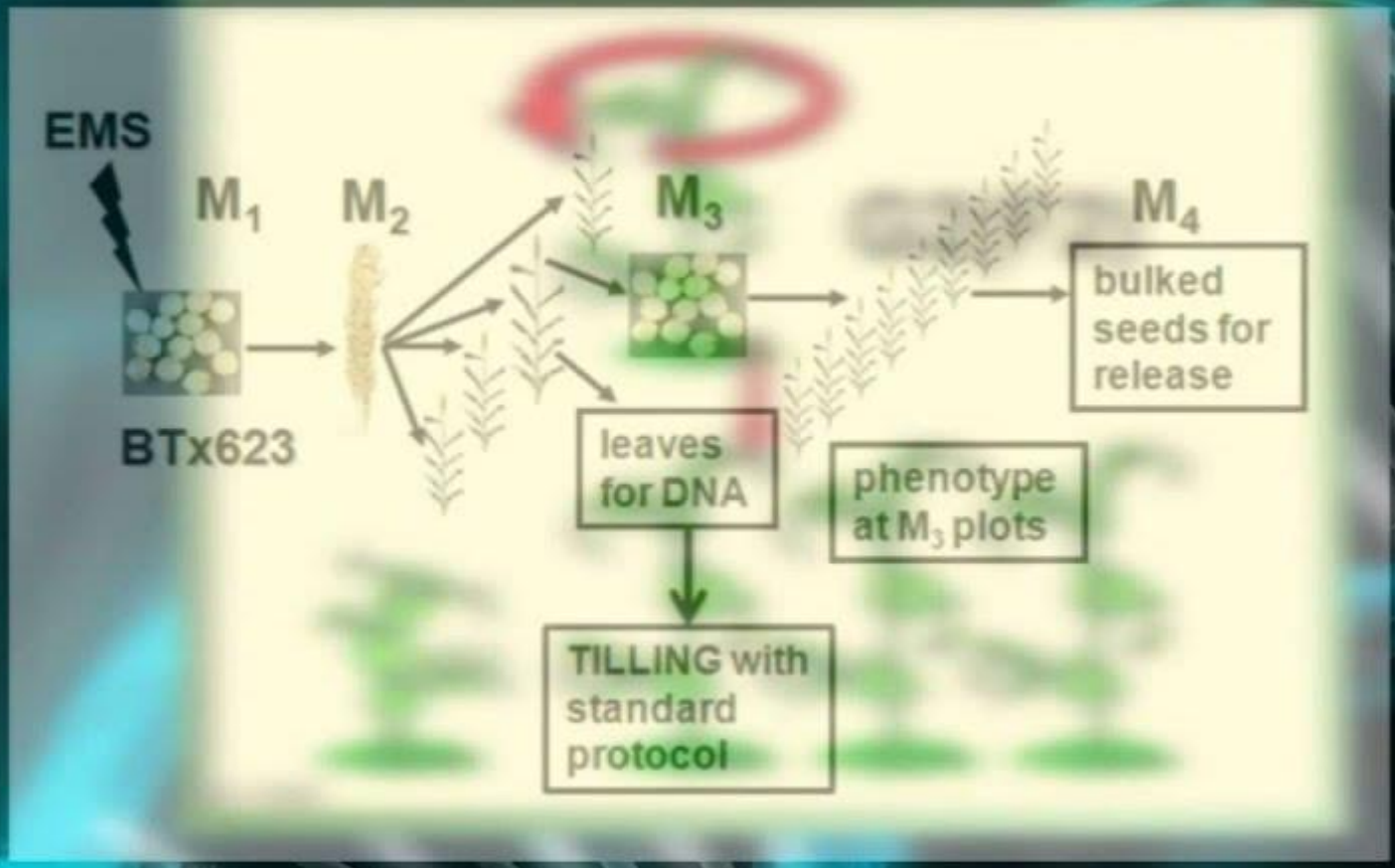
TILLING
(Targeting Induced Local Lesions In Genomes)



A shortcut in functional genomics

TILLING (Targeting Induced Local Lesions IN Genomes) was developed two decades ago as an alternative to insertional mutagenesis. It takes advantage of classical mutagenesis, sequence availability and high-throughput screening for nucleotide polymorphisms in a targeted sequence. The main advantage of TILLING as a reverse genetics strategy is that it can be applied to any species, regardless of its genome size and ploidy level. The TILLING protocol provides a high frequency of point mutations distributed randomly in the genome. The great mutagenic potential of chemical agents to generate a high rate of nucleotide substitutions has been proven by the high density of mutations reported for TILLING populations in various plant species. For most of them, the analysis of several genes revealed 1 mutation/200–500 kb screened and much higher densities were observed for polyploid species, such as wheat. High-throughput TILLING permits the rapid and low-cost discovery of new alleles that are induced in plants.

STRATEGIA	CARATTERISTICHE
1. Targeting locus-specifico	
• CoSoppressione, RNA asenso, RNAi, PTGS	dominante, efficace per famiglie geniche, richiede trasformazione
• Ricombinazione omologa	Richiede trasformazione
• Gene Editing	!!!!!!!!!!!! Richiede trasformazione
2. Mutagenesi genomica	
• Insertional mutagenesis	Richiede trasformazione
• "Fast neutron" mutagenesi	Non transgenici KOs di blocchi di geni
• EMS ↓ TILLING	Non transgenico Serie alleliche Alta efficienza Ottimo per geni di taglia piccola

• Per molte piante modello e molte piante di interesse agronomico, come il riso, la mutagenesi inserzionale è poco avanzata a causa:

- **di un bias nel sito d'inserzione del più diffuso trasposone, Tos-17,**
- **della bassa efficienza di trasformazione**

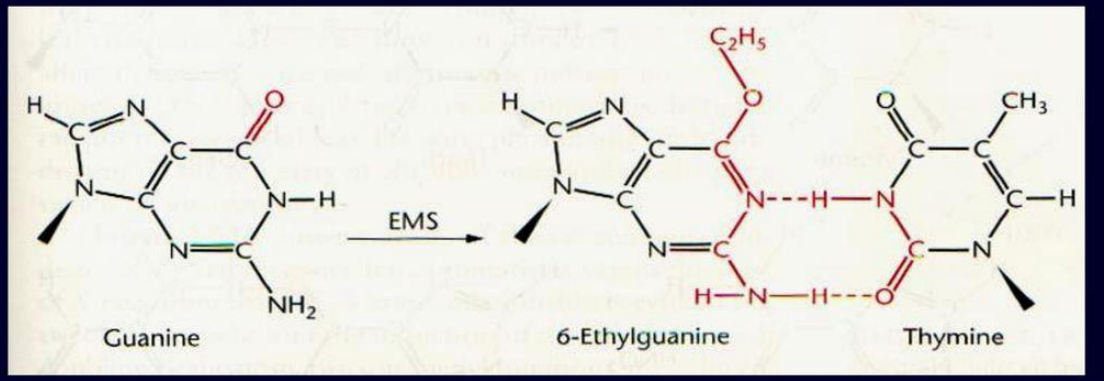
I metodi di mutagenesi tradizionale (radiazioni ionizzanti, mutageni chimici) sono applicabili con successo in reverse genetics, infatti:

- **non fanno uso di tecnologie transgeniche,**
- **sono poco costose**
- **sono facilmente realizzabili**

L'EMS (Etil Metan Sulfonato) provoca mutazioni puntiformi ed è stato ampiamente utilizzato per studi di genetica forward, sia nel mondo animale che vegetale.

Agente Alchilante EMS

Azione sulla guanina



Ethylmethane sulfonate (EMS) modifica guanina alla posizione N6 e altera la specificità di accoppiamento a favore della timina

Induce mutazioni a un tasso di $5x10^{-4}$ to $5x10^{-2}$ per gene

La mutagenesi EMS è ormai validata da lungo tempo per esperimenti di genetica forward sia in Drosophila che in Arabidopsis.

Livelli apparentemente simili di mutagenesi sono ottenuti in questi due organismi modello, nonostante l'enorme divergenza evolutiva. Ad esempio si ottengono mutanti letali recessivi con le stesse percentuali utilizzando l'EMS ai livelli max per entrambi gli organismi.

La taglia del genoma non sembra un fattore importante in quanto la densità di mutazioni/gene sembra molto simile per Arabidopsis e mais che ha un genoma circa 20 volte maggiore.

TILLING

(Targeting Induced Local Lesions In Genomes)

Il Tilling:

- **può fornire serie alleliche di mutazioni**
- **può essere applicato anche se la sequenza genomica è parziale**
- **è adatto per il targeting di piccoli geni**
- **ma permette il targeting di specifici domini in geni più grandi**
- **è assolutamente applicabile a diversi taxa**
- **non richiede tecniche transgeniche**

Tilling Mutagenesis

The general protocol for the creation of a TILLING platform in plants includes the following steps:

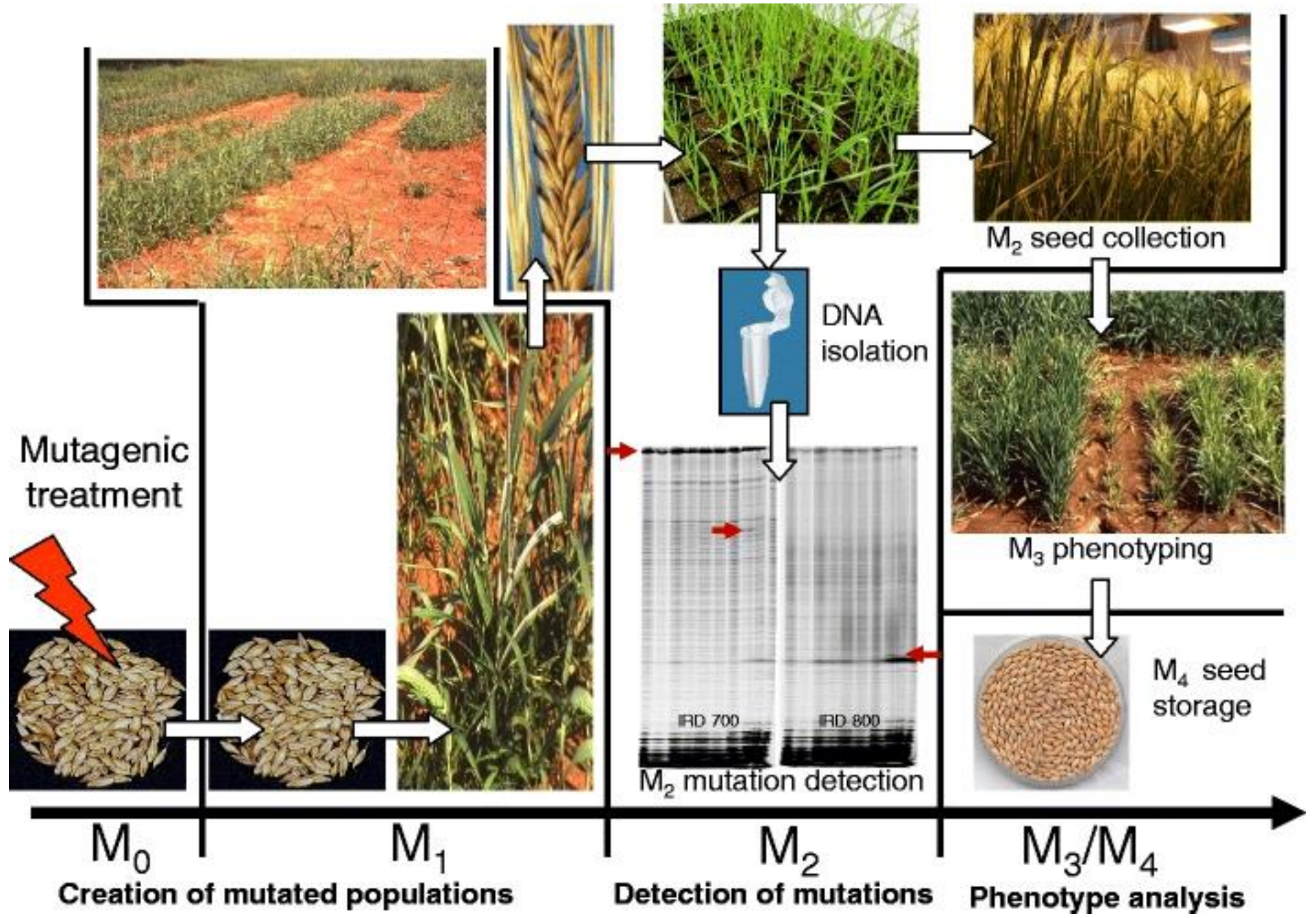
1- Creation of mutated populations

- Chemical mutagenesis
- Development of M_1 and M_2 generations
- DNA extraction from individual M_2 plants
- Creation of DNA pools of 5–8 M_2 plants
- Setting up an M_3 seed bank

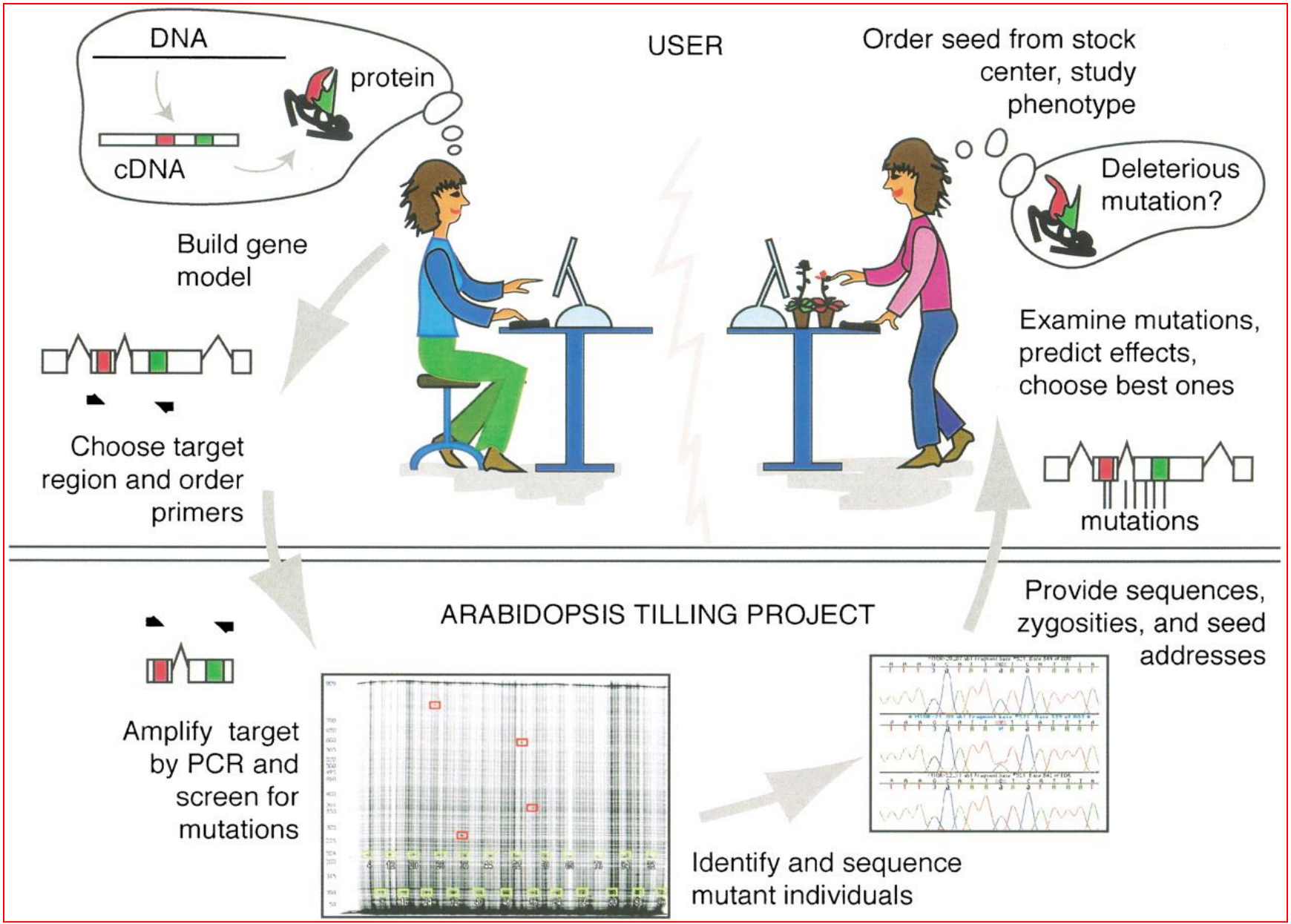
2- Detection of mutations in a targeted sequence

- PCR amplification of the targeted DNA segment (pooled DNA template)
- Detection of mutations using: ✓cleavage by specific endonuclease, ✓ DHPLC, ✓ high-throughput sequencing
- Identification of the individual M_2 plant carrying the mutation
- Sequencing the amplicon to confirm the mutation and to determine the type of nucleotide change

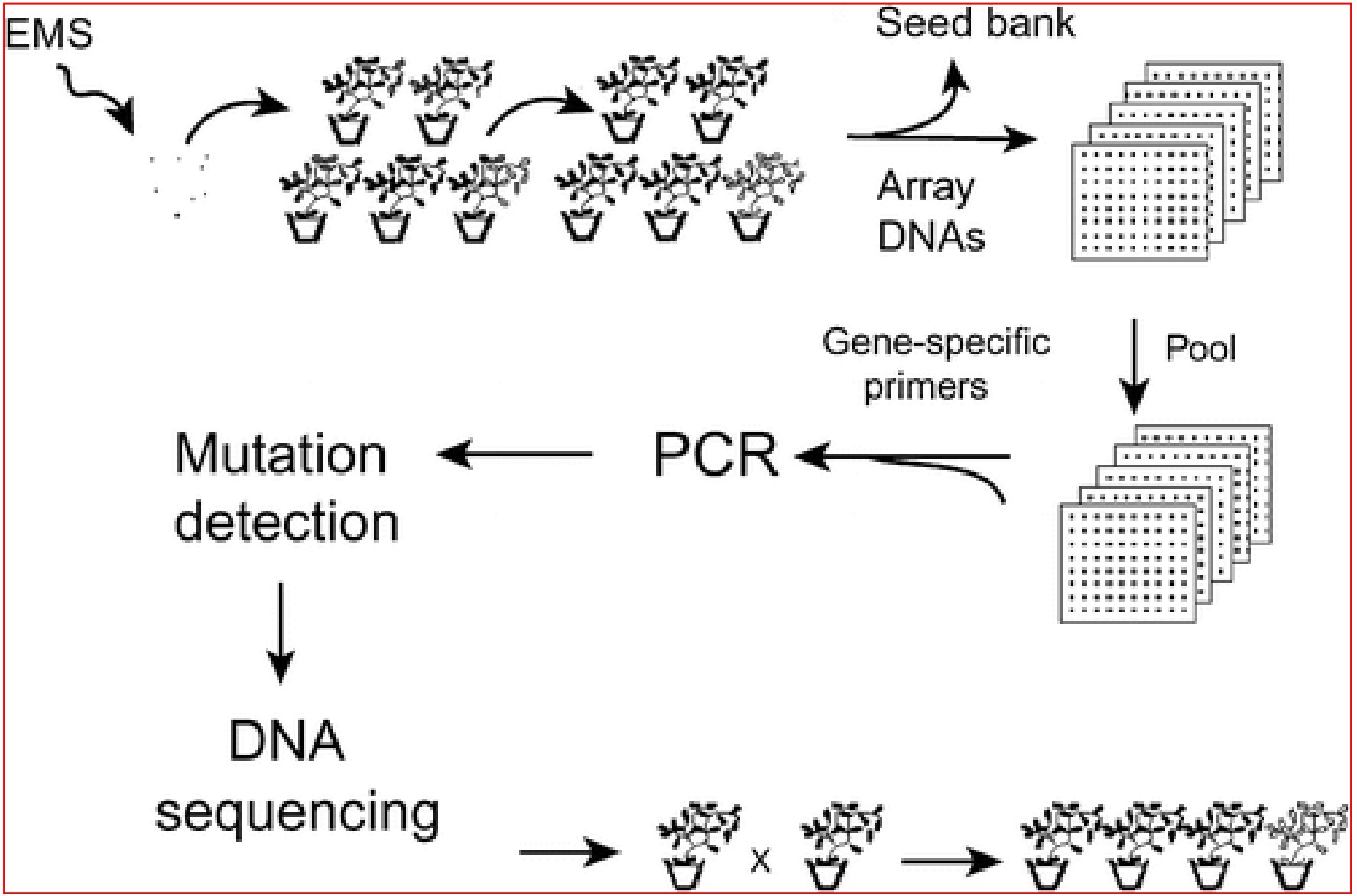
3- Analysis of the mutant phenotype


Mutagenic treatment
DNA isolation
M2 seed collection
M3 phenotyping
M4 seed storage
IRD 700
IRD 800
M2 mutation detection
M0
M1
M2
M3/M4
Creation of mutated populations
Detection of mutations
Phenotype analysis

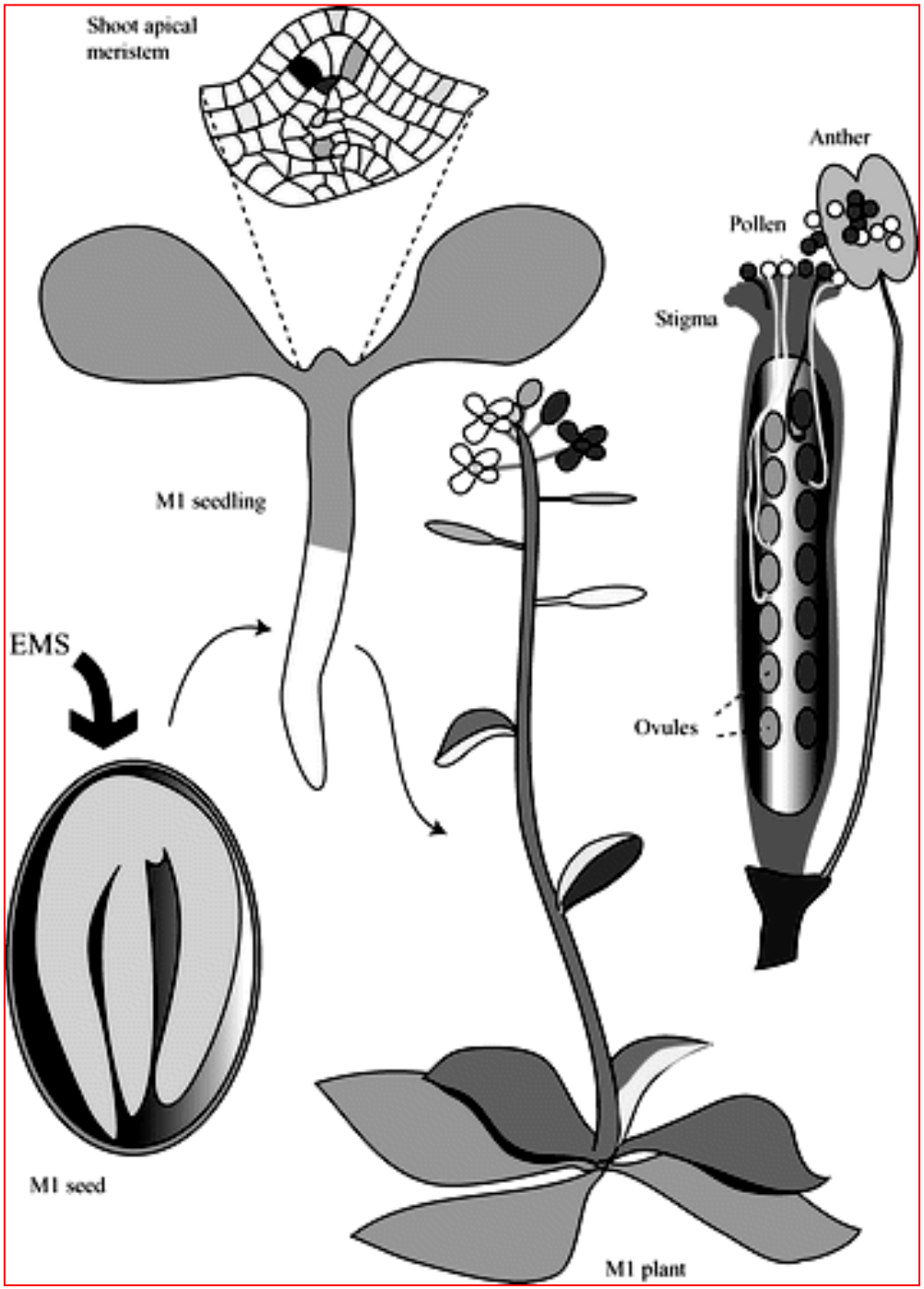
Outline of steps involved in the Arabidopsis TILLING Project



Tilling Strategy in Arabidopsis



I semi mutagenizzati per EMS originano delle chimere. Il trattamento induce diverse mutazioni, in eterizigosi. La pianta M1 mostrerà settori diversi, alcuni dei quali contribuiranno alla formazione degli organi riproduttivi



Heteroduplex Analysis

Dopo aver riunito i campioni di DNA in pool, questi sono amplificati per PCR con primers gene-specifici, poi i prodotti di PCR sono denaturati e riannilati. Come risultato un filamento mutante si riannilerà spesso con uno wt così una frazione del pool avrà un mismatch al sito della mutazione. Al crescere della taglia del pool, diminuisce la proporzione dell'heteroduplex e la sensibilità è ridotta.

SNP Discovery methods adapted for tilling

. Full sequencing

. DHPLC

(denaturing high pressure liquid chromatography)

. Endonuclease cleavage

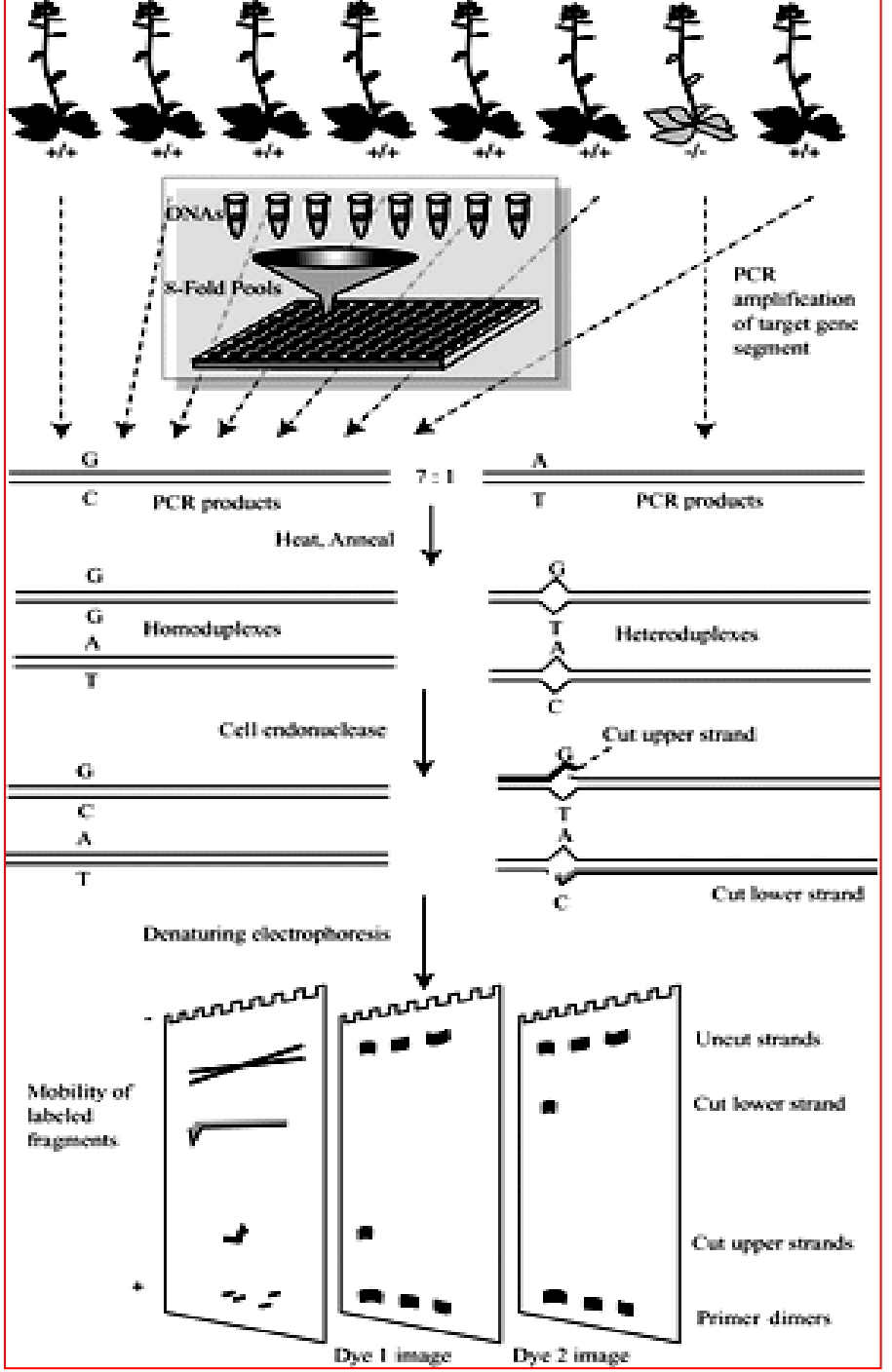
Nei progetti su larga scala ad alta efficienza, l'heteroduplex viene evidenziato per digestione con CelI, un'endonucleasi che taglia al 3' del mismatch producendo un duplex "nicked". L'elettroforesi su gel denaturante permette di separare una banda di taglia ridotta rispetto a quella WT.

Rispetto all'HPLC, questo metodo permette di posizionare la mutazione rispetto al frammento amplificato. Un altro vantaggio è che la taglia del frammento non rappresenta un limite per la sensibilità del metodo.

CelI, rispetto alla nucleasi S1, non presenta alcun bias di sequenza per l'efficienza del taglio del mismatch. L'enzima però attacca anche l'estremità 5' con attività esonucleolitica quindi va utilizzato non a saturazione per evitare la digestione dell'estremità marcata.

Formazione degli eteroduplex nei pool di DNA.

L ' amplificazione del locus target in un pool darà un prodotto eterogeneo che, a seguito di melting e riannealing, produrrà omo ed eteroduplex. Questi ultimi verranno tagliati dall' endonucleasi CEL1



Cel-I-based Tilling strategy

Genomic DNA library from the mutagenized fly lines

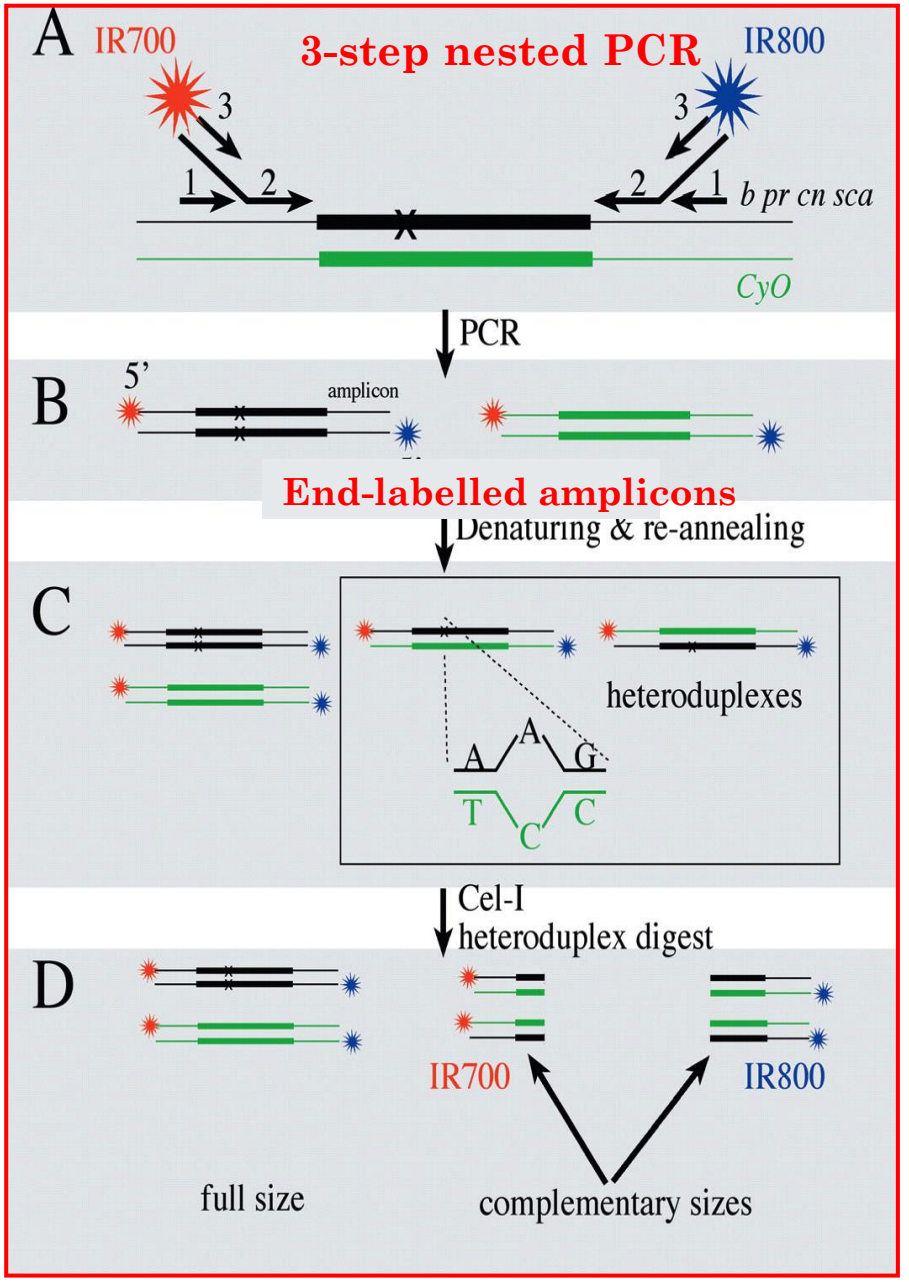
A **three-step (1,2,3) nested PCR reaction** (A) for a gene of interest. The third PCR included the **red and blue fluorophores** bound to the forward and reverse primers, thereby **differentially labeling the two ends of the amplicons** (B).

Denaturation and reannealing the fragments to generate wt/mutant heteroduplexes (C)

Digestion by Cel-I (D). When a SNP is present, cleavage of the heteroduplexes generates two fragments labeled by IRDye700 and IRDye800, respectively, which can be detected upon denaturing polyacrylamide gel electrophoresis.

A typical signal shows **two fragments with complementary sizes that must sum up the original amplicon size.**

Sequencing the PCR products to confirm.



Welcome to ATP

The Arabidopsis TILLING Project

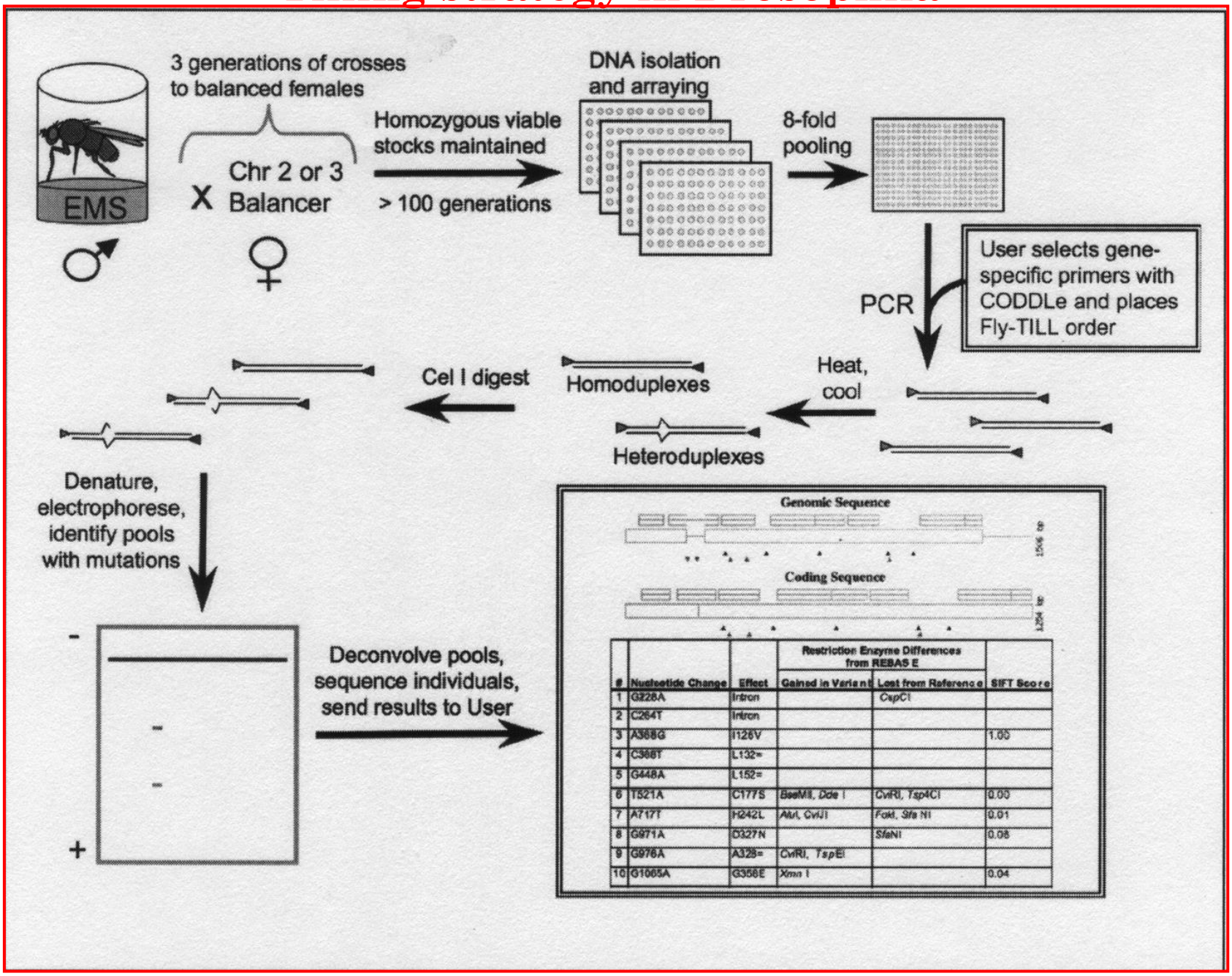
The goal of ATP is to rapidly deliver to the Arabidopsis community EMS-induced mutations in requested genes. For a description of our high-throughput reverse genetic screens used in TILLING, please refer to Till *et al.*, 2003. Below is a brief description of the steps involved in TILLING an Arabidopsis gene through ATP. We have outlined what will be done by ATP and what is required of the researcher making the request. To avoid inefficient uses of our resources and of your time, we ask that you read the following description and cautionary notes very carefully. For returning users, please note that our regular user fee is now $750 per order, and our policy is that what we deliver for the basic fee is influenced by gene target and outcome.

TILLING: A five step process

1. You decide whether your gene is worth TILLING.
2. You find the best the region to be targeted and place your order.
3. ATP screens the region for mutations.
4. ATP sequences the mutation and enters it in our public database.
5. ATP sends you a mutant report and you order seed.

STEP 1: You (the user) decide whether your gene is a good candidate for TILLING

Tilling strategy in Drosophila



#	Nucleotide Change	Effect	Restriction Enzyme Differences from REBASE		SIFT Score
			Gained in Variant	Lost from Reference	
1	G228A	Intron		*Csp*CI	
2	C264T	Intron			
3	A368G	I126V			1.00
4	C368T	L132=			
5	G448A	L152=			
6	T521A	C177S	*Bse*MII, *Dde* I	*Cvn*RI, *Tsp*4CI	0.00
7	A717T	H242L	*Ath*, *Cvn*I	*Fok*I, *Sfa* NI	0.01
8	G971A	D327N		*Sfa*NI	0.08
9	G976A	A328=	*Cvn*RI, *Tsp*EI		
10	G1065A	G358E	*Xmn* I		0.04

Target-selected mutant screen by TILLING in *Drosophila*

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The availability of the full *Drosophila* genomic DNA sequence prompts the development of a method to efficiently obtain mutations in genes of interest identified by their sequence homologies or biochemically. To date, molecularly characterized mutations have been generated in around 6000 of the ~15,000 annotated fly genes, of which around one-third are essential for viability. To obtain mutations in essential and nonessential genes of interest, we took a reverse genetics approach, based on the large-scale detection of point mutations by Cel-I-mediated heteroduplex cleavage. A library of genomic DNA from 2086 EMS-mutagenized lines was established. The library was screened for mutations in three genes. A total of 6.1 Mb were screened, and 44 hits were found in two different mutagenesis conditions. Optimal conditions yielded an average of one mutation every 156 kb. For an essential gene tested, five of 25 mutations turned out to cause lethality, confirming that EMS mutagenesis leads to high frequency of gene inactivation. We thereby established that Cel-I-mediated TILLING can be used to efficiently obtain mutations in genes of interest in *Drosophila*.