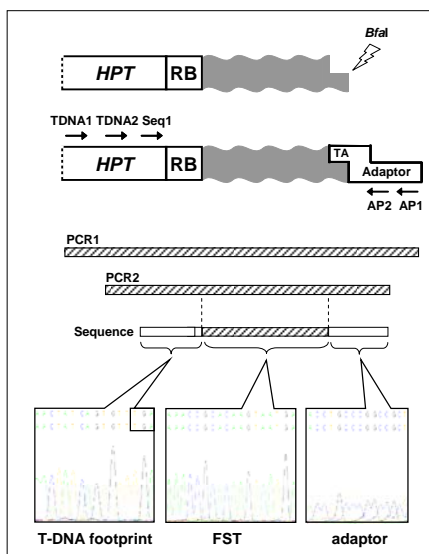




Protocol for *Brachypodium* FST analysis

[Vain P. et al. \(2008\)](#) Plant Biotechnology Journal, 6:236-245

[Thole V. et al. \(2009\)](#) Nature Protocols, 4:650-661.



Sequencing of the regions flanking the RB or LB of the T-DNA insert(s)

To sequence the region flanking the RB or LB of the T-DNA insert, set up the following reactions using the Seq1 or Seq2 primer, respectively.

1. Transfer approximately 250 ng of PCR2 product (see “BrachyTAG protocol for *Brachypodium* FST retrieval”) to a fresh tube/well in a final volume of 5.3 μ l.
2. Sequence with Applied Biosystems BigDye Terminator v3.1 cycle sequencing kit according to the manufacturer’s specifications. Add components 2, 3 and 4 sequentially to each PCR2 product (*i.e.* component 1).

Component	Amount per tube/well (μ l)	Final (in 10 μ l)
1	PCR2 product & H ₂ O	5.3
2	Big dye buffer (5x)	1.5
3	Seq1 primer (2 μ M)	0.75x
4	Big dye reaction premix	2.2
		0.44 μ M
		1

BrachyTAG



3. Cycle sequencing using a thermocycler and the following conditions:

Number of cycles	Temperature	Duration
1x	96°C	1 min
25x	96°C	10 sec
	50°C	5 sec
	60°C	4 min
1x	4°C	10 min
1x	10°C	pause

4. Purify extension products from Step 3 using Performa DTR V3 96-Well Short Plate Kit according to the manufacturer's instructions.
5. Collect samples from Step 4 to an Applied Biosystems MicroAmp™ optical 96-well reaction plate with barcode and seal with film using a heat sealer.
6. Sequence the samples from Step 5 on an Applied Biosystems 3730xl DNA analyser, 96 capillary, 50 cm array using pop7 according to the manufacturer's instructions.

Sequence analysis and identification of Flanking Sequence Tags (FSTs)

7. Identify sequence corresponding to T-DNA footprint (which can include part of the border repeat), flanking region and *Bfal*-adapter.
8. Analyse sequence of flanking regions using a BLASTn search routine against sequences of the binary vector(s), the Bd21 genomic sequence assemblies and all other existing sequences in online databases (e.g. GenBank, EMBL). Classify the flanking sequences as (1) not utilisable sequence (low quality, less than 30-bp long or without homology to any known sequence), (2) vector sequence (e.g. pVec8-GFP), (3) *B. distachyon* genomic sequence. The latter category (3) represents the Flanking Sequence Tag (FST) used to characterise the T-DNA insertion.
9. Compare the genomic location of FSTs adjacent to the LB and RB when retrieved from a single plant line.

This procedure is further detailed in [Thole V. et al. \(2009\) Nature Protocols](#), 4:650-661.