

Molecular Mapping of Auxin-Resistant 4 Enhancer Mutants

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Abstract

The plant hormone auxin plays an important role in plant growth and metabolism. This experiment is part of an effort to understand the biochemical pathways of transport of and response to auxin through studies of auxin resistant mutants of the plant *Arabidopsis thaliana*. *auxin-resistant4 (axr4)* is one such mutant. In this mutant, the auxin importer protein AUX1 is mislocalized, but the exact mechanism of AXR4 action is unknown. We are identifying proteins that function in the same pathway as AXR4 by isolating enhancer mutations of *axr4* and mapping them at the molecular level. The enhancers of the *axr4* mutations were isolated in the Columbia ecotype. In the F2 progeny derived from a cross between the enhancer mutant and a wild type plant from the Landsberg ecotype, the polymorphisms near the mutant alleles are likely to be from the Columbia ecotype since the polymorphisms of the Columbia parent and the mutations are linked. Hence, it was attempted to trace the enhancer mutation by finding the Columbia polymorphisms in the genome of the mapping population. DNA was collected from a phenotypically pre-screened population of 30 F2 enhancer mutants and amplified using PCR with primers specific to various markers on the *Arabidopsis* genome. Linkage was seen at the bottom of chromosome 5 between markers at 17.32 Mbp and 22 Mbp. This suggests the presence of the enhancer mutation at this locus on the 5th chromosome of *Arabidopsis thaliana*. Further mapping is in progress in order to define the position of the enhancer mutation more precisely.

Keywords: molecular mapping, SSLP, ecotypes, PCR, electrophoresis, *Arabidopsis*, auxin, *axr4*

1. Introduction

Arabidopsis thaliana is a small dicotyledonous plant that belongs to the Brassicaceae (mustard) family. *Arabidopsis* is very popular among geneticists for several reasons: it is very easy to grow; it has a short life cycle of approximately 2 months; it has a relatively small size (which means that many plants can be grown in a small area); and, is capable

of self-fertilization. Additionally, its small genome has already been completely analyzed¹.

The genetic approach is an important tool in identifying and understanding the function of genes that are necessary for a particular biological process. This experiment is part of a larger project that is investigating the biochemical pathway of the phytohormone auxin. Phytohormones are chemicals that control plant growth and metabolism and are active at extremely low concentrations. These chemicals are known to regulate the time for flowering, seed shape, root and leaf growth, leaf shape, lateral root formation, etc. Auxin is responsible for, among other processes, embryonic pattern formation, lateral root growth, and photo- and gravitropism. When auxin is present in small amounts, it stimulates plant growth, but at high concentrations it inhibits shoot and root growth, and can even lead to plant death.

Auxin resistant mutants have been isolated in order to understand the pathway underlying the auxin response. One such mutant, called *auxin resistant 4-2* (*axr4-2*), was isolated and characterized by Hobbie and Estelle (1995). Although the *AXR4* gene was sequenced and cloned², the biochemical function of the protein is still unknown. The *AXR4* protein is, however, known to contain an alpha-beta hydrolase fold and a single transmembrane domain. An important aspect of the *axr4* phenotype is that mutant plants mislocalize AUX1, a vital auxin influx protein in plant cells.

A classical genetic approach has been used in an attempt to try to find other genes that participate in the same pathway. Essentially, a multi-protein complex is envisaged and *axr4* is a mutation that renders this complex slightly dysfunctional. Now, through the classic genetic approach, an attempt is being made to find another gene that codes for a protein involved in this multi-protein complex. A mutation in this gene should further demolish the function of the complex. These modifier mutations are called enhancers since they increase the auxin resistance of the *axr4-2* mutant. For instance, enhancers should have longer roots than *axr4-2* single mutants on auxin. This method of studying enhancer mutations has been successfully and frequently used in plant genetics in an attempt to understand mutations in various genes in *Arabidopsis thaliana*. One such example is the *salt overly sensitive 3* gene whose enhancer mutation has been studied to understand the function of this gene. A genetic screen for putative enhancers and suppressors was performed and mutants with higher-than-*sos1-3* salt sensitivity were isolated³.

Similarly, the *axr4* enhancer mutants were developed (and later used for the molecular mapping of the enhancer mutation) by treating *axr4-2* seeds with ethylmethylsulfonate (EMS). EMS induces random point mutations in the genomes of the exposed seeds⁴. Thus, putative *axr4* enhancers and suppressors were created. The mutant seed population was self-crossed to yield M2 offspring. Subsequently, 20,000 M2 seeds were subjected to a genetic screen for enhanced/suppressed auxin resistance. Approximately 250 seedlings with roots longer-than-*axr4* were selfed to get M3 seeds. The M3s were retested at 4×10^{-7} M dichlorophenoxyacetic acid (a synthetic auxin), and the ones with long roots were crossed to wild type Columbia (wt Col). This backcross was done to eliminate other mutations in the genome. This gave us the F1 population. The F1 was selfed to yield F2 seeds.

The progeny of the cross between the mutant plant (Columbia plant) and wild type plant (Landsberg plant) yield F1. These progeny were allowed to self-fertilize to yield F2.

The F2 seedlings were then analyzed. The ones homozygous for the enhancer mutation (as well as for the *axr4-2* mutation) were identified. The double mutant seedlings had roots longer than the roots of the *axr4-2*. The chosen seedlings were then planted in pots. The resulting plants were allowed to self-fertilize and yield the F3 population, which was eventually used for molecular mapping. It is possible for mapping the plant gene to be attempted because in previous experiments, in the lab, mutant plants with defects in this gene have been identified. Molecular mapping comprises following the inheritance of the DNA sequence for a mutation of interest along with DNA markers.

A discussion of ecotypes (or varieties of strains) is imperative for the understanding of the rationale behind the molecular mapping. Essentially, there are numerous strains of *Arabidopsis*. The ones used are Columbia (Col) and Landsberg erecta (Ler). There are small differences in the genetic sequence between the two ecotypes in every chromosome. These are called molecular markers (polymorphisms). It is due to these polymorphisms that there are slight phenotypic differences between two strains. There are numerous sequence differences between Ler and Col at various points in their respective genomes⁵.

Consequently, it is easier to locate polymorphisms that are located near the mutations. This, in essence, is molecular mapping. The *axr4-2* mutant and the enhancer mutation both originated in the Columbia ecotype. When they are crossed to a wild type (Ler) plant, it can be expected for the polymorphisms very near the mutant alleles to be those in the Columbia ecotype. This is because the smaller the distance between a marker and mutant gene, the smaller the chance that crossing over will occur. This means that the DNA polymorphisms of the Columbia parent and the *axr4-2* and the enhancer mutations are linked.

Hence, chromosomal loci can be detected by finding the Columbia polymorphisms in the genome of the mapping population. The gene loci for inspection were amplified using Polymerase Chain Reaction (PCR). This helps to clearly identify the polymorphisms at various loci on the chromosomes. Agarose gel electrophoresis to view the polymorphisms were used.

When a certain locus, in the mapping population, shows a polymorphism found only in the Landsberg ecotype this means that the genes on that locus have come from the wild-type (Ler) parent and can, obviously, not contain the enhancer gene. If both Columbia and Landsberg markers are seen, the organism is said to be heterozygous at that locus. The DNA at that locus comes from parents of both strains. Columbia alleles in the mapping population are expected to be seen since it is known that the mutant allele came from the Columbia ecotype. Hence, the genes that only showed Columbia polymorphisms at a particular chromosomal locus were pinpointed. The Columbia linkage indicated the locus of the desired enhancer genes. When the enhancer gene is isolated and cloned, the protein this gene codes for, as well as its function, can be studied. Once this information is gathered and processed, clues regarding the role of the AXR4 protein pathway can be found.

2. Materials and Methods

2.1 seed sterilization and planting

F₃ seeds, along with controls from Landsberg and Columbia ecotypes (as well as *axr4-2* mutants), were sterilized⁶, spread on 2,4-D plates (containing nutrient 0.7% agar and

1% sucrose, and 4×10^{-7} M dichlorophenoxyacetic acid) and placed into a refrigerator at 4C. The sterilizing solution consisted of 30% bleach, water and 0.02% Triton X – 100. After 24 hours, the plates were moved to a growth chamber (maintaining constant temperature of 21C and 16 hours light/8 hours dark).

2.2 analysis and DNA extraction

Six days later, the F₃ seedlings were analyzed, along with the controls. Tissue was collected and DNA isolated from the seedlings that were homozygous for the enhancer mutation. DNA was isolated according to the procedure in Lukowitz et al., (2000).

2.3 polymerase chain reaction

PCR was performed using thermally stable DNA polymerase (Taq), 250µM DNA precursors (deoxyribonucleotide triphosphates), 250nM primers (two per reaction – one forward and one reverse), 1X PCR buffer (50 mM KCl, 10 mM Tris HCl, 1.5 mM MgCl₂), and 2.0mM MgCl₂ (required for polymerase reaction). PCR was carried out using the following program: 1) 5 min at 94C, 2) 30 sec at 94C, 3) 30 sec at 55C, 4) 30 sec at 72C, 5) repeat steps 2-4 39 more times, 6) 10 min at 72C.

2.4 agarose gel electrophoresis

The tested markers were at different locations from all five chromosomes. The sequences and locations of these primers are shown in Table 1. Agarose gel electrophoresis was performed⁶. The buffer used was 1X TBE⁷. The agarose concentration (for the gel preparation) was 3-4% (ultra pure agarose Amresco). Voltage used was 120V for a period of 55 min. After the period of 55min, the gel was removed and stained with ethidium bromide solution (1X TBE buffer containing 1µg/mL EtBr) for 15min. After that the gel was destained for 10min with TBE buffer. The last step involved exposing the gel to UV light and photographing the image.

Table 1. Molecular markers used

ID Letter Code	Chromosome/ Position (cM)	Forward primer	Reverse primer
AB	1.15	AAATGGGAGATGTTGATGTTCTTC	CCAAAGGGTCACGAGTGAAGTCTAG
AE	1.49	TTGGATTATCTCTTGAATGGGTTTGG	CCGTCTTTAGCATTAGGAACTCTGGTG
AQ	3.07	CATCCGAATGCCATTGTTC	AGCTGCTTCCTTATAGCGTCC
AS	3.53	TCAATGGCAGCAAAGTCCATTAATC	AACAGATACCAACTCCACAGGGACAA
CG	5.10	AAGTAGCCCAAAGCCGTACA	GCAGGGACAATCCGTAAAGA
BJ	5.60	Aattgtggaaggacaacaacaaaa	gagagagcacgtgagatgtcacaga
VC	5.88	TCGGAAAAAGTATGTTGGGAGT	TGTTCAACAATAGCTGCCAAA
VH	5.22	TCACCTTACTTAATTCAACTGCAA	CCAGATTCGATGTACTTCACTTTC
VP	5.23	AAAAGGTTCTGTCTAGGTACTGTC	AATATATGCGTATTTCTGCAAGC
VE	5.17	CCGATATAACTAAAGGTGCAGAGA	AAAATTCCTACCAAACGAAGCA
VG	5.18	GATTTGCTCTCTGCCCCAAA	AAAAATGACGGGACGAAAGT
VQ	5.24	CAAAACCAAACAAGGACGTG	GCTTGCACTCTCCTATTTAACAAA
VU	5.25	TGTGAGAGGTCAAAAATATAGATGAAAA	GCACCGCCTAAAGATTTTAC
VV	5.26	GGATCATATGACTGATGGAACCTCT	AGGAAGTACAATCTGAAGTGAAAAAC

3. Results

Pooled DNA from the enhancer mutation line FE 60.4.1xLer was tested for linkage with various markers across the 5 chromosomes. Markers that showed linkage to Columbia DNA were further tested with individuals from FE60.4.1. The number of Columbia/Landsberg alleles found are summarized in Table 2. In Fig 1 it can be seen that DNA from individuals shows Columbia linkage for all alleles at location 25.21 Mbp, and for 26 alleles at location 9.45 Mbp. This suggests that the enhancer mutant gene is closer to marker BO than to BJ.

Table 2. Results from individuals from line FE60.4.1xLer

Primer	Marker Name	Nt location from top of chromosome 5	Total # of chromosomes	Total # of individuals tested
BH	CA72	4.25 Mbp	Col: 26 Ler: 4	15
BJ	F2P16	9.45 Mbp	Col: 26 Ler: 4	15
VC	MMG4	17.32 Mbp	Col: 29 Ler: 1	15
VH	K18G13	22.00 Mbp	Col: 30 Ler: 0	15
VP	MRI1	23.39 Mbp	Col: 30 Ler: 0	15
VV	F1505	26.01 Mbp	Col: 30 Ler: 0	15
BO	MQB2	25.21 Mbp	Col:30 Ler: 0	15

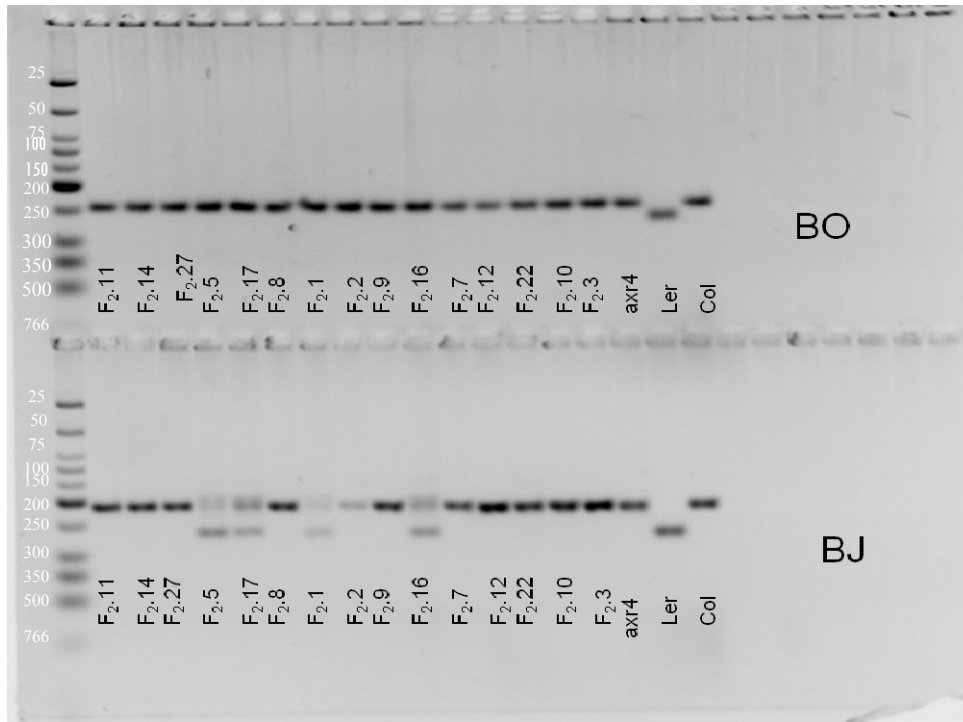


Fig 1. Individuals from line FE 60.4.1xLer tested with primer BO (with location 25,208,788 on chromosome 5) show tight linkage to Col. When tested with primer BJ (with location at 9,445,198 on chromosome 5) they show loose linkage to Col. The controls used were: axr4-2gl1, wtLer, and wtCol. The first column contains low weight molecular marker.

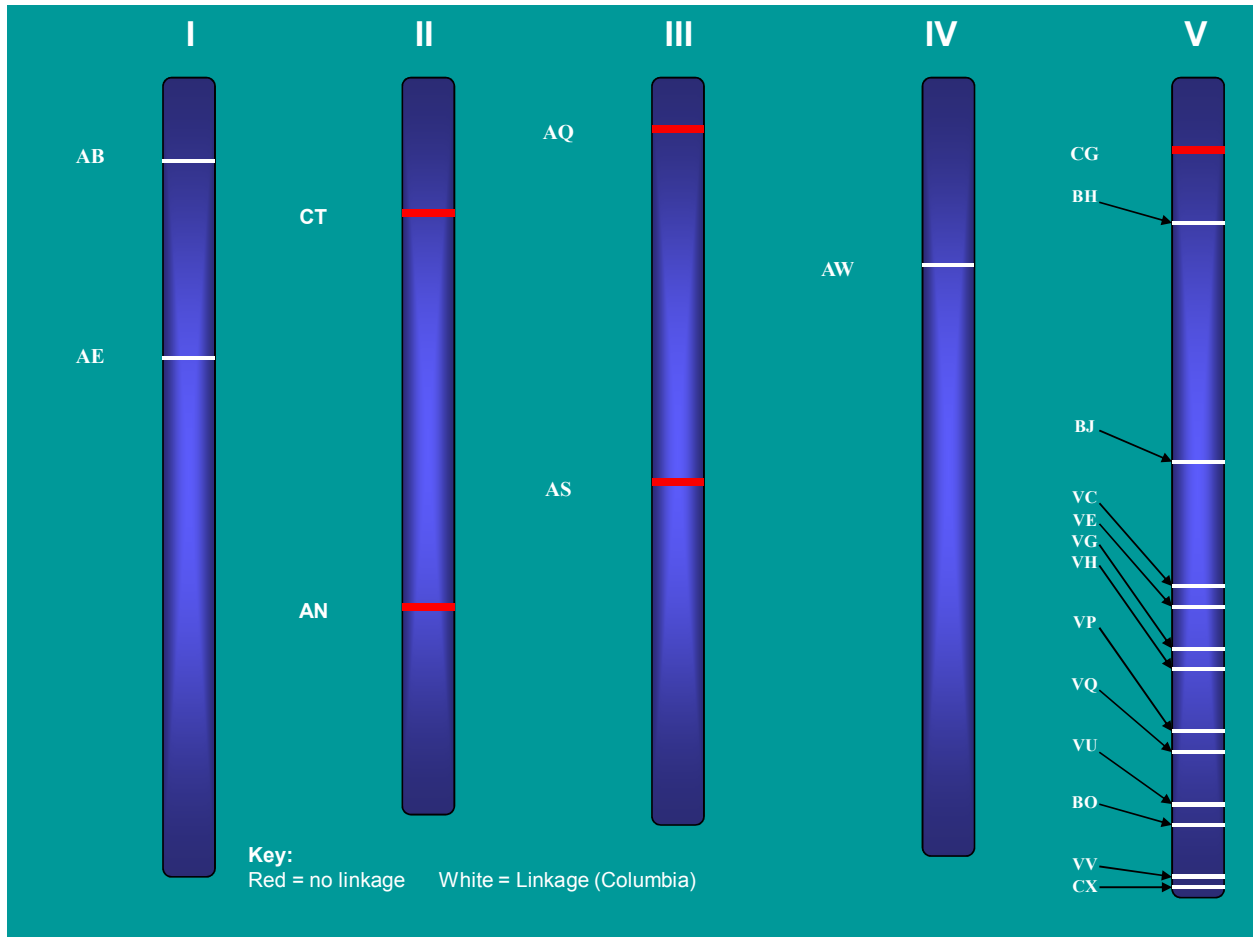


Fig 2. Diagram showing all markers tested and their relative position on the five chromosomes of *Arabidopsis thaliana*.

4. Discussion

Previous studies have used simple sequence length polymorphisms for pinpointing the locus of mutations (including enhancer mutations) in *Arabidopsis thaliana*⁸. This study has used the same technique and shown strong evidence for the presence of an *axr4* enhancer mutation in the lower part of chromosome 5. However, in order for these results to be verified and the enhancer gene located, more PCR reactions need to be performed.

For instance, the mapping population in this experiment consisted of 15 individuals. The selection of these individuals was based on phenotypic analysis of the F₃ population. This number of individuals is the minimal number needed for rough mapping. However, for high resolution mapping, 400-600 individuals are required⁹.

From the results based on the analysis of the gel electrophoresis pictures, it can be concluded that from the markers tested with individuals, there is linkage to Columbia with 7 of them: CA72 (BH), F2P16 (BJ), MMG4 (VC), K18G13 (VH), MRI1 (VP), MQB2 (BO), and F1505 (VV). Markers AE and CB, located on chromosome 1 at 1.49 cM and 1.83 cM respectively, also showed linkage to Col DNA. These results suggest linkage at more than one location. This, however, is expected since double mutants are being tested. They have both the *axr4* mutation and the enhancer mutation. Since there

are two mutations, it is expected for linkage to be seen to more than one position on the chromosomes. The position of the AXR4 gene is already known⁶ to be about 61 cM from the top of chromosome 1. The results are consistent with this information: primers AE and CB are just above and below that gene and they show the presence of Columbia DNA in the individual F₃ at these locations. The presence of second linkage (primers VC, VH, and VP with positions 5.88 and 5.113 cM) suggest presence of Columbia DNA at the bottom of chromosome 5. Since primer BJ (position 5.60) shows presence of both Col and Ler DNA, we could assume that the mutation is closer to VC than to BJ.

Future work would include testing more individuals with primers from chromosome 5 (a minimum of 300 individuals) to increase the resolution of the PCR study. PCR with primers from the other four chromosomes will also be repeated. This is needed so that it can be confirmed that there is no other place along the Arabidopsis genome where there is a linkage to Col. The next step is to isolate and clone the enhancer gene. Once the protein it codes for is identified, it will provide some understanding regarding the role of the AXR4 protein in auxin import.

5. Work cited

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