MATERIALS AND METHODS

Materials

Chemicals/ enzymes/ equipment	Company	Catalog number
[γ-32P]ATP (radioactive adenosine triphosphate)	Amersham	PB10204
384-well reaction plates	ABgene	AB-1384
96-well optical plates and optical covers	Applied Biosystems	4314320
96-well reaction plates	ABgene	AB-0900
96-well reaction plates	Corning	3754
Ampligase and buffer	Epicentre Technologies	A0102K
Antifoam A	Sigma	10794
Betaine	Sigma	B-2754
Bio-Gel HTP (hydroxylapatite)	Bio-Rad laboratories	130-0520
Bio-Rex 70 Resin	Bio-Rad laboratories	142-5852
DC Alufolien Kieselgel 60F_{254} chromatographic plates	Merck	5567
GF/F	Whatman	1825-090
Glass powder	Serva	22358
Igepal	Sigma	I-3021
$LiClO_4$	Fluka	62580
Ligase-65 and buffer	MRC-Holland	#Ligase-65
Ni-NTA His-Bind Resin	Novagen	70666-4
PEG 6000	Merck	807491
Pfu DNA ligase and buffer	Stratagene	600191
Plastic pistils	Roth	P987.1
DNeasy Plant Mini Kit	Qiagen	69104
Reference Dye, 100x	Sigma	R-4526
RNase A	Fluka	83831
T4 DNA ligase and buffer	New England BioLabs	M0202
T4 PNK and buffer	New England BioLabs	M0201
Taq DNA ligase and buffer	New England BioLabs	M0208
TaqMan PCR Core reagents kit	Applied Biosystems	N808-0228
Tth DNA ligase and buffer	ABgene	AB-0325
Ultrafree-MC HV centrifugal filter units	Millipore	UFC3 OHV NB

All other chemicals were purchased from Sigma.

Oligonucleotides

For the TaqMan assay, fluorescent probes labeled with TAMRA on the 3'-ends and Fam (Fluorescein) or Tet (Tetramethylrhodamine) on the 5'-ends were ordered from MWG Biotech (Ebersberg, Germany). Other oligonucleotides were purchased from TIB Molbiol (Berlin, Germany). Sequences of oligonucleotides used for this work are listed in Table 1 and Supplements 2 and 6.

DNA samples

Arabidopsis thaliana genomic DNA samples and plant material were provided by Dr. T. Altmann (MPI for Molecular Plant Physiology, Golm, Germany).

Human DNA samples representing three East European populations (Russians, Ukrainians, and Belorussians) were provided by Dr. N. Yankovsky (Institute of General Genetics, RAS, Moscow, Russia).

SNP loci

146 *Arabidopsis thaliana* SNP loci were examined in this work. Two loci ("G" and "H") polymorphic for accessions Columbia (Col-0) and Landsberg *erecta* (L*er*) were selected from the set of SNP markers described previously [Cho et al., 1999]. The other 144 loci (for accessions Col-0 and C24) were selected from the MASC-SNP database (http://www.mpiz-koeln.mpg.de/masc/). Genomic sequences adjacent to the SNP positions (30nt from both sides) are presented in Supplement 1.

One insertion/deletion polymorphism and 8 SNP loci associated with human disorders were chosen according to publications (see the section "Human SNP kit"). Genomic sequences adjacent to genomic variations (30nt from both sides) are given in Supplement 5.

Methods

Universal oligonucleotides

Sequences of universal (used for all loci) oligonucleotides are given in Table 1.

Common oligonucleotides (#L1, #L2 and #R) and adaptor oligonucleotides (#aL1, #aL2 and #aR) were involved in preparation of detector oligonucleotides.

PCR primers #PCR_L and #PCR_R were used for genotyping. Their sequences were checked for internal hairpins and dimer formation.

TaqMan probes #fam and #tet were designed according to established recommendations [Livak, 1999].

Name	Sequence	Length (nt)
#L1	TGCACAATTCACGACTCACGATCCACACGGTCTCGCACTGGC	42
#aL1	CCCGTGCCAGTGCGA	15
#L2	TGCACAATTCACGACTCACGATCATCCGCTCCGACGACACGA	42
#aL2	CCCGTTCGTGTCGTCG	16
#R	TAACGGGATAGCGTGGTGGTA	21
#aR	GCTATCCCGTTAGACCG	17
#PCR_L	GCACAATTCACGACTCACGA	20
#PCR_R	CCACCACGCTATCCCGTTAGAC	22
#fam	Fam-CCACACGGTCTCGCACTGGC-TAMRA	20
#tet	Tet-CATCCGCTCCGACGACACGA-TAMRA	20

Table 1. Sequences (5'→ 3' oriented) of universal oligonucleotides (see also Figure 15C).

Design of locus-specific oligonucleotides

Sequences of locus-specific oligonucleotides are listed in Supplement 2 (for *A. thaliana* SNP loci) and Supplement 6 (for human SNP loci).

Locus-specific oligonucleotides corresponding to Regions III and IV of the detector oligonucleotides (Figure 15B,C) were selected according to the following algorithm:

1. sequences adjacent to the SNP (~30nt from both sides of the SNP position) were blasted against the genome, and those showing strong homology to other genomic regions were excluded from consideration; sequences containing tandem repeats and long single base stretches were avoided when possible;
2. two allele-specific oligonucleotides corresponding to Regions III were designed to be at least 16nt in length (1-2nt difference was allowed), and to have melting temperatures close to 55°C (with ≤3°C difference between each other);
3. the oligonucleotide corresponding to Region IV was designed to have a melting temperature close to 60°C;
4. all detector oligonucleotides (DOs) were checked for internal hairpins ($\Delta G < -3$ was avoided).

For some loci, both strands were analyzed to choose the DO set most suited to the listed requirements. Estimation of melting temperatures was performed with the Vector NTI program (InforMax, USA) for 4nM primer and 50mM salt concentrations.

Locus-specific PCR oligonucleotides were designed complementary to the DOR (fully or partly complementary to Region IV) with melting temperatures of about 64°C (estimated for 0.4μM primer and 50mM salt concentrations). Oligonucleotides were analysed for internal hairpins and dimer formation with #PCR_L ($\Delta G < -3$ was avoided).

Preparation of detector oligonucleotides

The scheme of ligation-based synthesis (LBS) is shown in Figure 15C. Acetone precipitation was described previously [Daniliuk et al., 1986].

1. "Left" locus-specific oligonucleotides (#L1_01, …, #L1_n, and #L2_01, …, #L2_n) were phosphorylated: 1 nmol of each oligonucleotide was incubated at 37°C for 1 hour in 10μl of 1x T4 PNK buffer with 1mM ATP and 2.5u of T4 PNK. The PNK was then heat inactivated at 65°C for 20 minutes. Phosphorylated primers were used in LBS without any purification. Oligonucleotide #R, which is common for all loci, was phosphorylated during the phosphoramidite synthesis.
2. Ligation reaction involving 1 nmol of each of three oligonucleotides – common, locus-specific, and adapter – was performed for 1 hour at 20°C in 30μl of 1x T4 DNA ligase buffer with 15% PEG 6000 and 250U of T4 DNA ligase. T4 DNA ligase was then heat inactivated at 65°C for 15 minutes.
3. The "right" DO was phosphorylated for 30 minutes at 37°C directly in the ligation mixture by 2.5U of T4 PNK.
4. DO was separated from other oligonucleotides by electrophoresis in 10% denaturing PAGE.
5. After electrophoresis, DO band was visualized by UV shadowing on printer paper (or on the DC Alufolien Kieselgel 60F_{254} chromatographic plate) and cut out.
6. Gel fragment was crushed by centrifugation through a hole made by a hot needle in the bottom of a 0.5ml Eppendorf tube. Resulting pieces were incubated in 350μl of 2M $LiClO_4$ at 60°C for 2 hours or at room temperature (RT) overnight.
7. The liquid phase was separated by filtration through 0.45μm Ultrafree-MC HV centrifugal filter unit, mixed with 1ml of acetone and placed at -20°C for 1-2 hours.

8. After centrifugation (14000rpm) at 4°C for 30 minutes, the supernatant was discarded.
9. The pellet was washed with 200µl of cold acetone, dried at RT for 15 minutes and then dissolved in 15µl of H_2O. DO solutions were stored at -20°C.

T4 PNK buffer, 10x: Tris-HCl pH 7.6, 70mM; $MgCl_2$, 10mM; dithiothreitol, 5mM.
T4 ligase buffer, 10x: Tris-HCl pH 7.5, 50mM; $MgCl_2$, 10mM; dithiothreitol, 10mM; ATP, 1mM; BSA, 25 µg/ml.

Ligation detection reaction (LDR) – TaqMan SNP detection

"One tube – one locus" procedure

The overview of the procedure is shown in Figure 13.

1. The ligation reaction was performed on 0.4-150ng of *A. thaliana* or 2-50ng of human genomic DNA in the presence of 0.1nM (for *A. thaliana* loci) or 0.25nM (for human loci) DOs (DOL1, DOL2 and DOR – one locus set per tube) in 2.5µl of 1x *Pfu* DNA ligase buffer with 15% PEG 6000 and 0.125U of *Pfu* DNA ligase. The thermal profile of the ligation reaction was:

   95°C – 2 minutes
   95°C – 15 seconds
   65°C – 30 seconds } x 6 } x 20
   74°C – 10 seconds

2. 7.5µl of PCR mix (1.33x PCR Buffer, 0.27mM dNTPs, 2M Betaine, 532nM primers #PCR_L and #PCR_R, 400nM TaqMan probes #fam and #tet, and 0.4U of hot start *Taq* polymerase (AmpliTaq Gold /Applied Biosystems/, or homemade enzyme)) was added to ligation reaction. The thermal profile of the TaqMan assay was:

   95°C – 10 minutes
   95°C – 10 seconds }
   57°C – 10 seconds } x 40
   60°C – 10 seconds }

Reactions were assembled in 96-well optical plates sealed with optical covers (Applied Biosystems, USA) or in non-optical 96- or 384-well reaction plates (Corning) sealed with office tape.

Thermocycling with real-time and/or end point fluorescence detection was carried out on the ABI Prism7900HT system (Applied Biosystems).

Pfu ligase buffer, 10x: Tris-HCl, pH 7.5 20mM; KCl, 20mM; $MgCl_2$ 10mM; Igepal, 0.1%; ATP, 0.01mM; DTT 1mM.
PCR buffer, 10x: Tris-HCl pH 8, 100mM; KCl, 500mM; EDTA 0.1mM; Tween20 0.5%; Reference dye, 1x.

"One tube – many loci" procedure

The overview of the procedure is shown in Figure 14.

1. The only difference from the "one tube – one locus" scheme is that DO-sets for several loci were ligated simultaneously on 5-150ng of *A. thaliana* genomic DNA (concentration of each DO was 0.1nM).
2. 7.5µl of pre-amplification mix (1.33x PCR Buffer, 0.27mM dNTPs, 2M Betaine, 532nM primers #PCR_L and #PCR_R and 0.4U of hot start *Taq* polymerase) was added to ligation reaction. The thermal profile of the pre-amplification was:

   95°C – 10 minutes
   95°C – 10 seconds
   57°C – 10 seconds } x 10
   60°C – 10 seconds

3. The pre-amplification reaction was diluted with 90µl of water and 1µl was taken as a template for the locus-specific PCR-TaqMan.
4. Locus-specific PCR was performed in 10µl of 1x PCR Buffer, 0.2mM dNTP's, 1.5M Betaine, 400nM primers #PCR_L and #PCR_R, 300nM TaqMan probes #fam and #tet, and 0.4U of hot start *Taq* polymerase. The thermal profile and detection procedure for the TaqMan assay were the same as in the "one tube – one locus" procedure.

Determination of minimal concentrations of DOs

Genotyping was performed with 3.16x dilutions of locus "G" DOs (13, 41, 130, 410 and 1300pM) on 50ng of Col and L*er* DNA. All parameters of ligation and subsequent amplification were the same as in the "one tube – one locus" procedure, except for the different thermal profile of the ligation reaction (absence of sub-cycles and a shorter annealing time in cyclic ligation):

95°C – 2 minutes
95°C – 15 seconds } x 20
55°C – 20 seconds

Influence of PEG on the hybridization of DOs

Hybridization of 3.16x dilutions of locus "G" DOs (2, 6.3, 10, 63.2 and 200pM) on 1amol of the oligonucleotide #template (Supplement 2) was performed at 55°C for 2 hours in 5µl of 1x *Pfu* ligase buffer with or without 15% PEG 6000. Then 0.125U of

Pfu DNA ligase were added and the mixture was incubated at 55°C for 5 minutes. Amplification and detection were the same as in the "one tube – one locus" procedure.

Hot start Taq polymerase preparation

Purification of Taq polymerase.

The *Taq* polymerase expression construction was prepared as previously described [Engelke et al., 1990]. The purification protocol is a modified version of that suggested in the article.

1. Bacteria were grown in 1L of growth medium (TB, Ampicillin 100μg/ml, Glucose 2%) in a 2-5L flask at 37°C, 200-300rpm, up to OD_{600}=0.6-1.5.
2. The culture was centrifuged (4000rpm) at RT for 10 minutes, the medium was discarded, and the bacteria were washed with 100ml TB.
3. Bacteria were resuspended in 1L of the induction medium (TB, Ampicillin 100μg/ml, IPTG 0.5mM) and grown overnight at 37°C, 200-300rpm.
4. Bacteria were washed with 100ml of Buffer A, resuspended, and centrifuged (4000rpm) at 4°C for 10 minutes.
5. The pellet was resuspended in 20ml of Buffer A with 1mM PMSF. Then 20ml of Buffer B with 1mg/ml lysozyme was added and briefly but thoroughly mixed.
6. Bacterial lysate was incubated at 74°C for 45 minutes and then chilled in an ice-bath for ~10 minutes.
7. After centrifugation (10000-20000rpm) at 4°C for 20 minutes, the supernatant was filtered through a 0.45μm filter. *Taq* polymerase activity was estimated.
8. An equal volume of Buffer C with 50mM KCl and 0.5mM PMSF was added to the clarified lysate. The mixture was loaded on the Bio-Rex 70 column equilibrated with Buffer C containing 50mM KCl (~100 000U per 1ml of Bio-Rex 70).
9. The column was washed with 3 volumes of Buffer C containing 50mM KCl and 0.5mM PMSF.
10. *Taq* polymerase was eluted with 3 volumes of Buffer C containing 400mM KCl and 0.5mM PMSF, and the fractions with the protein peak were combined.
11. $MgCl_2$ and DNase I were added to the protein solution to the final concentration of 10mM and 2U/ml respectively. The mixture was incubated at 37°C for 40 minutes to destroy the DNA and then at 80°C for 15 minutes to inactivate the DNase.
12. *Taq* polymerase was dialyzed against three exchanges of ~10x volumes of Buffer D.

13. *Taq* polymerase activity was determined, and tests for DNA contamination (PCR without template) and for sensitivity in PCR reaction (PCR with decreasing amounts of template) were performed.

Taq polymerase was stored at -20°C or at -70°C (long term). Dilutions of *Taq* polymerase were prepared in Dilution Buffer.

TB medium: Bactotryptone, 1.2%; Bacto Yeast extract, 2.4%; Glycerol, 0.4%; KH_2PO_4 pH7.0, 100mM.
Buffer A: Tris-Cl pH7.9, 50mM; Glucose, 50mM; EDTA, 0.1mM.
Buffer B: Tris-Cl pH7.4, 10mM; KCl, 50mM; EDTA, 1mM; Tween 20, 0.5%; Triton X-100, 0.5%.
Buffer C: HEPES pH7.4, 20mM; EDTA, 1mM; Tween 20, 0.5%; Triton X-100, 0.5%.
Buffer D: HEPES pH7.4, 20mM; KCl, 100mM; Glycerol, 50%; PMSF, 0.5mM; DTT, 1mM.
Dilution Buffer: HEPES pH7.4, 20mM; KCl, 100mM; EDTA, 0.1mM; Glycerol, 50%; gelatin, 0.005%; DTT, 1mM.

Determination of Taq polymerase activity

1. The activity determination mixture (ADM) was prepared as follows: 1xPCR buffer, 2.5mM $MgCl_2$, dNTPs (dGTP, dCTP, dTTP – 1mM, dATP – 0.25mM), 0.3μM primer #188 (5'–GGTGCGGGCCTCTTCGCTATTACGCCA–3'), 78μg/ml single-stranded pBluescriptSK(+), ~0.06μCi/μl [α-^{32}P]dATP.
2. Serial dilutions of *Taq* polymerase were prepared in 1xPCR buffer with 100μg/ml BSA. 1 μl of diluted polymerase was added to 4μl of the ADM (in the ice-bath) and mixed.
3. Primer extension was performed:

   37°C – 1 minute
   72°C – 15 minutes
   0-4°C – 2 minutes

Reactions were placed on ice and the incorporation was determined by 10% TCA precipitation.

One unit is defined as the amount of *Taq* polymerase that incorporates 10 nmol of dNTPs in an acid-insoluble fraction at 72°C in 30 minutes. In this test, 100% incorporation corresponds to 0.2U/μl. To avoid saturation of the primer extension reaction, dilution series of *Taq* polymerase were analyzed. Activity was estimated when incorporation was proportional to the dilution.

PCR buffer, 10x: Tris-HCl pH 8.0, 10mM; KCl, 50mM; EDTA, 0.01mM; Tween 20, 0.05%.

Preparation of hot start Taq polymerase

1. *Taq* polymerase in Buffer E (20U/µl) was preheated to 37°C and mixed with formaldehyde (20µl per 1ml).
2. The mixture was incubated at 37°C for 30 minutes and then chilled in an ice bath for ~2 minutes.
3. Centrifugation (4000rpm) was performed at 4°C for 3 minutes.
4. The supernatant was mixed with an equal volume of 4M $(NH_4)_2SO_4$ and placed in an ice bath for 10 minutes.
5. *Taq* polymerase was pelleted by centrifugation (4000rpm) at 4°C for 10 minutes.
6. The pellet was washed with Buffer E containing 2M $(NH_4)_2SO_4$ and then dissolved in ~40µl Buffer E containing 50mM KCl.
7. The solution was centrifuged (14000rpm) at RT for 15 minutes.
8. The supernatant was dialized against three exchanges of ~10x volume of Buffer D.

Hot start *Taq* polymerase was stored at -20°C or at -70°C (long term). Dilutions of hot start *Taq* polymerase were prepared in Dilution Buffer.

Buffer E: HEPES pH7.4, 20mM; EDTA, 1mM.
Dilution Buffer: see the "Purification of Taq polymerase" protocol.

Estimation of the efficiency of formaldehyde inactivation

Activities of hot start *Taq* polymerase were estimated as described in *"Determination of Taq polymerase activity"*, except for the thermal profile, which either included the thermoactivation stage:

95°C – 15 seconds
37°C – 1 minute
72°C – 15 minutes

or not:

37°C – 16 minutes
72°C – 15 minutes.

Pfu DNA Ligase preparation

The open reading frame (ORF) of the *Pfu* DNA ligase gene was amplified from the genomic DNA of *Pirococcus furiosus*, cloned into the pET-11N vector (as wild type and N-His_6 variants), and expressed in BL21DE3 bacterial strain.

Purification of Pfu DNA ligase.

Steps 1-7 were the same as for purification of *Taq* polymerase. Remaining steps were as follows.

8. Filtered supernatant was loaded on Bio-Rex 70 column equilibrated with Buffer A:Buffer B (1:1) (1ml of Bio-Rex 70 per ~2mg of protein).
9. The column was washed with 4x volume of Buffer C containing 50mM KCl.
10. *Pfu* ligase was eluted with 2x volume of Buffer C containing 400mM KCl, and fractions with the protein peak were combined.
11. Bio-Gel HTP (hydroxylapatite), equilibrated with Buffer 25:25, was added to the combined fractions (1ml of Bio-Gel HTP per ~14mg of protein). *Pfu* ligase was loaded on to the Bio-Gel HTP with constant mixing at RT for 40 minutes
12. The Bio-Gel HTP was pelleted by centrifugation (1000rpm) at RT for 1 minute and washed twice with Buffer 25:25.
13. The Bio-Gel HTP was then packed in a column. *Pfu* ligase was eluted with Buffer 100:100 containing 400mM KCl, and fractions with the protein peak were combined.
14. His-tagged *Pfu* ligase was additionally purified on Ni-NTA His-Bind Resin:
    a) The combined fractions from step 13 were mixed with Ni-NTA His-Bind Resin, equilibrated with Buffer 300:50 (1ml of Ni-NTA His-Bind Resin per ~50mg of protein). *Pfu* ligase was loaded on to the resin with constant mixing at RT for 20 minutes.
    b) The resin was washed twice with Buffer 300:50 containing 15mM Imidazole.
    c) The resin was then packed in a column. *Pfu* ligase was eluted with Buffer 300:50 containing 100mM Imidazole.
15. The *Pfu* ligase was dialyzed against three exchanges of ~20x volumes of Dilution Buffer.

Pfu DNA ligase was stored at -20°C or at -70°C (long term).

Buffers A,B,C – see the "Purification of Taq polymerase" protocol.
Buffer 25:25: 25mM KCl, KH_2PO_4 pH 8.0, 25mM; PMSF, 1mM; β-mercaptoethanol, 2mM.
Buffer 100:100: 100mM KCl, KH_2PO_4 pH 8.0, 100mM; PMSF, 1mM; β-mercaptoethanol, 2mM.
Buffer 300:50: 300mM KCl, KH_2PO_4 pH 8.0, 50mM; PMSF, 1mM; β-mercaptoethanol, 2mM.
Dilution Buffer: Tris-HCl pH7.4, 50mM; EDTA, 1mM; Tween20, 0.1%; Igepal, 0.1%; DTT, 1mM; Glycerol, 50%.

Estimation of Pfu DNA Ligase activity

Serial dilutions of *Pfu* DNA ligase were prepared in 1x *Pfu* ligase buffer. 1µl of diluted polymerase was added to 4µl of 1x *Pfu* ligase buffer with 130ng of λ/*Bst*E II and mixed. The ligation was performed at 45°C for 40 minutes.

One unit of *Pfu* DNA ligase is defined by Stratagene as the amount of enzyme that ligates to completion 0.5µg of nicked pBluescript DNA in 15 minutes at 55°C. This amount of ligase should ligate 50% of the 12bp cohesive ends of 1µg of λ/*Bst*E II in 1 minute.

Pfu DNA ligase buffer, 10x: see the "One tube – one locus procedure" protocol.

T4 DNA Ligase preparation

The clone of T4 DNA Ligase was kindly provided by A.Montecucco. Purification was performed according to the established procedure [Rossi et al., 1997].

Genomic DNA purification

Plant Genomic DNA purification using CTAB buffer

This is a modified version of the protocol suggested previously [Rogers and Bendich, 1985].

1. 2-5g of plant tissue was pound in liquid nitrogen until a light-green powder was obtained.
2. The powder was quickly added to 25ml of CTAB Buffer 1 preheated to 65°C, and mixed thoroughly.

3. The mixture was incubated at 65°C for at least 20 minutes (usually for ~3 hours) and then chilled to RT.
4. An equal volume of chloroform:isoamyl alcohol (24:1) was added to the mixture.
5. After incubation with constant mixing at RT for 20 minutes, the mixture was centrifuged (5000rpm) at RT for 10 minutes and the aqueous phase was transferred to a clean tube.
6. The supernatant was mixed thoroughly with 0.2 volumes of CTAB Buffer 2 and incubated at 65°C for 10 minutes.
7. An equal volume of chloroform:isoamyl alcohol (24:1) was added to the tube. After mixing, the tube was incubated at RT for 10 minutes.
8. After centrifugation (5000rpm) at RT for 10 minutes, the aqueous phase was collected and transferred to a tube with an equal volume of Precipitation Buffer. The tube was left at RT for 1 hour (or overnight).
9. The tube was centrifuged (5000-8000rpm) at RT for 20 minutes.
10. The pellet was dissolved in 1-2ml HS-TE Buffer at RT or 65°C and precipitated by two volumes of ethanol at -20°C for 1 – 2 hours.
11. After centrifugation (8000rpm) at 4°C for 10 minutes, 200µl of H_2O and 100µl of 7.5M NH_4Ac (pH 7.5) were added to the pellet and the tube was incubated at 0°C for 20 minutes.
12. After centrifugation (13000rpm) at 4°C for 10 minutes, DNA was precipitated by two volumes of ethanol at -20°C for 1-2 hours.
13. The tube was centrifuged (13000rpm) at 4°C for 10 minutes.
14. The DNA pellet was washed with 80% ethanol and DNA was dissolved in TE.

Purified DNA was stored at -20°C.

CTAB Buffer 1: CTAB, 2%w/v; NaCl, 1.4M; TrisHCl pH 8.0, 100mM; EDTA, 20mM.
CTAB Buffer 2: CTAB, 5%w/v; EDTA, 350mM.
Precipitation Buffer: CTAB, 1%w/v; Tris-HCl, 50mM; EDTA, 10mM.
HS-TE Buffer: NaCl, 1M; Tris-HCl, 10mM; EDTA, 1mM.

Plant Genomic DNA purification using homemade silica spin-columns.

1. Leaves (~100mg of wet weight) were placed into a 1.5ml tube with ~50µl B1 Buffer, ~2µl of AntifoamA and ~20mg of glass powder. Then they were pound with a plastic pistil until visible pieces disappeared.

2. 4µl of RNaseA (100mg/ml) was added to the tube; the tube was mixed on the vortex and incubated at 65°C for 10 minutes, with mixing from time to time.
3. 400µl of chloroform:isoamyl alcohol (24:1) was optionally added.
4. 130µl of B2 Buffer was added to the tube. The contents were mixed and incubated at 0°C for 5 minutes.
5. The tube was centrifuged at RT for 5 minutes and the supernatant was carefully collected (addition of chloroform on step 3 facilitates the debris removal).
6. 1.5 volumes of B3 Buffer:ethanol (33%:67%) was added to the supernatant.
7. In several steps (~600µl per step), the mixture was loaded on to the 0.5ml GF/F column (with one filter, d=6mm), and centrifuged (30 seconds at RT). Flow-through was discarded.
8. The column was washed twice by adding ~650µl of WB Buffer and centrifuging for 30 seconds.
9. The column was centrifuged for 2 minutes to dry the filter.
10. The column was placed in a clean 1.5ml tube and ≥10µl EB Buffer was added to the column.
11. After incubation at 65°C for 5 minutes, DNA was eluted by centrifugation for 2 minutes at RT.

Purified DNA was stored at -20°C.

GF/F columns were prepared as described [Borodina et al., 2003].
B1 Buffer: MES pH5.3, 50mM; EDTA, 10mM; NaCl, 100mM; SDS, 1%.
B2 Buffer: CH_3COOK, pH 5.5, 3M.
B3 Buffer: MES pH5.5, 50mM; GuanidineCl, 8M.
WB Buffer: Tris-HCl pH7.5, 10mM; KCl, 100mM; ethanol, 70%.
EB Buffer: Tris-HCl pH8.5, 10mM.

Genomic DNA purification from saliva.

1. 2ml of saliva was collected by the "swish and spit" method [Hayney et al., 1995] and placed in 30ml of Rinsing Solution.
2. After centrifugation, (3000rpm) at 4°C for 5 minutes, the pellet was resuspended in 1ml of Resuspension Solution and transferred to a 1.5ml Eppendorf tube.
3. The tube was centrifuged (6000rpm) at RT for 5 minutes. The supernatant was discarded.
4. 50µl of Buffer B0 and ~20mg of glass powder were added to the pellet.
5. The pellet was pound with a plastic pistil until visible clumps disappeared.

6. Buffer B1 was added to create a total volume 400µl, and the tube was vortexed.
7. The tube was placed at 65°C for 10 minutes and mixed from time to time.
8. 400µl of chloroform:isoamyl alcohol (24:1) was added to the tube and the tube was vortexed.
9. 130µl of B2 Buffer was added to the tube. The tube was vortexed and kept at 0°C for 5 minutes.
10. The tube was centrifuged (12000rpm) at RT for 5 minutes.
11. The upper (DNA-containing) phase was collected and 1.5 volumes of B3 Buffer:ethanol (33%:67%) was added. The tube was vortexed.
12. The mixture was loaded on the 0.5ml GF/F column in several steps (~600µl per step), the column was centrifuged (12000rpm at RT for 30 seconds) and the flow-through was discarded.
13. The column was washed twice with 650µl of WB Buffer. Centrifugation (12000rpm) was performed at RT for 30 seconds.
14. The column was placed in a clean 2ml tube and centrifuged (12000rpm) at RT for 30 seconds to dry the filter.
15. The column was placed in a clean 1.5ml tube and 40µl of EB Buffer was added**.**
12. After incubation at 65°C for 5 minutes and DNA was eluted by centrifugation for 2 minutes at RT.

Purified DNA was stored at -20°C.

Rinsing Solution: NaCl, 100mM; TrisCl pH 8.0, 10mM.
Resuspension Solution: NaCl ,100mM; TrisCl pH 8.0, 10mM; EDTA, 10mM.
Buffer B0: MES pH 5.3, 50mM; EDTA, 10mM; NaCl, 100mM; RnaseA, 1µg/µl.
Buffers B1, B3, WB and EB were the same as in the "Plant Genomic DNA purification using homemade silica spin-columns" protocol.

Other procedures (acrylamide and agarose gel electrophoresis, phenol-chlorophorm purification of genomic DNA, etc.) were performed as described [Sambrook and Russel, 2001].