations during the first days did not show a marked difference between light and shadow-grown roots. However, 14 days later some differences were observed and were more striking afterwards. The main difference of shadowed versus light-grown root was elongation of the primary root. Also differences in the number of lateral roots were observed. Another difference was that shadowed roots seemed to cause less stress symptoms under P deficiency

than light-grown roots. However, chlorophyll and anthocyanin measurements were not done to confirm the visual evaluation. If stress responses are indeed reduced, is interesting to ask whether P is necessary to avoid or overcome stresses such as light in the roots, or if this phenotype is just the additive effect of different stresses. In nature, the roots of plants grow under the ground, and they normally do not receive daylight. Those plants show the symptoms associated with low P. The question of whether the lower susceptibility to P stress of shadowed roots is just an artifact also remains open.

Furthermore, differences of root morphology of L*er* plants in low and high P were easier to distinguish by using covered root experiments, and some candidate root mutants showed root differences when compared to the wild type. Those root morphology differences varied from just root length to altered responses to light or shadow.

Finally, the whole population (including the five subpopulations, but not including previously characterized or separated aerial mutants) was screened using an insoluble P source. Putative mutants were observed, but they must be analyzed and confirmed.

Genes that could help to enhance P solubilization and uptake when overexpressed would be the candidates to be found. Some examples would be those genes whose products cause changes in the medium pH or code for organic acids transporters, among others.

Interestingly, in the conditions used in the screen, many plants started to flower before 21 days. However, the CS controls flowered much later. These controls have a Col background, most of the population a Ws, and some a L*er* background, which can be the reason of the marked difference in flowering times. An interesting observation was that some plants of increased size with respect to the other plants in the plate seemed to have germinated under the medium, leaving a hole in the medium. It would seem that, in order to grow outside the medium, these plants grew bigger than the rest.

In conclusion, while problems were encountered to study root architecture, some promising mutants with altered architecture were observed, and should be further studied.

CONCLUSIONS

The main conclusions obtained from the work here presented are indicated below.

A) Regarding the transposon activation system.

1) Minimal transposon ends were functional for transposition in Arabidopsis (200 bp for the left Terminal Inverted Repeat and around 400 for the right, shorter than previously reported functional ends). 2) Transposon ends that were isolated from different loci were used to "rebuild" a functional transposon, and showed that the presence of a true direct repeat footprint is not necessary for transposition to occur, given the presence of the transposase. 3) When using the constructs showed here, the *in cis* strategy[4] was found to be more suitable to build large populations than the *in trans* strategy[5].

Transposon Activation Tagging produces "cleaner" insertions than T-DNA. In contrast to plants bearing T-DNA inserts where mutations that are not directly caused by the insertion are produced due to the transformation process, most of the mutant phenotypes obtained by Transposon Activation Tagging (TAT) were found to be due to transposon insertion. The frequency in which aerial morphological altered phenotypes were observed in the TAT population was around 1%, in comparison to the reported frequency of 0.1% in a T-DNA Activation Tagging population (Weigel, D. *et al.*, 2000). *In short, and according to published data, Activation Tagging using transposons is more efficient than using T-DNA as the tag.* Moreover, the use of transposons as activation tags can open the possibility of using Activation Tagging in crops where transformation methods are not efficient enough to produce high numbers of T-DNA-containing plants.

B) Regarding the mutants obtained with the TAT system.

[4] *I* transposon and *En* transposase source introduced to the plant in a single vector.
[5] *I* transposon and *En* transposase source introduced to different plants in separate vectors.

The aerial morphological mutants studied, of dominant nature, showed that the *I/Spm* Activation Tagging transposon system can be effectively used to produce insertion activation mutants. When the mutants were studied, overexpressed genes adjacent to the AIE were found in almost all cases. From these mutants, some are affected in genes found by T-DNA Activation Tagging, in genes that belong to gene families also identified by T-DNA Activation Tagging, and some in genes that have not been described before. Different kind of genes were affected in the mutants identified in the TAT population, some of them being enzymes and some being transcription factors. In some cases, the overexpression caused by the enhancer inside the AIE element seemed to be ectopic, and in other cases not totally.

Finally, the TAT population was used to search for genes involved in postembryonic root development and growth using an insoluble phosphate source. Candidate mutants with root morphology alterations and candidate mutants with enhanced capabilities for growth in insoluble phosphate sources have been found in the population generated with Activation Tagging transposons, indicating the applicability of this system to diverse kind of screens.

PERSPECTIVES

Strategies like Activation Tagging have shown their value as tools for new gene function discovery. Improvements, like the use of the transposon system described here, can make Activation Tagging even more valuable. Further improvements to this kind of activation systems might involve the use of fine tuned enhancers. Transactivation systems and chemically inducible systems have already been built and are in use, as indicated in the introduction. Inducible systems allow the recovery of lethal or very severe dominant mutations, and aid in the study of gene functions, for example, of those genes that provoke early phenotypes that mask their roles in later stages when constitutively overexpressed. However, the inducible and transactivation systems reported to date use T-DNA as a tag, and might be improvable with the use of transposons.

Furthermore, other kind of inducible systems could be used as well, not necessarily chemically inducible ones. An example would be a combination of tags, preferably transposons, containing *in cis* binding elements, and virus-based vectors with the corresponding DNA-binding-activating proteins. This kind of system would have the advantage that no transformation would be required, just virus infection to induce the system. The DNA-binding-activating protein in the virus could also be expressed with differently regulated promoters, allowing fine induction. Plants containing exactly the same tag could be infected with different viruses where the DNA binding-activating protein is expressed in different tissues or times, and the role of the activated gene could be studied in more detail. The main disadvantage of the system –at least until now, but maybe can be solved in the future- would be that it might not be effective in meristems, where viruses can not reach. Endogenous enhancers could also be used for Activation Tagging. Moreover, though not exactly for Activation Tagging, maybe even the use of very specific endogenous repressors can provide interesting results, diminishing or preventing expression of genes in specific times or tissues, in contrast to knock-outs or gene silencing, where the silencing signal can travel and would have broader effects.

In general, the Transposon Activation Tagging system has a wide variety of applications that can range from the pure scientific search for new gene functions to the direct generation of commercially valuable interesting crops. The system described here, or more refined Transposon Activation Tagging populations could be used for a very broad number of studies: screens for visual developmental/morphological alterations, metabolic screens, and screens for resistance/tolerance to biotic, physical or chemical stresses. Moreover, studies of plant growth in unusual conditions (without gravity, for example) or to search for altered responses to different stimuli of diverse nature (light, nutrients, other organisms, controlled environments, signaling compounds, etc.) can also make use of this kind of strategy.

Furthermore, alterations in regulation of gene expression like in gene-silencing processes or altered expression of reporter genes fused to promoters of interest can also be looked for using Transposon Activation Tagging. Other kind of regulation alterations in the genome, transcriptome or proteome could be studied, given a suitable system to monitor changes.

Additionally, the use of transposons, as mentioned before, can be of great help in studying gene functions in non-model plants of human interest, for which suitable transformation systems have not been developed, an interesting example could be medicinal plants. Either an activation system with "constitutive" or with specific or inducible enhancers could be used then.

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KASUZA	http://www.kazusa.or.jp/kaos/

APPENDIX I. Phenotype of *cloudy* F1s.

Event (female x male)	F1 Plants Mutant phenotype	F1 Plants Wild type phenotype
C55 self	7	4
C55 x Ws	2	6
C56I self	6	5
Ws WT x C56I	0	8
Col WT x C56I	0	8
C56I x Col	6	0
C57 self	7	5
C57 x Ws	6	2

APPENDIX II. Candidate root mutants from population 2, obtained under P deficiency conditions.

Mut.	Root phenotype	Aerial phenotype	Tray #
11	No lateral roots	Multiple thin stems	7 & 8
17	No lateral roots		
19	No lateral roots	Multiple thin stems	9 & 10
22	No lateral roots	Multiple thin stems, 15 cm., infertile	1 & 2
26	No lateral roots	Multiple thin stems, 15 cm., cup-like cotyledons	1 & 2
29	No lateral roots	Multiple thin stems, 15 cm.	1 & 2
33	No lateral roots	Multiple thin stems, 15 cm.	1 & 2
37	No lateral roots	Multiple thin stems, 15 cm.	1 & 2
38	No lateral roots	Multiple thin stems, 15 cm.	1 & 2
39	No lateral roots	Multiple thin stems, 15 cm.	1 & 2
41	No lateral roots	Multiple thin stems, 15 cm., cup-like cotyledons	1 & 2
52	Abundant root hairs	No trichomes, slow development, 16-19 cm., infloresences are formed in the tip of the stem, shiny green siliques. The progeny did not show the phenotype again.	17
68	No lateral roots	Many stems, 30 cm.	20

Note: Wild type controls could reach 45 cm. in height, and had several lateral roots. 5 seeds of each candidate mutant were assayed. Other selected mutants (not included in this table) were #55, #81, and #83

APPENDIX III. Aerial phenotype of candidate root mutants.

Mutant	Phenotype
D	Medium size (showing basta resistance segregation)
G3	Variegated
H	Wild type
LL	Short (SU1 phenotype)
N	Short (SU1 phenotype)
Q	Short (SU1 phenotype)
11 and 19	Normal
22-41	Same phenotype as mutant *8-2 (surco)*
52	L*er*
68	Normal

APPENDIX IV. Seedling and adult phenotype of candidate mutants obtained during a screen using variable P and light conditions.

A. Mix 1, 1 mM P, light				
75 seed in 9 plates (14 did not germinate).				
WT	**Root length (cm)**	**Comments**	**Final heigth (cm)**	**Ramifications & comments**
Ws	8	>4 LR, > 4 true leaves	>35	Medium
Mut	**Root length (cm)**	**Comments**	**Final heigth (cm)**	**Ramifications & comments**
A1	2.4	No LR, true leaves begin, small cots.	17	few
A2	4.3	No LR, less developed	20	
A3	7.8	Normal green, more LR (?)		Basta sensitive
A4	5.6	Long root, 1 LR, pale plant	29	Medium
A5	5.6	Like A4	21	few
A6	4	Two primary roots (4 and 1.7 cm.), smaller than WT	15	Medium
A7	4.6	1 LR, smaller than WT	22	Medium
A8	2.7	2 roots, 4 LR, dwarf (looks like L*er*)	11 Ler	One branch 22 cm
A9	7.2	1 LR (1.7cm) with subramifications	21	Many
A10	5.3	Dwarf, no LR	27	Medium
A11	5.6	Dwarf, no LR	16	Medium
A12	4.3	Small, L*er*-like, 2 LR	14	Medium, Ler(?), reduced size siliques

B. Mix 1, 0P, light				
65 seed in 7 plates (11 did not germinate)				
WT	**Root length (cm)**	**comments**	**Final heigth (cm)**	**Ramifications & comments**
WT Ws	2.3	6-9 LR, subramified	>35	Medium
L*er*	3.6-4.5	10-12 LR		
Mut	**Root length (cm)**	**comments**	**Final heigth (cm)**	**Ramifications & comments**
B1	0.4	4 LR, small plant	11	Many
B2	0.9	4 small LR, small plant	34	Few
B3	3.7	Only 4 LR without ramifications	16	Few ramifications. Very small siliques, easy to open.
B4	1.2	4 LR		Did not survive
B5	0.6	3 small LR, small plant	30	Medium
B6	3.6	4 very small LR	20 L*er*	Medium
B7	1.5	3 small LR, pointed, elongated (alargadas) leaves17	22 L*er*	Few ramifications. Small siliques.
B8 *	4.5	3 very small LR, very small plant.	17	Few ramific. Medium sized siliques. Maybe L*er*.
B9	1	4 LR, Normal root morphology but shorter.		Basta sensitive
B10	1	As B9		
B11	1	As B9		

C. Mix 2, 1 mM P, light					
21 plates,10-12 seed per plate, 9 did not germinate.					
WT	**Root length (cm)**	**Width Plant (cm)**	**# LR & comments**	**Final heigth (cm)**	**Ramifications & comments**
Ws	8	1	> 4 LR, > 4 true leaves	>35	Medium
Mut.	**Root length (cm)**	**Width Plant (cm)**	**# LR & comments**	**Final heigth (cm)**	**Ramifications & comments**
C1	0.6	2	Bigger plant (hanging downwards)		Basta sensitive
C2	0.6	0.3	Serrated leaves		No plant
C3	0.3	0.5			No plant
C4	1.3	0.3	No LR, many root hairs	24	Medium ramif. Small siliques. Similar to B7. Still green after 3.5 months. Maybe L*er*.
C5	2.5	0.3	No LR		Did not survive
C6	2.5		Medium/small plant, 2 long LR, no visible trichomes		
C7 *			In a contaminated plate, where most plants are big, this plant remained small.		
C8	3	0.3	No LR, wavy root, small plant	22	
C9	1.9		Like C8, only 2 cotyledons	18	
C10		0.2	Like C8 and C9. 2 concave	27	

			cotyledons.		
C11			Like C8, C9 and C10		Basta sensitive
C12	2.4	0.3		30	
C13	2.5	0.4		18	
C14	2.5	0.4	Pale plant	27	
C15	5.3	0.7	1 LR, 2 cots, 2 leaves. Seems "normal", but the new leaves are concave.		Basta sensitive
C16	10.5	1.5	6 LR,		Basta sensitive
C17	4.3	0.6	3 long LR	17	Medium. Maybe L*er*.

Group of mutants C18-C27 reduced plant size, primary root medium size, no LR							
Mut	**Root length (cm.)**	**Lateral Root (LR) #**	**Plant width (cm.)**	**Leaf #**	**comments**	**Final heigth (cm)**	**Ramifications & comments**
C18	5.5	0	0.5	4		16	Many
C19	5	0	0.5	6		28	Medium
C20	6.5	1 small in the top	0.5	6		10	Many
C21	5.5	0	0.5	6		19	Many
C22	5	0	0.5	4		24	Few
C23	3.5	0	0.4	4	2 small leaves		Did not survive
C24	3.7	0	0.4	4	2 small leaves	20	Medium
C25	5.4	0	0.5	4	2 small leaves	21.5	Medium
C26	5.3	1 very little	0.4	4		20	Medium
C27	6.4	0	0.4	6	2 small leaves	10	Extremely ramified.

E. Mix 2, 1 mM P, shadowed roots							
72 seed in 7 plates (6 did not germinate, 4 in a contaminated plate).							
Almost all the candidate mutants showed the same phenotype: reduced size, shorter main root and no lateral roots.							
Mut	**Root length (cm.)**	**LR #**	**Plant width (cm.)**	**Leaf #**	**comments**	**Final heigth (cm)**	**Ramifications & comments**
E1	1.8	0	0.2	2	minuscule leaves		Did not survive
E2	3	0	0.5	2			Did not survive
E3	2.5	0	0.4			30	Medium
E4	3.5	0	0.4	4		11	Many
E5	3.8	0	0.4			24	
E6	2.6	0	0.3	2	Minuscule leaves		Did not survive
E7	1.6	0	0.2	2	Only 1 cot., minuscule leaves, reduced plant size		Did not survive
E8	2.3	0	0.3	2	Minuscule leaves		Did not survive
E9	1.6	0	0.5	2	Minuscule leaves	29	Medium
E10	2.6	12	0.4		In a contaminated plate (but very reduced plant size)	25	Medium

F. Mix 2, O P, Shadowed roots
79 seed in 7 plates (8 did not germinate)

WT	Root length (cm.)	LR #	Plant width (cm.)	comments	Final heigth (cm)	Ramifications & comments
Ws	6.2	12	0.7	2 mini-leaves	>35	Medium
L*er*	7	11	0.6-0.7			
Mut	**Root length (cm.)**	**LR #**	**Plant width (cm.)**	**comments**	**Final heigth (cm)**	**Ramifications & comments**
F1	1.2	2	0.3	LR small		Basta sensitive
F2	0.4	4	0.3	Very small LR in the root tip	23	Few ramifications. Main stem is not straight.
F3	3.5	0	0.4		24	Medium
F4	3.7	2	0.3	1 LR small. There was another plant of similar size, but with long root (~6 cm)	25	Medium
F5	2.2	0	0.2			Did not survive
F6	1.7	2	0.3			Basta sensitive
F7	1.8	9	0.4	2 very small leaves	30	Medium
F8	1.7	3	0.3		22	Medium. One of the stems is odd-looking.
F9	6.4	1	0.3 – 0.4	LR above		Basta sensitive
F10	2.4	1	0.3		27	Medium.

I. Mix 2, 0P, light						
304 seed in 26 plates (10 seed did not germinate).						
4 groups of mutants						
WT	**Root length (cm.)**	**LR #**	**Plant width (cm.)**	**comments**	**Final heigth (cm)**	**Ramifications & comments**
WS	2-2.5	7-11	0.5	Short lateral roots, higher LR density in the root tip than above.	>35	Medium
L*er*	3.4 to 5.7	7-9	0.3-0.4	The LR density is lower in the root tip (see **figure 29**)		
Group 1. Roots shorter than the controls						
Mut	**Root length (cm.)**	**LR #**	**Plant width (cm.)**	**comments**	**Final heigth (cm)**	**Ramifications & comments**
I1	0.6	3	0.2	Normal Ws root architecture		Did not survive
I2	0.6	0	0.1	Only 1 cotyledon (pale)		Did not survive
I3	0.5	2	0.25		25	Medium
I4	0.7	4	0.2-0.3	Normal Ws root arch.		Sensitive to basta
I5	0.9	4	0.3	Swab/broom-like root	30	Medium
I6	0.7	4	0.2	Normal arq.	28	Few
I7	0.6	2	0.2	Relatively normal arq.	24	Few
I8	0.3	4	0.2	Swab/broom-like root	26	Few
I9	0.2	0	0.1	Seems like just starting to germinate	27	Few
I10	0.8	5	0.3		26	Few
I11	0.9	5	0.3	Normal arq.	20	Few

Group 2. Roots that are longer or with different lateral root number than the wild type						
MUT	**Root length (cm.)**	**LR #**	**Plant width (cm.)**	**comments**	**Final heigth (cm)**	**Ramifications & comments**
I12	~10	15	0.6		28 L*er*	Small siliques
I13	~3.2	5	0.5	Wider cotyledons	11	Very ramified (siliques slightly vertically oriented)
I14	6	5	0.5	3 extremely small LR	27	Medium
I15	6	7	~0.5	Very small LR	25	Medium
I16	5.7	1	0.4	LR with 1 subramification	13	Medium
I17	7.2	5	~0.5	Taller shoot than Ws	27	Medium
I18	1.1	0.1-0.2		Very pale and only 2 cotyledons.		Did not survive

Doubtful candidate mutants						
Group 3. Medium-sized roots (shorter roots than the wild type)						
Mut	**Root length (cm.)**	**LR #**	**Plant width (cm.)**	**comments**	**Final heigth (cm)**	**Ramifications & comments**
I19	0.9	8	0.2		16	Medium
I20	1.1	3	0.3		29	Few
I21	1	8	0.3		13	Medium
I22	0.8	7	0.2		15	Medium
Group 4. Candidate mutants with initially lower number of lateral roots, that later developed more lateral roots.						
I23	2	7	0.4		40	Few
124	2.5	8	0.5		40	Few
I25	3.5	8	0.5			Basta sensitive
I26	1.8	5	0.4			Basta sensitive

Notes: Mutants A, B and C1-C11 were measured after growing for 16 days in the growth chamber. Mutants C12 onwards, E, and F after 17 days, and mutants I after 18 days in the growth chamber. Ws and Ler wild type seedlings were used as controls. Letters D and G were not used deliberately to avoid confusions with previous mutants named with those letters. Plants can also have reduced size due to late germination.