Identification of Solanum Immune Receptors by Bulked Segregant RNA-Seq and High-Throughput Recombinant Screening

Solanum tuberosum

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https://doi.org/10.1007/978-1-0716-1609-3_15

Chapter 15

Identification of *Solanum* Immune Receptors by Bulked Segregant RNA-Seq and High-Throughput Recombinant Screening

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Abstract

The identification, understanding, and deployment of immune receptors are crucial to achieve high-level and durable resistance for crops against pathogens. In potato, many *R* genes have been identified using map-based cloning strategies. However, this is a challenging and laborious task that involves the development of a high number of molecular markers for the initial mapping, and the screening of thousands of plants for fine mapping. Bulked segregant RNA-Seq (BSR-Seq) has proven to be an efficient technique for the mapping of resistance genes. The RNA from two bulks of plants with contrasting phenotypes is sequenced and analyzed to identify single-nucleotide polymorphism (SNPs) markers linked to the target gene. Subsequently, the SNP markers that are identified can be used to delimit the mapping interval. Additionally, we designed an in vitro recombinant screening strategy that is advantageous for analyzing a large number of plants, in terms of time, space, and cost. Tips and detailed protocols, including BSR-Seq, bioinformatic analysis, and recombinant screening, are provided in this chapter.

Key words Pattern recognition receptors (PRR), Receptor-like proteins (RLP), Receptor-like kinases (RLK), Nucleotide-binding site leucine-rich repeat (NLR), Resistance genes (*R* genes), Effectors, Genetic mapping, Bulked segregant analysis (BSA), Mapping, Effectoromics

1 Introduction

Potato (*Solanum tuberosum* L.) is a staple food for 1.3 billion people [1], and its global production is close to 400 Mt yearly [2]. Unfortunately, potato is host to many pathogens that can affect all organs of the plant and diminish its production [3]. There are 35 economically important bacterial, fungal, and oomycete diseases of potato worldwide [4]. Among these, late blight, caused by the oomycete *Phytophthora infestans*, is the most pernicious, especially in developing countries [5]. It is estimated that the total cost due to losses and to control this disease is close to €9.4 billion per year

David Dobnik et al. (eds.), *Solanum tuberosum: Methods and Protocols*, Methods in Molecular Biology, vol. 2354, https://doi.org/10.1007/978-1-0716-1609-3_15,

[6, 7]. Therefore, the development of new cultivars that are resistant to economically important diseases is essential.

Plants have evolved a recognition-based immune system to defend themselves against pathogens [8–10]. Pattern recognition receptors (PRRs) that are located on the cell surface recognize microbial associated molecular patterns (MAMPs) or apoplastic effectors and activate defense responses. Intracellular nucleotide-binding site leucine-rich repeat (NLR) proteins that are encoded by resistance (*R*) genes recognize cytoplasmic effectors, also called Avirulence (Avr) proteins [8]. In case of late blight, potato breeders and researchers have relied mainly on the use of *R* genes. More than 20 *R* genes have been cloned [11], and many of them have been introgressed into cultivars with initial success [12]. The first and only characterized PRR in potato is the receptor-like protein (RLP) ELR (elicitin response) that recognizes INF1 elicitin from *P. infestans*. *ELR* overexpression results in enhanced resistance to the pathogen, although the resistance level is not as strong as most *R* genes [13]. The suitable deployment of immune receptors in crop plants, particularly by stacking multiple receptors, seems to be the key to obtain a durable resistance against pathogens [14–17].

Traditionally, wild and cultivated relatives of potato have starred as plentiful sources of resistance in potato breeding programs [18]. For many years, techniques as field, greenhouse, and laboratory assays have been used to screen *Solanum* species for disease resistance [3]. In the last years, the effectoromics strategy has come onto the scene to accelerate identifying of resistance and understanding of plant immunity [11]. Effectoromics is a high-throughput functional genomic approach that uses mainly two *Agrobacterium*-based functional assays, agroinfiltration and PVX agroinfection, to transiently express effectors in plant cells and monitor for the occurrence of macroscopic cell death responses [19, 20]. Additionally, for apoplastic effectors or microbe-associated molecular patterns (MAMPs), it has been shown that infiltration of effector proteins or peptides also represents an efficient way to screen responses [21].

The map-based cloning strategy has commonly been used to identify *R* genes in potato [12, 22–24], and, in combination with the effectoromics approach, it has allowed the rapid cloning of various *R* genes and a surface immune receptor against late blight, like *Rpi-sto1*, *Rpi-pta1*, and *ELR*, respectively [13, 25]. The central procedure of map-based cloning is the genetic mapping of the target gene, which is performed in a segregating population, using molecular markers distributed along each chromosome to identify markers delimiting the genetic region that contains the target gene [26]. Then, new markers are developed within the region and used to identify recombinants from a much larger population. These recombinants are then phenotyped/tested for response/resistance to narrow the genetic interval down in a

process called fine mapping [26]. The final goal of fine mapping is to determine a sufficiently small interval that allows the prediction of candidate genes. For potato, the whole process used to take several years, but fortunately, with the emergence of next-generation sequencing (NGS) technologies [27, 28] and the availability of the potato genome sequence [29–31], new strategies can be used to shorten this process [32].

Bulked segregant analysis (BSA) is an efficient method to identify markers linked to any gene or genomic region. In BSA, two bulks of plants with contrasting phenotypes for a specific trait are generated and analyzed to identify markers that discriminate the bulks [33]. BSA is a flexible approach that can be assisted by NGS technologies for the development of molecular markers [34]. Bulked segregant RNA-Seq (BSR-Seq) was developed as a new genetic mapping strategy by combining BSA and RNA-Seq [35] and has recently been used to accelerate the mapping of genes of interest [36–38]. RNA-Seq is commonly used for gene expression profiling but also for identification of single-nucleotide polymorphism (SNPs) that can be used as molecular markers [39, 40]. Compared to whole-genome sequencing, RNA-Seq requires a lower sequencing depth and additionally provides useful gene expression data from the candidate genes [34]. When required, this strategy is followed by fine mapping. To gather the necessary number of recombinants, a large number of individuals from the population (usually a few thousand) have to be genotyped with flanking markers. This is typically time consuming and requires a large amount of space and labor in a greenhouse facility. By screening for recombinants in vitro before transferring plants to the greenhouse, the efficiency of this process can be much improved. In this chapter, we describe a detailed protocol for BSR-Seq and the subsequent high-throughput recombinant screening under in vitro conditions to facilitate and accelerate the fine mapping of immune receptors in potato.

2 Materials

2.1 Sowing of Potato Seeds Under In Vitro Conditions and Micropropagation of Plants

1. 70% Ethanol.
2. 1.5% Sodium hypochlorite.
3. Sterilized tap water.
4. MS20 medium: 4.4 g of Murashige and Skoog basal salt mixture (including vitamins) and 20 g of sucrose dissolved in 900 mL of double-distilled water (ddH_2O). Adjust pH to 5.6 using KOH or NaOH, and complete the volume to 1 L. Add 8 g of micro agar and autoclave.
5. 100,000 ppm Gibberellic acid (GA_3) stock solution: 1 g of GA_3 dissolved in 10 mL of ddH_2O. Filter-sterilize.

2.2 RNA Isolation

1. RNeasy Plus Mini Kit, Qiagen.
2. Spectrophotometer for DNA/RNA quantity/purity evaluation.

2.3 DNA Isolation

1. CTAB buffer: 100 mL 1 M Tris–HCl (pH 7.5), 140 mL of 5 M NaCl, 20 mL of 0.5 M EDTA (pH 8.0), 740 mL of Milli-Q water, and 20 g of cetyl trimethylammonium bromide (CTAB) in 1 L of ddH_2O.
2. RNase 20 mg/mL (add 1 μL/mL of CTAB buffer just prior to use).
3. β-Mercaptoethanol (add 2 μL/mL of CTAB buffer just prior to use).
4. Chloroform:isoamyl alcohol (24:1 [v/v]).
5. Isopropanol.
6. 70% Ethanol.
7. Milli-Q water.
8. TissueLyser II and steel beads (3.2 mm).
9. Polypropylene cluster tubes: 1.2 mL 8-tube strips and strip caps, assembled in 96-tube racks.

2.4 Genetic Mapping

1. LightScanner System (BioFire) or any other suitable device for high-resolution melting analysis.
2. LC Green Plus (BioFire).
3. Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific).
4. White-96-well plates (4titude).
5. Restriction enzymes (*see* **Note 1**).
6. QIAquick PCR purification kit (Qiagen).
7. Genomic resources: CAPS Designer (Sol Genomic Network, https://solgenomics.net/tools/caps_designer/caps_input.pl), Potato reference genomes of DM1-3 516 R44 (http://solanaceae.plantbiology.msu.edu/pgsc_download.shtml), and Solyntus (https://www.plantbreeding.wur.nl/Solyntus/).

3 Methods

3.1 Bulked Segregant RNA-Seq

1. Identify diploid *Solanum* genotypes that respond to a specific effector or that are resistant to a certain pathogen (R), as well as genotypes that do not respond or that are susceptible (NR).
2. Generate a mapping population by crossing the R and NR genotypes (*see* **Note 2**).
3. Select 30 R and 30 NR individuals (*see* **Note 3**).

4. Sample leaves from the R parent, NR parent, 30 R plants, and 30 NR plants. Freeze the leaves in liquid nitrogen and store at −80 °C until use.
5. Isolate RNA using the RNeasy Plus Mini Kit from Qiagen following the manufacturer's instructions, and remove genomic DNA using the gDNA eliminator columns (part of RNA isolation kit) or by on-column DNase digestion using RNase-free DNase Set (Qiagen). Evaluate the quantity and purity of the RNA using a spectrophotometer, and use 1 μL of sample for gel electrophoresis to evaluate RNA integrity (*see* **Note 4**).
6. Prepare four samples: (1) R parent, (2) R bulk (mix equal amounts of RNA from the 30 R plants), (3) NR parent, and (4) NR bulk (mix equal amounts of RNA from the 30 NR plants) (*see* **Note 5**).
7. Send the RNA samples for sequencing (*see* **Note 6**).

An overview of the BSR-Seq strategy is shown in Fig. 1.

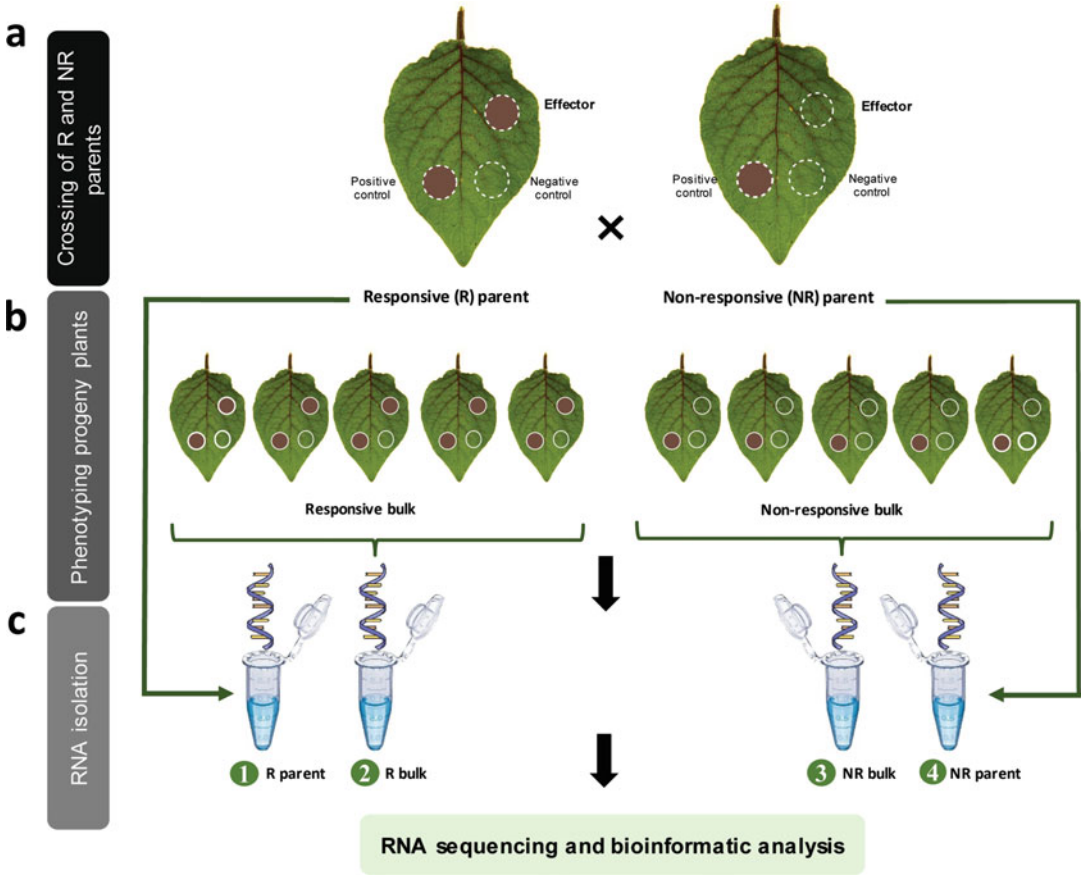

Fig. 1 Bulked segregant RNA-Seq strategy to map S*olanum* immune receptors. (**a**) Selected responsive (R) and nonresponsive (NR) genotypes are crossed to generate a segregating population. (**b**) Progeny plants with clear and confirmed phenotypes are selected to form the R and NR bulks. (**c**) Leaves are collected to form four samples, i.e., R parent, R bulk, NR parent, and NR bulk; the RNA is isolated and sent for sequencing

3.2 Bioinformatic Analysis

1. Process the fastq files to remove adapter sequences, bad reads, and low quality bases using software such as fastp [41].
2. Map RNAseq reads to the reference of choice using a splice-aware aligner like STAR [42].
3. Use the alignment files for variant calling and identification of SNPs (VarScan) [42].
4. Filter for putative SNPs linked to response or resistance (heterozygous in the responding/resistant parent and progeny pool, while absent or homozygous in the non-responding/susceptible parent and progeny pool).

3.3 Genetic Mapping and Marker Development

From identified SNPs (*see* Subheading 3.2), develop molecular markers covering the complete mapping interval (*see* **Note 7**):

1. Design primers to target the previously identified SNPs, and test them in the parents and a small set of R and NR plants using one of the methods described below (*see* **Note 8**).
2. Analyze the population with the selected markers to identify the flanking markers.

3.3.1 High-Resolution Melting (HRM) Markers

1. Design a PCR primer set producing ideally an 80–120 bp (base pair) fragment (*see* **Note 9**).
2. Prepare a 10 μL PCR reaction: 5.0 μL Milli-Q water, 2 μL Phire Reaction Buffer, 0.4 μL 5 mM dNTPs, 0.25 μL of each 10 μM primer, 1 μL LC-Green® Plus, 0.1 μL Phire Hot Start II DNA Polymerase, and 1 μL of genomic DNA (10–20 ng).
3. Dispense the solution in white-well PCR plates, and add 20 μL of mineral oil.
4. Use the following PCR conditions: 98 °C for 30 s followed by 40 cycles of PCR amplification (98 °C for 5 s, Ta (annealing temperature) for 10 s, and 72 °C for 15 s) and terminated by an incubation at 72 °C for 60 s and 25 °C for 30 s.
5. Run the samples in the LightScanner and perform the HRM analysis to genotype the samples.

3.3.2 Cleaved Amplified Polymorphic Sequence Markers (CAPS)

1. Design a PCR primer set producing ideally a 500–1,000 bp fragment. Target informative SNPs or another region inside the mapping interval.
2. Prepare a 15 μL reaction: 11.3 μL Milli-Q water, 1.5 μL reaction buffer, 0.6 μL 5 mM dNTPs, 0.3 μL of each 10 μM primer, 0.06 μL DreamTaq DNA Polymerase, and 1 μL of genomic DNA (10–20 ng). Dispense the solution in standard PCR plates.
3. Use the following PCR conditions: 95 °C for 3 min, followed by 35 cycles of PCR amplification (95 °C for 30 s, Ta for 30 s,

and 72 °C for 1 min), and terminated by an incubation at 72 °C for 10 min.

4. Run 3 μL of PCR product on a 1% agarose gel, and verify the presence of single bands for the parents. Purify and sequence the PCR products of both parents using the QIAquick PCR purification kit or any other.
5. Find the most suitable restriction enzymes using the CAPS Designer tool (*see* **Note 10**).
6. Digest all the PCR products using the selected restriction enzyme, and run 5 μL of the digested sample on a 1% agarose gel.
7. Determine the marker polymorphisms and genotype the plant samples. For each marker, analyze the band pattern of the parents and score the population accordingly.

3.4 High-Throughput Recombinant Screening

To narrow down the mapping interval, the screening of a few thousand of plants from the population is required. The plants are genotyped using the previously identified flanking markers to find the plants with recombination events (recombinants). This results in new flanking markers and therefore a smaller mapping interval (Fig. 2).

3.4.1 In Vitro Sowing of Seeds

1. Rinse seeds three times with tap water.
2. Soak seeds in 200 mL of 70% ethanol for 1 min.
3. Inside a flow cabinet, remove ethanol and soak the seeds in 1.5% sodium hypochlorite for 15 min (*see* **Note 11**).
4. Rinse seeds three times with sterile tap water to remove the sodium hypochlorite.

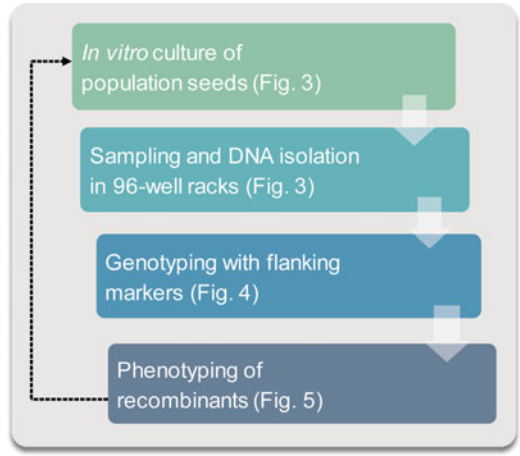

Fig. 2 Workflow for the high-throughput recombinant screening

5. Sow the seeds on Murashige and Skoog [43] medium supplemented with 20 g/L sucrose (MS20) and 100 ppm GA_3 and incubate them in a climate room in the dark for 2 weeks (*see* **Note 12**).
6. Transfer the first emerging seedlings to a new pot with fresh MS20 medium. Place 24 plants in each pot and assign a unique number to each plant (Fig. 3).
7. Incubate the plants in a climate room for 2 weeks.

3.4.2 DNA Isolation in 96-Tube Racks

1. Fill a rack with two steel beads per well.
2. Inside a flow cabinet, take one leaf from the first plant of the first pot and place in position A1 of the rack, and maintain the rack on ice during sampling. Fill each position in the rack with the samples from four pots (Fig. 3) (*see* **Note 13**).
3. Grind the samples using a TissueLyser at 25 Hz for 90 s.
4. To add buffers, use a repeating pipette, and to transfer the aqueous phase to new tubes use an 8-channel pipette.
5. Add 250 μL of CTAB buffer to each sample, incubate at 65 °C for 60 min (mix by inversion occasionally), and cool the samples down on ice for 15 min.
6. Add 250 μL of chloroform:isoamyl alcohol (24:1). Mix by inversion and centrifuge at 4,000 rcf for 15 min. In the meantime, prepare a new rack with clean tubes and add 200 μL of isopropanol to each tube.
7. Transfer 200 μL of the aqueous phase to the new tubes containing isopropanol, mix by inversion, and incubate at −20 °C for 15 min.
8. Centrifuge for 15 min at 4,000 rcf, remove the supernatant, and wash the DNA pellets with 500 μL of 70% ethanol.
9. Remove the ethanol and dry the pellets (*see* **Note 14**).
10. Add 100 μL of Milli-Q water. To evaluate the purity and quantity of the DNA, analyze some random samples using a spectrophotometer (*see* **Note 15**).
11. If needed, dilute the DNA to a final concentration of around 20 ng/ μL.

3.4.3 Genotyping

1. Analyze the entire rack with the two previously identified flanking markers (Subheading 3.3) to identify the recombinant plants (Fig. 4).
2. Design more molecular markers between the flanking markers.
3. Analyze the recombinants (*see* Subheading 3.4.4) and identify new flanking markers to narrow the mapping interval down.

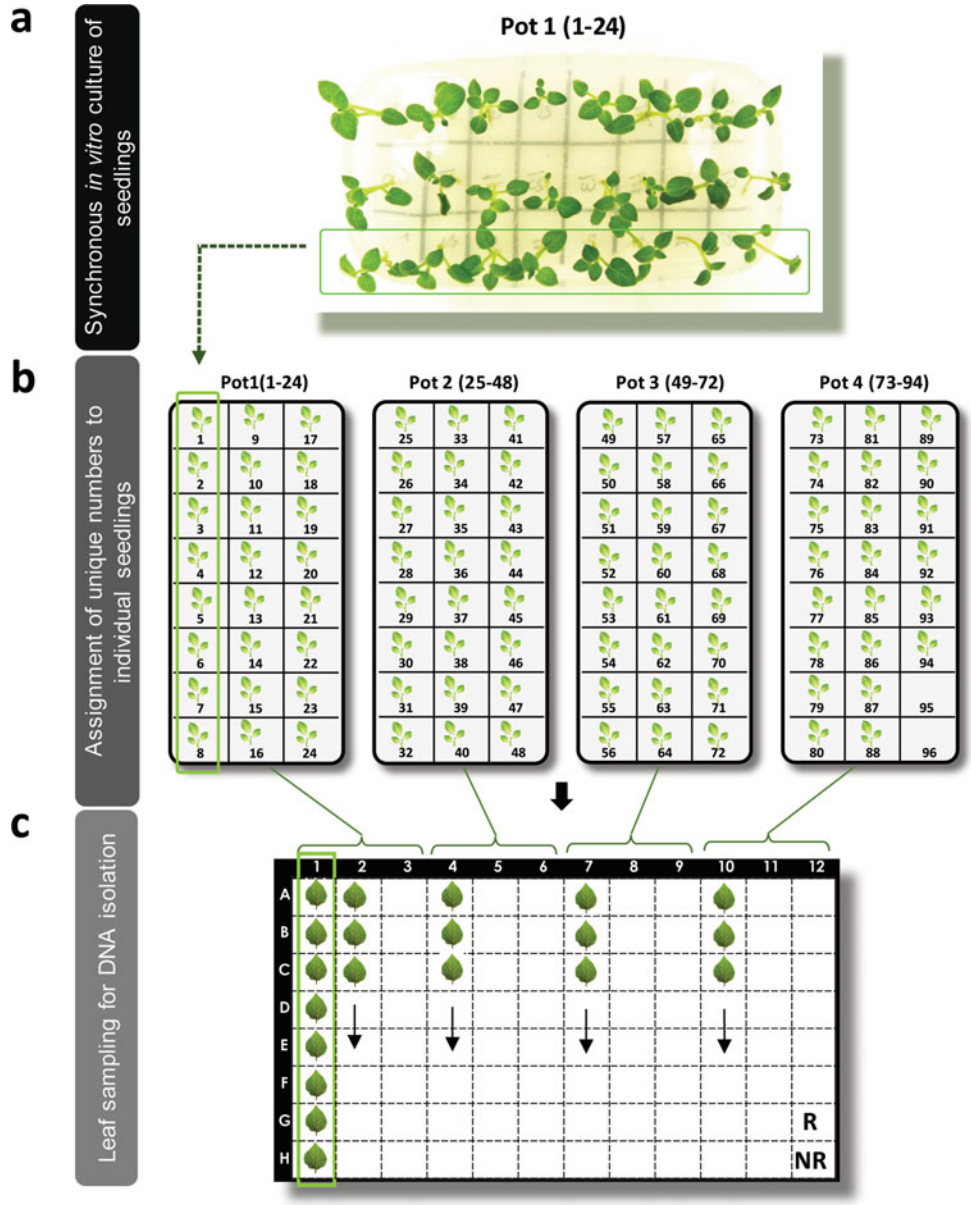

Fig. 3 In vitro culture and sampling for the high-throughput recombinant screening. (**a**) Seedling is transferred to pots with MS20 medium and organized in groups of 24 plants to grow them synchronously. (**b**) Each plant is assigned a unique number to track them along the process. Every four pots will form a 96-well rack. (**c**) For each individual seedling, one leaf is collected for DNA isolation and transferred to a rack. The two last wells are filled with samples from the two parents (R and NR)

4. If needed, return to Subheading 3.4.1. This process should be iterated to fine map the immune receptor, until the mapping interval is small enough for selecting candidate genes (*see* **Note 16**).

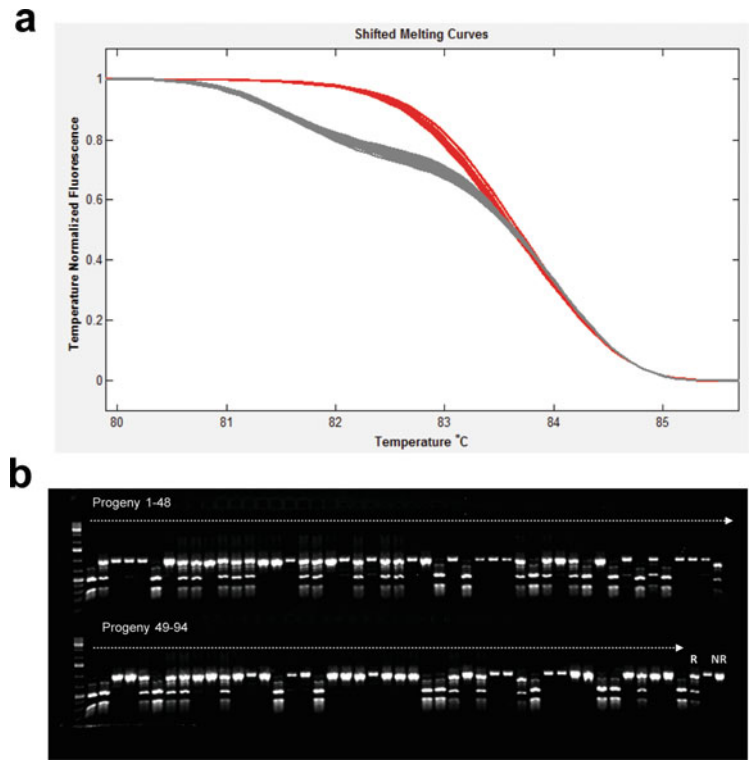

Fig. 4 Genotyping of the mapping population (plants 1–94 and parents). (**a**) HRM marker showing two patterns of melting curves (red or gray). The two different patterns indicate two groups with genotypes corresponding to the R or NR parent. (**b**) Gel image of the same plants tested with a CAPS marker, showing two different band patterns: three bands for the R parent (and progeny) and one band for the NR parent (and progeny)

3.4.4 Phenotyping of Recombinant Plants

1. Transfer the recombinant genotypes to pots containing fresh MS medium.
2. Micro-propagate and transfer two plants per genotype to the greenhouse.
3. Phenotype the recombinant plants using the method of your choice (Fig. 5).
4. Combine the genotypic and phenotypic information to determine the position of the recombination in relation to the flanking markers and target locus (Fig. 6).

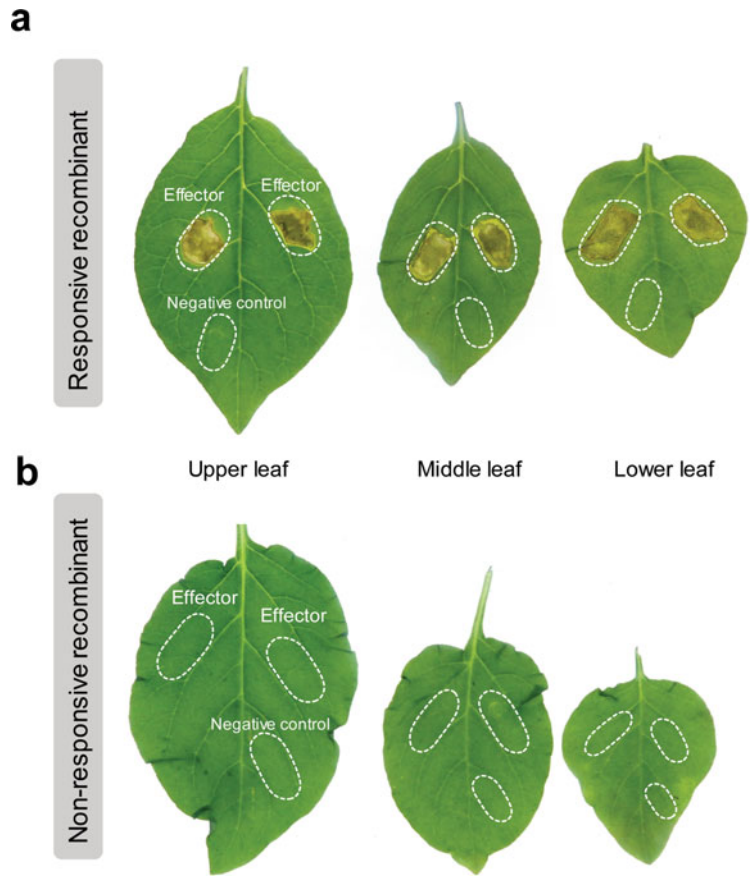

Fig. 5 Phenotyping of recombinant plants for response to an effector. Three leaves from a recombinant are infiltrated with the effector in duplicate. A negative control is included. (**a**) Responsive recombinant that shows a clear hypersensitive response (HR) to the effector. (**b**) Nonresponsive recombinant, unable to recognize the effector, and no HR is observed

4 Notes

1. The required restriction enzymes entirely depend on the sequence of the target. Commonly used enzymes are *Eco*RI, *Bam*HI, *Hin*dIII, *Rsa*I, and *Dra*I, among others.
2. To obtain the mapping population, sow around 100 seeds from the F1 in the greenhouse and phenotype them for the response to the effector or pathogen. If the F1 segregation is compatible with a 1:1 ratio, it indicates that the R parent has the immune receptor and that it is in heterozygous state. If the F1 does not show segregation, a backcross population (BC1) is required, a R genotype from the progeny should be crossed with the NR parent, and the segregation ratio should be determined. If the segregation is indeed 1:1 in the BC1, continue with this population.

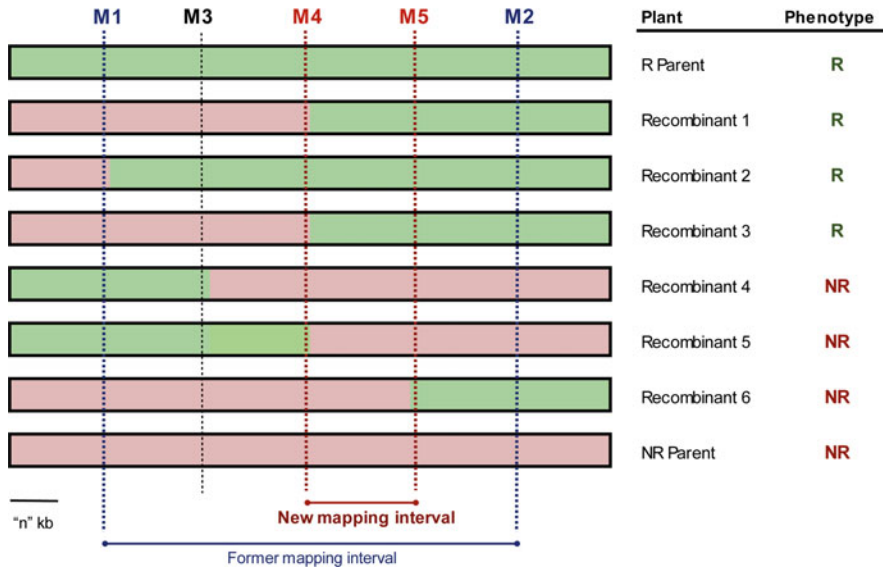

Fig. 6 Fine mapping. The markers M1 and M2 are used as flanking markers to identify recombination events and six recombinant plants (1–6) are identified. Then, new markers (M3, M4, and M5) are designed between the flanking markers and tested on the six recombinants and the parents. In parallel, the recombinants are phenotyped. Now, new flanking markers (M4 and M5) are identified and the mapping interval is narrowed down

3. The size of the mapping interval obtained from the BSR-Seq depends on the number of individuals per bulk, the sequencing depth, and the density of polymorphisms of the mapping population. For each parameter, more is better [35]. In our hands the BSR-Seq worked well using between 15 and 34 individuals per bulk.
4. RNA purity is determined measuring the OD ratios 260/280 and 260/230, which should be above 2.0. The RNA integrity can be determined on an agarose gel, by evaluating the 28S and 18S rRNA bands.
5. For RNA isolation, it is possible to mix all the samples that form a bulk and then isolate the RNA from one sample. However, we consider it more appropriate to isolate the RNA from each sample, and then mix equal amounts of each to form the bulk RNA sample.
6. Generally, around 2 μg of RNA is required for sequencing. The depth of the sequencing will depend on the number of individuals used per bulk. For 30 individuals per bulk, we used around 40 million paired-end reads (150 bp) per sample, corresponding to 12 Gbp of raw sequencing data.

7. The number of required molecular markers varies between experiments. The aim is to obtain markers that cover the complete mapping interval.
8. Before analyzing the entire population, test the markers in the parents and progeny with a known phenotype (use a subset of the plants included in the bulks). The selected markers are those that discriminate between R and NR plants. This test also gives information about the marker linkage phase.
9. For the HRM analysis, it is possible to genotype SNPs in bigger amplicons; however small amplicons allow for a better differentiation between variants. With bigger amplicons it is possible to detect additional mutations, which can result in more complex melting patterns that make it difficult to distinguish the variants [44].
10. Select the restriction enzyme that leads to clear differences in size between the cleaved and uncleaved PCR products.
11. After sterilizing the seeds of the mapping population, all the subsequent steps should be performed under sterile conditions.
12. Potato seeds are dormant directly after harvest, but will usually germinate rapidly after a period of cold storage for 6 months. The use of gibberellin hastens the onset of germination and can obviate the need for storing the seeds before sowing [45]. The timing and rate of germination depend on the population. In our hands, the required time for germination after GA treatment varies between 2 weeks and a few months, and the germination percentage is between 30% and 90%.
13. Once the leaf sampling is done, keep the pots in a cold climate room (18 °C) until the recombinants are identified. In this way, the plants grow slowly, and it will be easier to keep them separated.
14. Dry the DNA pellet leaving the tubes open inside a fume hood, until residual ethanol has evaporated.
15. The DNA purity is determined by measuring the OD ratios 260/280 and 260/230, which should preferably (but not necessarily) be around 2.0.
16. The final mapping interval should be small enough to end up with a manageable number of candidate genes for the next cloning steps.

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