Appling of novel subtracted method Genetically Directed Differential Subtraction Chain (GDDSC) in plant genomes.

Ewa Siedlecka, Aneta Hromada-Judycka, Magdalena E. Pawełkowicz, Rafał Wóycicki, Monika Rakoczy-Trojanowska & Zbigniew Przybecki

Department of Plant Genetics, Breeding and Biotechnology, Warsaw University of Life Sciences, Nowoursynowska 159, 02-776 Warsaw, Poland. Correspondence should be addressed to E.S. (ewa_siedlecka@sggw.pl).

ABSTRACT

We present a simple subtraction procedure of GDDSC as a modification of the original DSC and GDRDA methods. Genetically Directed Differential Subtraction Chain (GDDSC) is a process by which highly related genomes are compared in order to isolate tags carrying the polymorphisms. To detect specific DNA fragment (tag), we can then monitor offspring plants for efficient molecular breeding. The GDDSC protocol was applied to isolation of new sex related clones from cucumber plants (*Cucumis sativus* L.) and new tissue culture response clones from rye (*Secale cereale* L.). The newly identified tags, obtained by GDDSC represent pools of candidate genes and other sequences, which could serve as potential markers for requested traits.

Key words: cucumber (*Cucumis sativus* L.), rye (*Secale cereale* L.), GDDSC, subtraction, tester, driver

BRIEF INTRODUCTION

In 1999, Luo *et al.*[1] elaborated a new subtractive hybridization method - differential subtraction chain (DSC) which enabled the subtraction of the complex genomes. DSC was developed by modifying representational difference analysis (RDA, Lisitsyn *et al.*[2]). The modification relied on a slight but particularly significant change: the successive exchange of the tester into the driver sequence during the subsequent subtraction rounds which extremely simplified the method and shortened the procedure about ten times. Scanning the whole genome, with both DSC as well as RDA were able to generate the polymorphic fragments without defying regions of origin. Lisitsyn *et al.*[3] took a next step and elaborated genetically directed representational RDA (GDRDA), a method generating genetic markers linked to a gene/trait of interest from the small genome region. A genetic direction of the subtraction seems to be an

especially interesting idea when it would be combined with any simple subtractive method, e.g. DSC. That was the reason to elaborate the genetically directed DSC (GDDSC)[4,5,6]. In GDDSC, the low quantity of isolated polymorphic fragments arise in accordance with number of subtraction rounds . Theoretically, the big advantage of this method is possibility to isolate the gene/genes of interest (directing gene). Enhancing the number of rounds in GDDSC, the subtracted mix become more saturated in polymorphic sequences. Moreover, the set of the GDDSC fragments should be a subset of the DSC ones, but the pool after GDDSC shouldn't consist difference caused by the plant individual variantion

In this paper, we describe the use of GDDSC method to identify genomic differences associated with sex phenotype in cucumber plants (*Cucumis sativus* L.) and with tissue culture response of rye (*Secale cereale* L.).

PROCEDURE

1/ Construction of segregating populations and subtracting DNA pools.

A. Cucumber (*Cucumis sativus* L.). To generate genetic markers tightly linked to the sex gene in cucumber (*Cucumis sativus* L.) we prepared separate pools of genomic DNAs isolated from F2 generation plants differeing in sex phenotype. In order to find genetic markers of two sex genes in cucumber (*M* – male organs development and *Gy* - female organs development) we have used two different F2 populations coming from the cross of Near Isogenic Lines (NILs). One line pair has different alleles at loci *m*, Gy3 (a dominant female line with an *MMFFGyGy* genotype), and HGy3 (an isogenic hermaphroditic line with an *mmFFGyGy* genotype). The other pair of lines has differing alleles at the loci *Gy*, B10 (a monoecius Borszczagowski line with an *MMffGyGy* genotype), and 2gg (a recessive female with an *MMffgygy* genotype
isogenic to B10). Their hybrid F1 generations were self pollinated, and the segregating F2 generations
were used to generate sex GDDSC marker. F2 plants were evaluated regarding to sex phenotype and then divided into groups according to flower sex type but with establish manner regard to pair set. The genomic DNAs were isolated from young leaves and then pooled according to sex phenotype divisionThose bulks were used as a pool for GDDSC method. Each bulk was used also as a tester and as a driver but respectively for *M* and *Gy* gene.

B. Rye (*Secale cereale* L.). To isolate DNA regions linked to the investigated trait (response in tissue culture), the 102 individuals from 7th generation of recombinant inbred lines (RIL) were used. They were developed by single seed descent selection from the cross between already characterized in *in vitro* culture parental lines: L318 (regenerating plants) and L9 (unable to regenerate plants). The isolated DNA from young rye leaves was divided into four groups (bulks), differing in respect to the tissue culture response and embryogenic callus production efficiency from immature embryos. Those bulks were coupled as follows: R (DNAs from RILs regenerating plants) with NR (DNAs from RILs non-regenerating plants) and E>90 (DNAs from RILs with percentage of immature embryos producing embryogenic callus above 90%) with E<25 (DNAs from RILs with percentage of immature embryos producing embryogenic callus below 25%).

Each bulk was used both as a tester and as a driver. The negative control was made from NR pool digested with *Hind*III, *Bgl*II and *Bam*HI and was used as a tester and the driver in the same reaction.

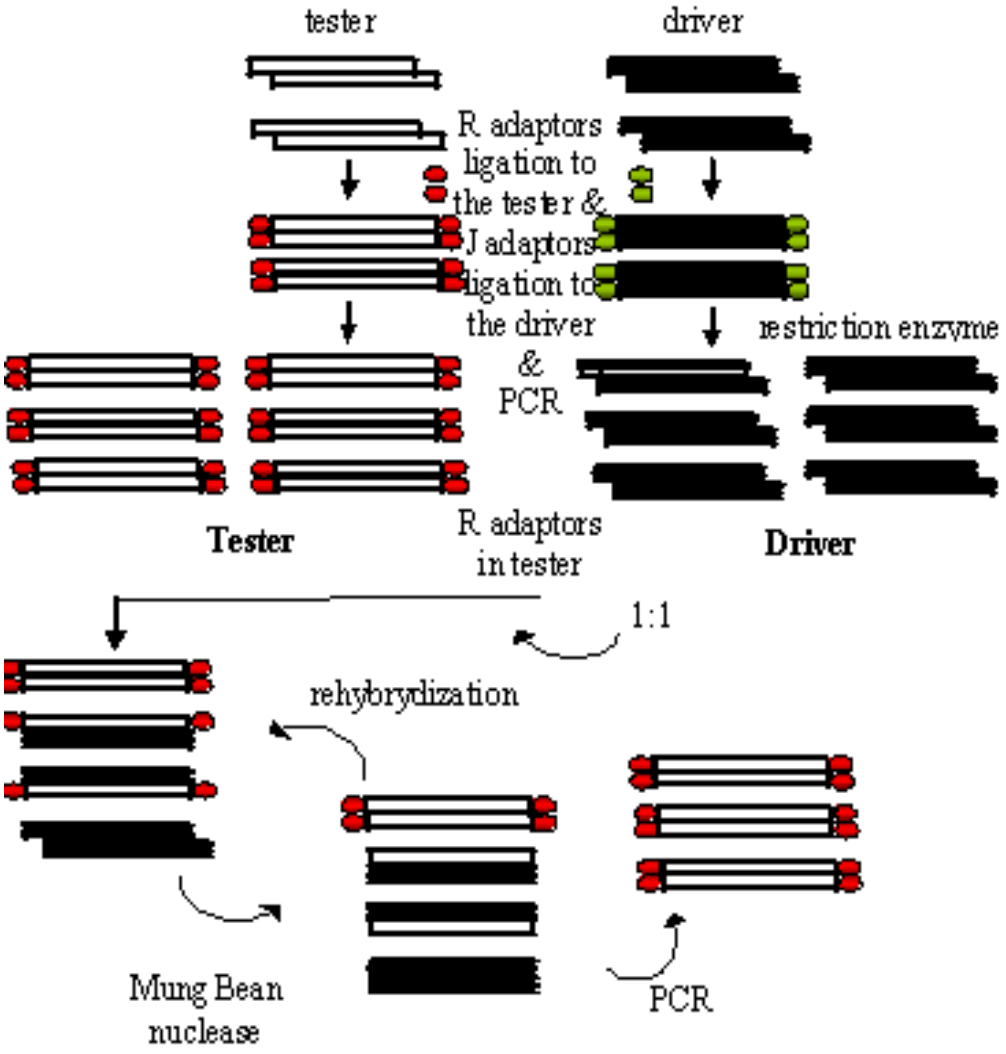
▲ CRITICAL STEP

To obtain the clearly differentiating markers from the GDDSC results, one should conduct the extensive and precise phenotypic observations of the plant populations used in the study

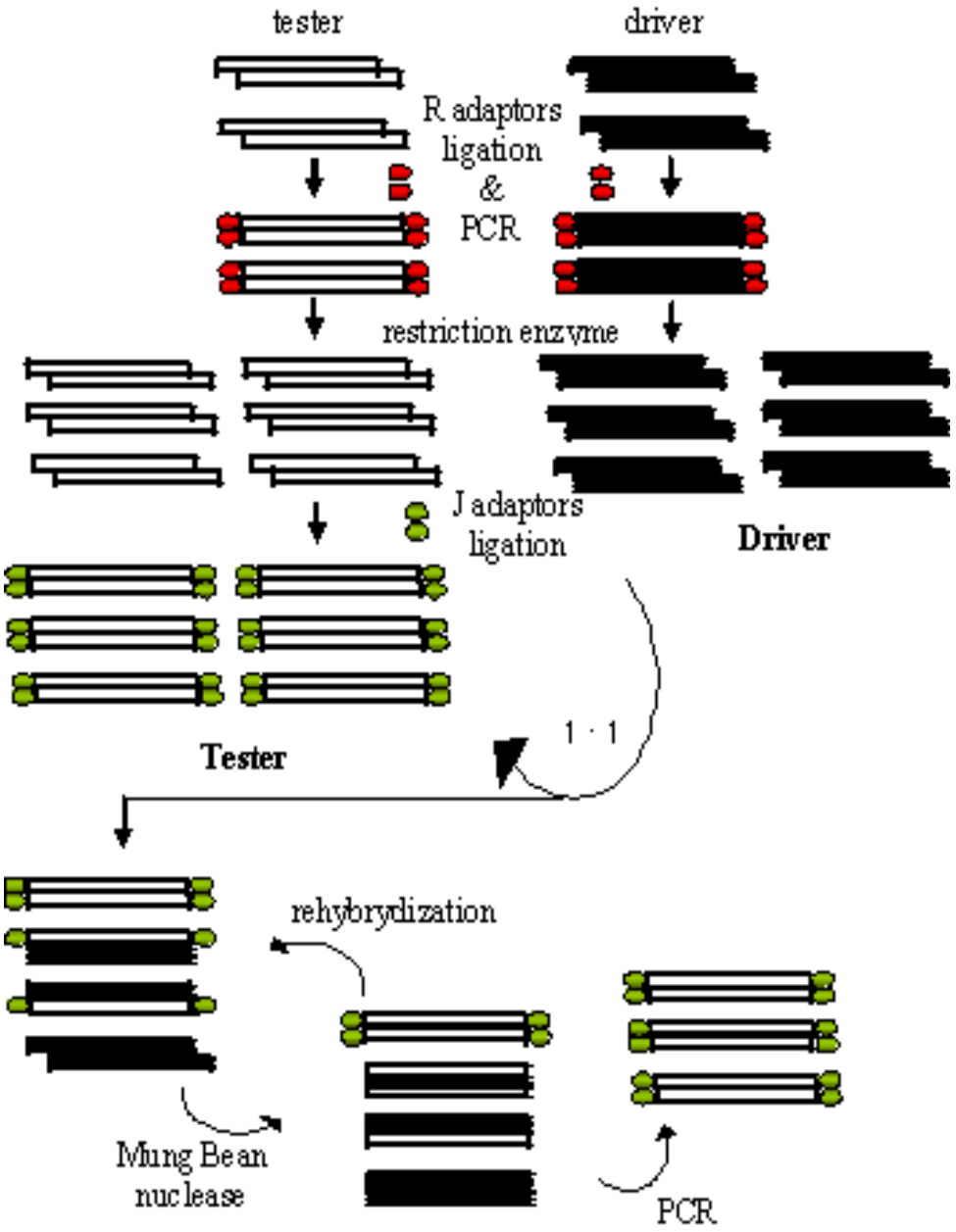
▲ CRITICAL STEP

Genetically similar nearly isogenic lines (NILs), differing only at a small genome fraction associated with the trait locus, are useful in genomics studies, molecular characterization and mapping of the gene of interest.

Schema 1 and 2 show the GDDSC methods for cucumber and rye, respectively.



Schema 1 shows an overview of the GDDSC protocol according to the original DSC protocol in regard to the adaptors ligation reaction (Luo *et al*., 1999). R adaptors were ligated to the tester and J adaptors were ligated to the driver DNAs to generate representations. Adaptors were removed only from representation of the driver.



Schema 2 shows an overview of the GDDSC protocol with optimization step. R adaptors were ligated to both, tester and driver DNAs to generate representations. After amplification adaptors were removed by restriction enzyme. Prior to subtractive hybridization, J adaptors were ligated only to the tester representation.

2/ Genomic DNA isolation. From one to five micrograms of genomic DNA were isolated according to CTAB procedure (*Secale cereale* L.) or GenElute PlantGenomicDNA miniprep Kit

by SIGMA-ALDRICH procedure (*Cucumis sativus* L). The pipette tips with aerosol barriers should be used for all pipetting steps and H_2O should be deionized and sterilized before it's use for solutions preparation.

▲ CRITICAL STEP

The quality of genomic DNA is very important for successful subtraction. Degraded DNA will result in the isolation of artificial bands. You may use any commercially available kit for plant genomic DNA isolation.

▲ CRITICAL STEP

In principle it is possible to create amplicons from minuscule quantities of material, including single cells. However, problems can arise when maintaining a truerepresentation of DNA from very small amounts of starting material.

3/ Restriction enzyme digestion. This step generates short, sticky-ended dsDNAs for subtractive hybridization. The final volume of digestion is 200 µl. Add the following reagents into a 1.5 ml microcentrifuge tube:

Genomic DNA (2 µg)	x µl	
10X Restriction Buffer	20.0 µl	
*Bgl*II or *Bam*HI or *Hind*III (10 units/µl)	2.0 µl	(see **Box 2**)
Deionized H_2O up to 200 µl	y µl	

Mix gently and then centrifuge briefly. Incubate at 37°C for16 - 18 hours. To check quality of digestion, set aside 10 µl of the digestion mixture and run 1% agarose gel with undigested DNA.If the range of digested band in agarose gel is between x-x bp the digestion is completed The size od=f band in gel is also depending on the type of enzyme used to the reaction. To precipitate cuted DNA Add 0.1 volume of 3 M NaOAc (pH 5.5) and 2 volumes of 99.8% ethanol. Mix gently and store at -20°C over night. Centrifuge for 1 hour at 14,000 rpm, 4°C. Remove the supernatant. Gently wash the pellet with 70% ethanol, centrifuge at 14,000 rpm for

15 min. Air dry the pellet for 2-5 min. Carefully remove the supernatant and dissolve the pellet in 5 µl of H_2O and then store at -20°C.

▲ CRITICAL STEP

Terminate the reaction only if you are satisfied with the digestion results..

4/ Oligonucleotide adaptors ligation. For adaptor ligation, 2 µg of digested tester and driver DNAs (from step 3) are mixed in a total volume of 20 µl with 7.5 µl of each a 12-mer and 24-mer oligonucleotide adaptors (adaptor pairs: **Box 1**) from a 62 pmol/µl stock solution. The mixture is heated to 72ºC for 3 min to release unligated oligonucleotide adaptors and next combined with a Ready-to-Go Ligation kit (according to the manufacturer's recommendations of Amersham Pharmacia, USA). To anneal adaptors, the ligation mixture is incubated at 16°C for 30-45 min in a thermal block, after that step the ligation mixture is immediately placed at 70°C (inactivation of a ligation reaction) for 10 min. The reaction tubes are incubated on ice for 5-10 min.

▲ CRITICAL STEP

Oligonucleotide adaptors used during ligation reaction should be purified by HPLC, since T4 DNA ligase is easily inactivated by traces of chemicals used for oligonucleotide synthesis, to avoid low yields of the tester and driver representations.

▲ CRITICAL STEP

Note that it is essential to use unpfosphorylated oligonucleotide adaptors. The 12-mer oligonucleotide provides a splint to allow the ligation of the 24-mer. The 12-mer does not have a phosphate group on its 5'end so it is not ligated itself and dissociates during the PCR reaction.

The ligation products were purified by DNA purification spin column (A & A Biotechnology, Gdansk, Poland). The maximum volume of the probe should be no higher than 150µl. The

column capacity is 10 µg DNA. The purification protocol (A & A Biotechnology kit) is presented below (please read the instructions carefully) :

a) Add 5 volumes of G buffer to the DNA sample (e.g. 250µl of G buffer to the 50µl of DNA sample). Mix gently by inversion or vortexing.
b) Centrifuge it short in order to remove the rest of the mixture from the walls of tube.
c) Pour whole liquid onto the column and centrifuge at 10,000-15,000 rpm for 30 seconds.
d) Remove the column from the tube, spill out the filtrate and place again the column in the tube.
e) Add 0,6 ml of A1 buffer onto the column and centrifuge at 10,000-15,000 rpm for 30 seconds.
f) Repeat the step d) and add 0,3 ml of A1 buffer onto the column.
g) Centrifuge the column at 10,000-15,000 rpm for 2 minutes.
h) Place the dry column in a new tube and add 30 µl to 50 µl of TE buffer (10mM Tris-HCl, 1mM EDTA pH 8,0) or water. Make sure that the liquid reaches the blue ring inside the column, otherwise the elution will be not efficient. Notice that, the more solvent you use, the lower DNA concentration you obtain, but the efficiency of the elution is greater. Finally we eluted the DNA with 30 µl of water.
i) Incubate it for 3 minutes at room temperature and centrifuge at 10,000-15,000 rpm for 1 minute.
j) Remove the column and store the DNA at -20°C until further analysis.

BOX 1. DNA POOLS PREPARATION

The first step of GDDSC is the preparation of „representations" from DNA populations. Typically, a representation is a set of restriction endonuclease fragments of a limited size range amplified by PCR (using oligonucleotide adaptors). Restriction endonuclease-based representations have major advantages over other RR (reduced representatios) methods(1) their complexity can be regulated by the choice of restriction enzyme, (2) they are highly reproducible.

Restriction enzymes. In the GDDSC, similar to the RDA and GDRDA, enzymes like *Bam*HI, *Hind*III or *Bgl*II are typically used, as they generate considerably larger fragments. Additionaly, using those enzymes, a 100 fold reduction of complexity was estimated for GDDSC genomic libraries (similar to RDA).

(For each organism, the average fragment lengths can be calculated using the dinucleotide frequencies).

Oligonucleotide adaptors. GDDSC require two pairs of adaptors, each formed by one 12-mer and one 24-mer oligonucleotide. We ligated two pairs of adaptors in the two following ways (1) The R pair was ligated to the digested DNA used for preparing tester, the J pair was ligated to driver. Only the driver amplicon was then digested again with restriction enzyme while the R pair of tester amplicon remained unchanged for subtraction cycles (according to the DSC protocol, Luo *et al*., 1999).(2) The R pair was ligated to the digested DNA used for preparing tester and driver in representation. Tester and driver amplicons were then digested again with restriction enzyme and the J pair was ligated to tester for the subtraction cycles

Table1 . Sequences of oligonucleotide adaptors used for GDDSC

Oligonucleotide adaptors sequences *	**Name**	**Enzyme**
5 '-AGCACTCTCCAGCCTCTCACCGCA- 3 '	*RBgl*24	*Bgl*II
5 '-GATCTGCGGTGA- 3 '	*RBgl*12	
5 '-ACCGACGTCGACTATCCATGAACA- 3 '	*JBgl*24	

5 ’-GATCTGTTCATG- 3 ’	*JBgl*12	
5 ’-AGCACTCTCCAGCCTCTCACCGAG- 3 ’	*RBam*24	*Bam*HI
5 ’-GATCCTCGGTGA- 3 ’	*RBam*12	
5 ’-ACCGACGTCGACTATCCATGAACG- 3 ’	*JBam*24	
5 ’-GATCCGTTCATG- 3 ’	*JBam*12	
5 ’-AGCACTCTCCAGCCTCTCACCGCA- 3 ’	*RBgl*24	*Hind*III
5 ’-AGCTTGCGGTGA- 3 ’	*RHind*12	
5 ’-ACCGACGTCGACTATCCATGAACA- 3 ’	*JBgl*24	
5 ’-AGCTTGTTCATG- 3 ’	*JHind*12	

5/ PCR of representations

Set up the following 250 µl reactions in 0.5 ml thin-walled PCR eppendorfs. Combine five reactions into 1.5 ml microcentrifuge tube:

10x PCR buffer	5,00 µl
10 mM dNTPs mix	1,00 µl
50 mM $MgCl_2$	1,50 µl
20 µM adaptor (24-mer)	1,25 µl
DNA	1,25 µl
Taq DNA polymerase	0,25 µl
Water	39,75 µl

The longer adaptor is also used as primer for DNA amplification after adaptor ligation.
Preheat the thermal cycler to 72°C. Place PCR tubes in the thermal cycler for 3 minutes to allow 12-mer oligonucleotide adaptor to dissociate. Fill in 3’ ends of ligated DNA/adaptors by adding 0.25 µl *Taq* DNA polymerase to each PCR tube in the thermal cycler and mix by pipetting up and down. Incubate for 5 minutes at 72°C.
Follow this PCR program:
72°C for 3 min; 72°C for 5 min; 20cycles, 95°C for 1 min and 72°C for 3 min; 72°C for 10 min.
After a final extension cool samples at 4°C.

▲ CRITICAL STEP

The PCR amplification step to generate the representations is critical one for a successful GDDSC. To generate representations that truly represent the original DNA, the PCR needs to be optimized by careful titration the number of cycles and template concentration for each sample.

▲ CRITICAL STEP

After adding *Taq* DNA polymerase into the reaction tube, the probes have to be heated for at least 3 minutes at 72ºC. Therefore, you should mix enzyme as fast as possible or you can use a PAUSE function on your thermal cycler.

■ PAUSE POINT

Store at 4°C for up to several months.

We suggest to use the DNA purification spin column (A & A Biotechnology kit). The procedure is described below. After using the DNA spin column DNA is eluted in 30 µl .

Analyze 10 µl of each PCR product by electrophoresis on a 2% agarose/EtBr gel to check the range and quality of representations. **Figure 1A, B and C.**

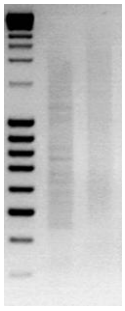


Figure 1A.

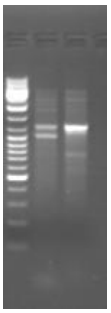


Figure 1B.

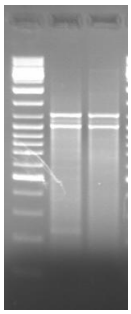


Figure 1C.

Figure 1 A-C. Characterization of results of different representation preparation strategies by electrophoresis on 2% agarose/EtBr gel. A traditionally strategy to generate tester and driver representations in rye. **B** traditionally strategy for generation of tester and driver

representations in cucumber. **C** shows the results modified strategy for generation of tester and driver representations in cucumber.

▲ CRITICAL STEP

A smear of genomic representations ranging in size from 150 – 1500 bp should be seen.

Digest the tester and driver DNA in 200µl reaction volume with the proper restriction endonuclease. In the case of rye genomic subtraction, only the driver DNA was digested as above.

▲ CRITICAL STEP

To avoid loss of unique sequences, the original DSC strategy (Luo *et al*.1999) was used. That approach assume the utilization the different adaptors, ligated to the tester and driver DNAs. There is no need to cut off and ligate other adaptors to the tester pool of DNA. Such approach caused the low probability that the unique tester sequences will be excluded from further analysis because of the incorrect ligation of the new adaptors to the already amplified and digested tester representation.

Purify the digested driver representation using DNA purification spin column (A & A Biotechnology kit). The final volume of DNA is 30 µl in water

Precipitate the amplified DNA representations of tester and driver. Add 0.1 volume of 3 M NaOAc (pH 5.5) and 2 volumes of 99.8% ethanol. Mix gently and store at -20°C by over night. Centrifuge for 1 hour at 14, 000 rpm, 4°C. Remove the supernatant. Gently wash the pellet with 70% ethanol, centrifuge at 14,000 rpm for 15 min. Air dry the pellet for 2-5 min. Carefully remove the supernatant and dissolve the pellet in 5 µl of H_2O (tester representation) and in 4 µl of 3x EE Buffer (SIGMA-ALDRICH).

■ PAUSE POINT

Incubation at -20°C over night.

6/ Exchanging oligonucleotide adaptors in representations.

A. Cucumber (*Cucumis sativus* L.). In order to ligate J pair adaptors, digested DNA of tester and driver representations (about 10µg) are mixed in a total volume of 20 µl with 7.5 µl of 12-mer and 24-mer oligonucleotide adaptors (adaptor pairs: **Box 1**) from a 62 pmol/µl adaptor stock solution. The mixture was heated at 72ºC for 3 min to release unligated oligonucleotide adaptors and next combined with a Ready-to-Go Ligation kit (according to the manufacturer's recommendations of Amersham Pharmacia, USA). To ligate new set of adaptors , the ligation mixture was incubated at 16°C for 30-45 min. in a thermal block, after that, the ligation mixture was immediately placed at 70ºC for 10 min. The reaction tubes were incubated on ice for 5-10 min.

To precipitate DNA with new adaptors add 0.1 volume of 3 M NaOAc (pH 5.5) and 2 volumes of 99.8% ethanol. Mix gently and store at -20°C over night. Centrifuge for 1 hour at 14, 000 rpm, 4°C. Remove the supernatant. Gently wash the pellet with 70% ethanol, centrifuge at 14,000 rpm for 15 min. Air dry the pellet for 2-5 min. Carefully remove the supernatant and dissolve the pellet in 4 µl of 3 x EE Buffer (SIGMA-ALDRICH) by vortexing for about 2 min. Centrifuge briefly.

B. Rye (*Secale cereale* L.). mixed in a total volume of 20 µl. In case of rye probes only DNA of driver representation was undertaken to digestion. Total procedure of ligation and precipitation was described above.

■ PAUSE POINT

Precipitation step.

7/ Denaturation and hybridization of tester and driver representations.

Mix the tester and driver representations in ratio 1:1, overlay each representation with two drops of mineral oil, even if you use thermocykler with heated lid. Heat samples at 98°C for 3-4 min in a heating block for complete denaturation of the DNA strands. Carefully add 2 µl of 5M NaCl directly to representations mixture under the oil on the bottom of the microcentrifuge tube. Incubate at 67°C for 16-20 hours to allow complete hybrydyzation of complementary DNAs.

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▲ CRITICAL STEP

Excess of NaCl may cause inhibition of DNA association. It is very important to thoroughly but carefully mix NaCl with the subtraction mixture.

After removing drops use the DNA purification spin columns (A & A Biotechnology kit) to clean the DNA from any remaining contaminants and then of resuspend the DNA pellet in 43 µl water.

Digest the subtraction reaction with Mung Bean Nuclease. Mung Bean Nuclease cut off the sticky ends (overhangs) on double stranded DNA. Set up the following reaction in the total volume of 50µl and incubate at 30ºC for 25 minutes. Inactivate the enzyme by adding 0,5µl of 10% SDS.

Mung Baean Nuclease digesting reaction:

DNA probe	43µl
Mung Bean Buffer	5µl
Mung Bean Nuclease (10U/µl)	2µl

We suggest the use of a DNA purification spin column (A & A Biotechnology kit). We elute the DNA in 30 µl water.

8/ PCR subtracts – selective amplification.

10x PCR buffer	5,00 µl
10 mM dNTPs mix	1,00 µl
50 mM $MgCl_2$	1,50 µl
20 µM primer (24-mer)	1,25 µl
DNA	1,25 µl
Taq DNA Polimerase	0,25 µl
Water	39,75 µl

Follow this PCR program:

43 cycles, 95°C for 1 min and 72°C for 3 min; 72°C for 3 min

To check the PCR results, analyze 2 µl of the reaction after the first round of subtractive hybridization on a 2% agarose/EtBr gel.

Before the next round of subtractive hybridization which includes subsequent hybridization and representations amplification, mixture was reheated to 98ºC.

We performed five rounds of subtractive hybridization in cucumber (**Figure 2**) and nine rounds of subtractive hybridization in rye (**Figure 3**). The products differing tester and driver pools, after five or nine rounds of hybridization were used for direct cloning.

Figure 2

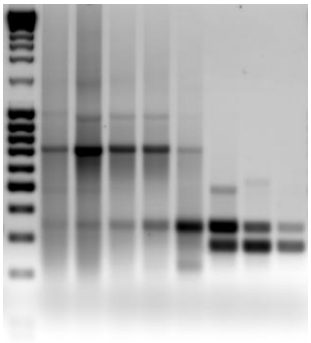
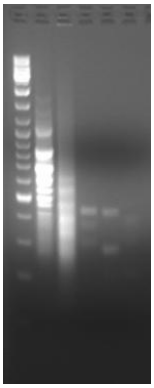


Figure 3



Figures 2 and **3** show rounds of subtractive hybridization carried on for rye and cucumber, respectively.

■ PAUSE POINT

Subtraction step.

▲ CRITICAL STEP

No additional driver representation was added after the first round of GDDSC.

Follow the procedure for hybrydyzation and selective amplification as described above. The negative experiment control have been conducted with identical tester and driver DNAs(tester and driver prepared from identical genomic DNA sample). In both cases: cucumber (after five rounds) and rye (after nine rounds) there was no differentiating product observed.

Figure 4

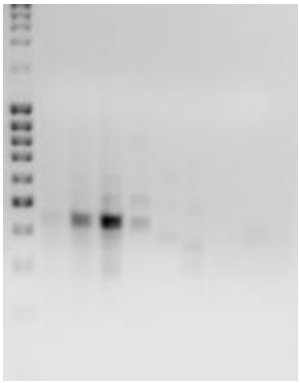
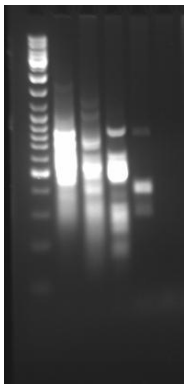


Figure 5



Figures 4 and 5 show the negative control in rye (9 round) and cucumber (5 round), respectively.

▲ CRITICAL STEP

Comparison of representations and differing products by 2% agarose/EtBr gel electrophoresis, verified the expected appearance of distinct bands in later rounds of hybridizations, compared to

the relative smear in the initial representations. The differing products from five (cucumber) or nine (rye) round were used for direct cloning.

9/ Analysis of subtracts. (subtracted clones).

- **DNA inserts analysis by electrophoresis.**

Run electrophoresis on the 6% polyacrylamide/$AgNO_3$ gel prepared according to Pillen et al. (2000) using the products from the last round of subtraction.

Cut the bands from the dry polyamide gel and suspend them separately in the 10µl of sterile water.

■ PAUSE POINT

Incubation at 4ºC over night.

- **DNA inserts analysis by PCR**

Prepare a master mix for PCR reactions (number of reactions equals number of bands cut from polyacrylamide gel):

10 x PCR reaction buffer	5,00 µl
Primer 1	1,25 µl
dNTPs mix (10 mM)	1,00 µl
H_2O	39,75 µl
Taq Polimerase	0,25 µl
Total volume	50,00 µl

Pour 48,75 µl of the master mix into each tube. Transfer 1,25 µl of each band (insert) to each tube. Perform PCR, using the following conditions:

1 cycle: 95°C, 1 min., then 43 cycles: 95°C, 1 min. ; 72°C, 3 min.

Analyze 5 µl from each reaction on a 6% polyacrylamide/$AgNO_3$ gel.

Figure 6.

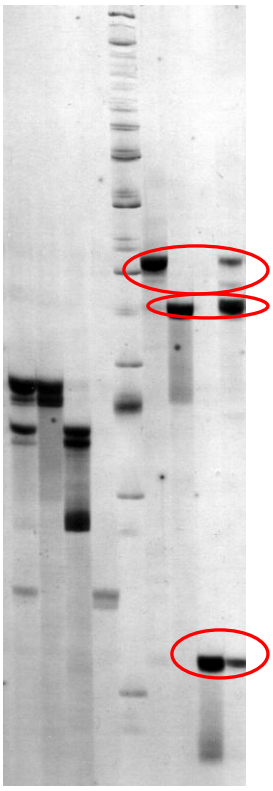


Figure 6. Inserts screening analysis on a 6% polyacrylamide/$AgNO_3$ gel.

▲ CRITICAL STEP PCR amplification of inserts, could be done using the primers flanking the insertion site of the vector.

TROUBLE SHOOTING

Product PCR is invisible.	**Add higher volume of template.** **Increase of cycle number** **Do PCR from PCR mixture**
To many PCR products are visible.	**Rise the annealing temperature of PCR program.** **Add less volume of template**

- **Cloning of subtracted DNAs.**

There are two way of cloning: cutting the band directly from polyacrylamide gel and after band amplification cloning it to the vector or to clone the whole mix after the last round of subtraction. The second approach cause the loss of information of band size and their quantity. During

separation mixture on the gel, there is a possibility to evaluate quantity and size of obtained subtracted DNA fragments. Note the product validation is not full informed because on one band in the polyacrylamide gel could be different sequence of similar The cloning procedure was used into TOPO vector (TOPO TA Cloning Kit, Invitroge) according to the manufacture instruction.

- **Sequence analysis of subtracted clones.**

The obtained band after subtraction should be sequenced and basic bioinformatic analysis shoul be performed. In case of cloning band from gel, at leats two bacterial colony should be taken to sequencing of insert in TOPO vector. Sequence comparison in e.g. Blast2Seq algorithm to elucidate if there was one or more sequences present in one band. To evaluate function of the sequence use other bioinformatics algorithm available on webpage eg

blastn and blastx algorithms, at the e-value below 0,01, using the relevant nucleotides and ESTs databases, respectively.. In our analysis, only GDDSC products with the similarities above 85% were taken into consideration.
Choose the interesting sequences and design internal primers (e.g. using Primer 3 program).

- **Real-Time analysis.**

To conduct our investigations the RNA was isolated from callus produced by L318 and L9 lines after 2, 4, 8 and 12 weeks culture on the induction media and after 2 days of culture on the regeneration media. Also RNA from immature embryos (21 days after self-pollination) of both lines was isolated as above, using Trizol kit (Invitrogen). The RNA concentration was checked using the spectrophotometer..

Use the Transcriptor High Fidelity cDNA Synthesis Sample Kit (Roche) to make the first strand of cDNA preparation (according to the manufacturer protocol). Real-Time PCR analysis was carried on the LightCycler 480® Multiwell Plate 96 (Roche) with LightCycler 480® SYBR Green I Master (Roche). In the case of rye *in vitro* response analysis, relative abundance of the GDDSC transcripts to the RUBISCO small subunit sequence was determined (**Figure 7**).

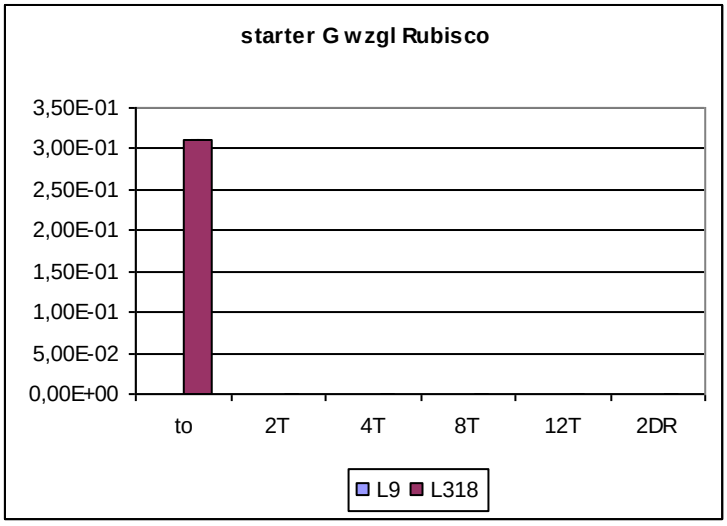


Figure 7 shows a relative abundance of GDDSC transcripts (R 450 Bam) in calli tissue produced by immature embryos of L318 and L9 lines after 2 (2T), 4 (4T), 8 (8T) and 12 (12T) weeks culture on the induction media and after 2 days (2DR) culture on the regeneration media and immature embryos (t_0) at the beginning of the culture.

Source

This protocol was provided directly by the authors.

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