APPENDIX S4. KAPA3G Optimization 2.

This optimization procedure may be used to reduce non-specific amplification. Program 1 is the recommended cycling protocol for the KAPA3G Plant PCR Kit, using the optimal annealing temperature determined during Optimization 1. Program 2 reduces the extension time per cycle. Program 3 is a touchdown program, with decreasing annealing temperature, while Program 4 is a reverse touchdown (or touch-up), with increasing annealing temperature. Programs 3 and 4 may be more effective for certain gene regions like the *psbA-trnH* spacer. Mixes A–D are the same master mixes presented in optimization 1.

The second round of optimization with *Linum*, *Anethum*, and *Senna* for *rbcL* produced mixed results. The extension time in Program 2 was too short for most samples and resulted in little or no amplification. Programs 3 and 4 produced the best PCR products overall (brightest bands on a 1% agarose gel). Sequence data (Q20 values) for samples produced with Programs 3 and 4 are presented in Appendix S5. No clear differences emerged among the master mixes ($MgCl_2$ concentration, Enhancer presence/absence).

Program 1: 95°C 10 min; 40 cycles: 95°C 20 s, 58°C* 15 s, 72°C 90 s; 72°C 90 s.

Program 2: 95°C 10 min; 40 cycles: 95°C 20 s, 58°C* 15 s, 72°C 45 s; 72°C 90 s.

Program 3: 95°C 10 min; 11 cycles: 95°C 20 s, 58–48°C* 15 s, 72°C 90 s; 29 cycles: 95°C 20 s, 48°C 15 s, 72°C 90 s; 72°C 90 s.

Program 4: 95°C 10 min; 11 cycles: 95°C 20 s, 48–58°C* 15 s, 72°C 90 s; 29 cycles: 95°C 20 s, 58°C 15 s, 72°C 90 s; 72°C 90 s.

*Annealing temperature should be the highest temperature from Optimization 1 that worked for all samples, including those that need Enhancer. For the touchdown programs, choose a 10°C range from the optimal temperature.

Mix A—1.5 mM $MgCl_2$, with Enhancer (50 μL reaction volume)

PCR-grade water	20.1 μL
KAPA3G Plant PCR Buffer (2×)†	25 μL
Forward primer (10 μM)*	1.5 μL
Reverse primer (10 μM)*	1.5 μL
KAPA3G Plant DNA Polymerase (2.5 U/μL)	0.4 μL
KAPA Plant PCR Enhancer (100×)	0.5 μL
Template	1.0 μL

Mix B—1.5 mM $MgCl_2$, without Enhancer (50 μL reaction volume)

PCR-grade water	20.6 μL
KAPA3GPlant PCR Buffer (2×)†	25 μL
Forward primer (10 μM)*	1.5 μL
Reverse primer (10 μM)*	1.5 μL
KAPA3G Plant DNA Polymerase (2.5 U/μL)	0.4 μL
Template	1.0 μL

Mix C—2.0 mM $MgCl_2$, with Enhancer (50 μL reaction volume)

PCR-grade water	19.1 μL
KAPA3G Plant PCR Buffer (2×)†	25 μL
Forward primer (10 μM)*	1.5 μL
Reverse primer (10 μM)*	1.5 μL
KAPA3G Plant DNA Polymerase (2.5 U/μL)	0.4 μL

$MgCl_2$ (25 mM)	1.0 μL
KAPA Plant PCR Enhancer (100×)	0.5 μL
Template	1.0 μL

Mix D—2.0 mM $MgCl_2$, without Enhancer (50 μL reaction volume)

PCR-grade water	19.6 μL
KAPA3G Plant PCR Buffer (2×)[†]	25 μL
Forward primer (10 μM)*	1.5 μL
Reverse primer (10 μM)*	1.5 μL
KAPA3G Plant DNA Polymerase (2.5 U/μL)	0.4 μL
$MgCl_2$ (25 mM)	1.0 μL
Template	1.0 μL

[†]Includes dNTPs at a final concentration of 0.2 mM each.

*If primer stocks are at a different concentration than 10 μM, include the appropriate volume of each primer for a final concentration of 0.3 μM each, and adjust the volume of water accordingly (for a reaction volume of 50 μL).