3

Jan 12, 2022

Genotyping Arabidopsis T-DNA lines V.3

Lynn Doran[1], Steven J Burgess[2]

[1]Realizing Increased Photosynthetic Efficiency (RIPE); [2]University of Illinois at Urbana-Champaign

1

dx.doi.org/10.17504/protocols.io.b2hzqb76

Burgess Lab UIUC

Steven Burgess
University of Illinois at Urbana-Champaign

This protocol is used for genotyping Arabidopsis seedlings to test for the presence of a transfer DNA (T-DNA) insertion. By using two primer sets it is possible to determine whether a seedling is homozygous, heterozygous or azygous for an insertion in the predicted genomic location.

To identify lines with T-DNA insertions in a gene of interest, you need the Arabidopsis Genome Identifier (AGI) number corresponding to the genomic locus (e.g. RCS1A = AT1G67090), then visit the Salk Institute T-DNA Express site to find all the mapped insertions at your locus of interest.

Genotyping primers have been pre-designed for each T-DNA line, these can be retrieved from the Salk Institute T-DNA primer site, and ordered at any supplier of DNA oligonucleotides before starting the protocol.

In the US T-DNA lines can be purchased from the Arabidopsis Biological Resource Center (ABRC) and in the UK and EU from the European Arabidopsis Stock Center (NASC).

Recommended reading

- http://signal.salk.edu/tdnaprimers.2.html

Setting up the PCR reaction

Genotyping is performed with the Phire Plant Direct PCR Mix, this includes the polymerase, nucleotides and salts necessary for amplification. We use the “dilution protocol” which involves taking a small leaf disk and homogenizing it in dilution buffer using a gel tip (see manufacturer’s instructions for more details.)

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Lynn Doran, Steven J Burgess 2022. Genotyping Arabidopsis T-DNA lines.
protocols.io
https://dx.doi.org/10.17504/protocols.io.b2hzqb76
Lynn Doran

Realizing Increased Photosynthetic Efficiency (RIPE) that is funded by the Bill & Melinda Gates Foundation, Foundation for Food and Agriculture Research, and the U.K. Foreign, Commonwealth & Development Office
Grant ID: OPP1172157

protocol

DOI: 10.1126/science.1086391

Updated Phire Direct to PCR to be specific to the Phire Plants Direct to PCR protocol.

Arabidopsis, Genotyping

protocol ,

Dec 01, 2021

Jan 12, 2022

Jan 12, 2022 Steven Burgess University of Illinois at Urbana-Champaign

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- Phire Plant Direct Master Mix (Thermo Fisher Scientific; F160S)
  Note: There is also a Phire Tissue Direct Kit. This kit may not be able to break down cell walls to properly release DNA.
- Genotyping primers (SALK Border: LBb1.3 ATT TTG CCG ATT TCG GAA C, SAIL Border: Lb1 C/418-451 of pCSA110-pDAP101_T-DNAs GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC)
- Swiss Line Core Sampling Tool 0.5 mm, 0.50 mm I.D., 0.80 mm O.D. (Fisher Scientific; NC1310089) /(Electron Microscopy Sciences; 69039-05)
- 100bp ladder (New England Biolabs; N0467S)
- 10x Tris-Acetate-EDTA (TAE) Buffer (see Sigma website for recipe)
- 10,000x SYBR^{TM} Safe DNA stain (Thermo Fisher Scientific; S33102)
- 6x Gel Loading Dye (New England Biolabs; B7024S)

Prepare primer working solution

1 Re-suspend lyophilized primers in dH_2O to a stock concentration of 100 mM. Note: primer sequences can be obtained from SALK T-DNA express if you have the T-DNA accession number (http://signal.salk.edu/tdnaprimers.2.html)

2 Create a 10 mM working solution of the primer by diluting 10 µL of the stock into 90 µL of DNase-free water.

Preparing the PCR template

3 Using a 0.5 mm sampling device take a single leaf punch and add to 20 µL of Phire dilution buffer.

> Phire Plant Direct to PCR Kit is very sensitive. Sampling devices should be thoroughly sterilized in 70% Ethanol between samples to avoid cross-contamination.

4 Mash the sample with the end of a pipette tip or toothpick until the dilution buffer turns green.

5 Incubate sample at room temperature for 5 mins

6 Spin down sample for 1 min max speed in a mini centrifuge

Setting up the PCR reaction

7 Add the following components to a PCR tube. Set up one reaction with the diluted template from the putative T-DNA insertion line and one with WT arabidopsis as a negative control. The reaction will result in amplification if the T-DNA insert is present.

A	B
Component	**Amount**
2x Phire Master Mix	10 µL
Diluted template	0.5 µL
LBb1.3 primer [10 mM]	1 µL
RP [10 mM]	1 µL
dH2O	7.5 µL

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- Note: LBb1.3 (ATTTTGCCGATTTCGGAAC) is the recommended sequencing primer for SALK lines. If you have SAIL, Wisc_Lox or GABI-KAT lines you will need to use a different border primer. Results have successfully been obtained for SAIL lines using SAIL Border: Lb1 C/418-451 of pCSA110-pDAP101_T-DNAs (GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC)

8 In a second PCR tube set up the following reaction. Set up one reaction for the putative T-DNA insertion line and one for WT arabidopsis as a positive control. This reaction tests for the absence of a TDNA insertion. If a T-DNA insertion is present there will be no amplification product as the distance between the L and R primers. The WT control should give a product, and heterozygous lines will also give a product as there is one WT allele.

A	B
Component	**Amount**
2x Phire Master Mix	10 µL
Diluted template	0.5 µL
LP [10 mM]	1 µL
RP [10 mM]	1 µL
dH2O	7.5 µL

9 Run the following PCR program

A	B	C	D
Step	**Temperature**	**Time**	**Cycles**
Initial denaturation	98	5 min	1
Denaturation	98	5s	40
Annealing	*	5s	
Extension	72	20s	
Final Extension	72	1 min	1

*indicates annealing temperature to be adjusted to primer

Primers obtained from Salk Institute TDNA Site come with a recommended annealing temperature. Be aware that the annealing temperature will be different when using Phire taq polymerase. Recommended annealing temperature for primers when using Phire taq polymerase can be calculated using Thermo Fisher's Tm Calculator.

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Optimization of annealing temperature may still need to be conducted experimentally.

10 Analyze the resulting products by gel electrophoresis

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