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TABLE OF CONTENTS

1. INTRODUCTION

Conventional plant breeding usually identifies genetic variation by visual or chemical selection. Certain important traits such as nutritional value, alkaloid levels and fatty acid composition, which lead to very subtle changes in morphological appearance, are always challenges for plant breeders. The process of developing new crop varieties can take up to 25 years. However, with the advancements in molecular biology, the duration has been considerably shortened to 7-10 years. One of the important techniques that makes it efficient for scientists to select plant traits is Marker-Assisted Selection (MAS).

Molecular technologies enable us to identify changes in DNA or gene sequences associated with the phenotype variation. Various types of molecular markers have been used to label and map these molecular changes through linkage analysis with segregating populations. Genetic linkage describes the fact that molecular markers and genes of interest proximal to each other are inherited together during meiosis. A desirable attribute for a user-friendly marker is close linkage with the gene corresponding to phenotype, which significantly precludes false-positive selection due to recombination (or cross-over) events. Other qualities expected by breeders for genetic markers are: (1) co-dominance; (2) representing high level of genetic polymorphism (can be easily discerned by users); (3) low cost during application; (4) high stability (able to be confirmed and duplicated by colleague researchers), and (5) ease of use.

In this review, we have surveyed the most commonly used molecular techniques in tobacco. These include RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), SSR (Simple Sequence Repeat), SCAR (Sequence Characterized Amplified Region), CAPS (Cleaved Amplified Polymorphic Sequences), dCAPS (derived CAPS), and KASP™ (Kompetitive Allele Specific PCR). The different types of molecular markers are explained in detail in the forthcoming sections. Further, the traits for which markers for tobacco are available are outlined in Table 1, and detailed information regarding primers and/or sequences are provided in supplementary Table S1. Quantitative Trait Loci (QTLs) and the markers used to map the QTL are summarized in Table 2, and detailed information is provided in supplementary Table S2.

2. TYPES OF MOLECULAR MARKERS

2.1 RFLP Markers

Restriction Fragment Length Polymorphism (RFLP) markers were first used to distinguish two ecotypes and to construct a genetic map in *Arabidopsis* (Chang *et al.* 1988); (Liu *et al.* 1996). Even though RFLP markers are co-dominant, meaning that distinct patterns are obtained for plants that are homozygous or heterozygous for the target alleles, the main disadvantage posed for the routine application of RFLP markers in plant breeding is that it requires relatively large amounts of DNA, which is subjected to digestion of restriction enzymes and southern blotting. RFLP markers are no longer commonly used, having been replaced by the more user-friendly PCR-based DNA markers.

2.2 PCR-based DNA Markers

PCR-based DNA markers are the most widely used because they need only a small amount of DNA sample (about 10-20 ng per reaction) and no time-consuming DNA blotting or hybridization is required. PCR-based DNA markers are grouped into two classes based on the type of information they provide; anonymous markers and Sequence-Tagged Site (STS)

markers. The most commonly used anonymous markers include Amplified Fragment Length Polymorphism (AFLP) markers (Vos *et al.* 1995) and Random Amplified Polymorphic DNA (RAPD) markers. STS markers include Sequence Characterized Amplified Region (SCAR) markers (Paran and Michelmore 1993), Simple Sequence Repeat (SSR) markers (Litt and Luty 1989) and CAPS and dCAPS markers based on Single Nucleotide Polymorphisms (SNPs) (Konieczny and Ausubel 1993; Neff *et al.* 1998).

2.2.1 Anonymous Markers

2.2.1.1 Amplified Fragment Length Polymorphism

The AFLP technique involves digestion of genomic DNA or cDNA with restriction enzymes, followed by ligation of adaptors to digested fragments. A subset of fragments is selected to be amplified under high stringency conditions. Hence, this technology combines the power of RFLP with the flexibility of PCR-based markers and provides a universal, multi-locus marker tool that can be applied to complex genomes from any source. In addition, based on the use of endonucleases, there are several modified AFLP techniques, such as single endonuclease AFLP (Boumedine and Rodolakis 1998), three endonuclease-AFLP (van Der Wurff *et al.* 2000) and second digestion AFLP (Knox and Ellis 2001).

Radio-active detection has been replaced with silver staining, fluorescent tag or agarose gels for single endonuclease AFLP. This method is remarkably reliable, consistent, and as a result, it has been used to specifically address genetic variation, phylogenetic relationships, homoplasy, etc.

2.2.1.2 Random Amplified Polymorphic DNA

RAPD technology exploits arbitrary primers of short synthetic oligonucleotides to amplify fragments. It is simple, time-saving, and needs only small amount of template DNA without the requirement of the sequencing. Due to these advantages, RAPD technology has been widely used in localization of target genes, genetic mapping and evolution genetics. However, RAPD markers are inherently unstable, which limits their wide application among laboratories.

2.2.2 Sequence Tagged Site markers

2.2.2.1 Sequence Characterized Amplified Region markers

To overcome the limitations associated with anonymous markers, RAPD and AFLP markers can be converted into SCAR markers (Paran and Michelmore, 1993). In general, the target bands are extracted from agarose gels, followed by ligation into sequencing vectors to obtain the full sequence of the insert. Eventually, primers are designed to specifically amplify the band of interest based on its sequence. Through this process, the RAPD and AFLP markers can be transformed into simple and robust PCR markers. This procedure also improves the reproducibility of anonymous markers and avoids the occurrence of non-homologous markers with equal molecular weight. The cloned and sequenced DNA fragments can then be used for the development of dominant markers, or co-dominant CAPS markers.

2.2.2.2 Simple Sequence Repeats

SSRs are tandem repeated sequences of one to six nucleotide long DNA motifs that occur frequently in all eukaryotic and prokaryotic genomes. SSR markers can be developed directly from genomic DNA libraries or from libraries enriched for specific microsatellites (Zane *et al.* 2002). SSR markers are characterized by high variability,

reproducibility, co-dominance, locus specificity, amenability to automation and random dispersion throughout most genomes; therefore SSR markers can be easily and reproducibly detected by PCR. The disadvantage of SSR markers is that they require a labor- and cost-intensive development process particularly when screening and sequencing genomic DNA libraries enriched by repeated motifs. Interestingly, once they have been developed for a plant species, SSR markers are almost always the first choice for genetic mapping because of low start-up costs.

2.2.2.3 Single Nucleotide Polymorphisms

Single Nucleotide Polymorphisms (SNPs) have emerged as the most abundant and universal form of genomic polymorphism. In breeding applications, utilization of SNPs is still in its infancy, mainly because other marker-related applications do not require the massively parallel, genome-wide genotyping necessary for SNPs. The availability of draft genome sequences of the tobacco varieties TN90 (Burley), K326 (flue-cured) and Basma Xanthi (Oriental) (Sierro *et al.* 2014), followed by optical mapping, enabled an improved assembly of the K326 genome (Edwards *et al.* 2017), which has laid the foundation for gene mapping and trait discovery in tobacco. The release of the SNP-based high density genetic map, *N. tabacum* 30k Infinium HD consensus map 2015 (Edwards *et al.* 2017) also provides the tobacco genetic research community with resources to fine map their trait of interest (https://solgenomics.net/cview/map.pl?map_version_id=178). The database can also be used to convert some of the previously used SSR, AFLP, SCAR markers etc., to closely linked SNP markers from the genetic map.

The use of SNPs for genotyping is based on fluorescent detection of SNP-specific hybridization probes on PCR products such as Molecular Beacons, Taqman™, KASP™ (Semagn *et al.* 2013), and Invader enhanced by array technology (Olivier 2005; Tapp *et al.* 2000). The end point detection of SNP genotypes requires either the PCR product to be read by a plate reader, or a real time PCR instrument. For simple and accurate genotyping to detect SNPs without sophisticated equipment, CAPS and dCAPS techniques provide a solution by using endonuclease. The CAPS assay consists of digestion of PCR amplicons with one or several restriction enzymes, and separation of the digested DNA on agarose or polyacrylamide gels. CAPS markers are co-dominant and locus specific and have been indispensable for fine mapping of target genes in map-based cloning. Even though SNPs can be converted into size polymorphisms through the CAPS assay, the disadvantage of this technique is that sometimes the SNP cannot be recognized by any endonuclease. To overcome this limitation, a variant of the CAPS assay, known as dCAPS, was proposed by Neff *et al.* (1998). Combined with dCAPS, CAPS can be used to detect virtually any SNP at a lower cost than that of other assays. The CAPS technique has been proved to be very useful for genotyping known point mutants in segregating populations. However, the process is time consuming and not efficient for large breeding programs. If the initial cost of instrumentation is not a barrier, then genotyping technologies based on end point detection, such as KASP™ are efficient, cost effective and reliable.

3. CONCLUSIONS

Our recommendations for tobacco breeding programs heavily weigh in favour of SNP markers. SNP or INDEL (Insertion Deletion) markers in the form of KASP™ and Taqman™ are codominant and reliable, and can be used for tracking trait-specific mutations or as closely linked markers. They are easy to use and efficient, especially for breeding programs handling a large volume of samples. The CAPS and dCAPS markers are also useful markers, because they are reliable, relatively inexpensive, and co-dominant markers that can be gene-specific. They can be used only when there are different PCR products from the two parents (e.g. they cannot be used where one allele results from a deletion). It is more time consuming to go from DNA to results, when compared to KASP™ or Taqman™, making it inefficient for breeding programs handling large volume of samples. The next most useful markers are closely-linked SCAR markers; they are reliable, but they are dominant markers and so less useful to breeders because they cannot identify heterozygotes. They are used when the sequence is known, but there is PCR product from only one parent. AFLP and RAPD markers are not ideal, but they can be used when the sequence information is unknown. RAPD markers are unreliable because they are not always repeatable. However, the procedure is simple and uses reagents and equipment routinely found in marker laboratories. AFLP markers are more reliable and give more repeatable results than RAPD markers, but they are not as convenient. The procedure is more expensive, takes longer and uses specialised equipment not routinely used in most marker laboratories.

Table 1: Available markers for major genes or traits in tobacco (*Nicotiana tabacum* L.)

Trait	Gene	Marker	Linkage	Dominance	Publication
Alkaloid locus B	*Nic2*		gene-specific	dominant	Shoji *et al.* (2010)
Black root rot resistance (ex *N. debneyi*) *Thielaviopsis basicola*		RAPD	closely-linked	dominant	Bai *et al.* (1995)
Black root rot resistance (ex *N. debneyi*) *Thielaviopsis basicola*		SCAR	1 cM	dominant	Julio *et al.* (2006)
Black root rot resistance (ex *N. debneyi*) *Thielaviopsis basicola SS192650*		CAPS	closely-linked	co-dominant	Qin *et al.* (2018)
Black shank race 0 resistance (ex *N. longiflora*) *Phytophthora nicotianae*	*Phl*	SCAR	closely-linked	dominant	Unpublished, personal communication
Black shank race 0 resistance (ex *N. plumbag*) *Phytophthora nicotianae*	*Php*	RAPD	closely-linked	dominant	Johnson *et al.* (2009)
Black shank race 0 resistance (ex *N. plumbag*) *Phytophthora nicotianae*	*Php*	RAPD	closely-linked	dominant	Johnson *et al.* (2009)
Black Shank Race 0 resistance (*P. parasitica* var. *nicotianae)*	*Ph*	SCAR	closely-linked	co-dominant	Bao *et al.* (2019)
Black Shank Race 0 Coupling Phase	*Php*	SCAR	closely-linked	dominant	Unpublished, personal communication
Black Shank Race 0 Repulsion Phase	*Php*	SCAR	closely-linked	dominant	Unpublished, personal communication
Blue mold resistance *Peronospora tabacina*		SCAR	closely-linked	dominant	Milla *et al.*(2005)
Blue mold resistance *Peronospora tabacina*		SCAR	closely-linked	dominant	Julio *et al.* (2006)
Blue mold resistance *Peronospora tabacina*		SCAR	closely-linked	dominant	Wu *et al.* (2015)
Broomrape resistance *Orobanche ramosa*	*Nt14*	SSR	closely-linked	co-dominant	Julio *et al.* (2019)

Trait	Gene	Marker	Linkage	Dominance	Publication
Brown spot (*Alternaria*) resistance *Alternaria alternata*		RAPD	closely-linked	dominant	Zhang *et al.* (2008)
Cis-abienol synthase; diterpene synthase	*abs cps2*		gene-specific	dominant	Sallaud *et al.* (2012)
Demethylase e10	*CYP82e10*	CAPS	gene-specific	co-dominant	Li *et al.* (2012)
Demethylase e4	*CYP82e4*	dCAPS	gene-specific	co-dominant	Li *et al.* (2012)
Demethylase e5	*CYP82e5*	dCAPS	gene-specific	co-dominant	Li *et al.* (2012)
Demethylase e10	*CYP82e10*	KASP	mutation specific	co-dominant	Unpublished, personal communication
Demethylase e4	*CYP82e4*	KASP	mutation specific	co-dominant	Unpublished, personal communication
Demethylase e5	*CYP82e5*	KASP	mutation specific	co-dominant	Unpublished, personal communication
Low alkaloid production	*MYC2a*	CAPS	gene specific	co-dominant	Burner *et al.* (2022)
Multiple Disease Resistance: Black shank and bacterial wilt (*Phytophthora nicotianae* and *Ralstonia solanacearum)*	*Phn7.1*	CAPS	12.6 cM	co-dominant	Shi *et al.* (2021)
N-Gene (*N. Glutinosa* Virus Resistance)	*N-gene*		gene specific	dominant	Whitham *et al.* (1994)
Potyvirus susceptibility	*eiF4E1.S*		gene specific	dominant	Dluge *et al.* (2018)
Powdery Mildew resistance	*NtMLO1/2*	CAPS	closely-linked	co-dominant	Komatsu *et al.* (2020)
PVY resistance Potato virus Y	*va*	SCAR	5.1 cM	dominant	Julio *et al.* (2006)
PVY resistance Potato virus Y	*va*	RAPD	closely-linked	dominant	Lewis (2005)
PVY resistance Potato virus Y	*eIF4E1.S*	Bi-PASA	gene specific	co-dominant	Lin *et al.* (2021)
PVY resistance Potato virus Y	*eIF4E-2*	KASP	closely-linked	co-dominant	Michel *et al.* (2020)
PVY resistance Potato virus Y	*va (eIF4E)*		gene specific	dominant	Julio *et al.*(2015)

Trait	Gene	Marker	Linkage	Dominance	Publication
PVY RBV resistance	*eIF(iso)4E*	dCAPS	mutation specific	co-dominant	Takakura *et al.* (2018)
Powdery mildew resistance *Golovinomyces cichoracearum* var. *cichoracearum*	*NtMLO1* *NtMLO2*	SSCP	gene specific	co-dominant	Fujimura *et al.* (2018)
Root-Knot resistance *Meloidogyne incognita*	*Rk*	RAPD	closely linked	dominant	Yi *et al.* (1998)
Tobacco mosaic virus (TMV) resistance	*N*	SCAR	gene-specific	dominant	Lewis (2005)
Tomato spotted wilt virus (TSWV) resistance		AFLP SCAR	<5cM	dominant	Moon *et al.* (2008)
Wildfire (race 0) resistance *Pseudomonas syringae* pv. *tabaci* tox-		RAPD	closely-linked	dominant	Yi *et al.* (1998)
Yellow Burley (Yb) trait Yb24-2	*YB1*	KASP	closely-linked	co-dominant	Edwards *et al.* (2017)
Yb24-4	*YB1*	KASP	closely-linked	co-dominant	Edwards *et al.* (2017)
Yb24-6	*YB1*	KASP	closely-linked	co-dominant	Edwards *et al.* (2017)
Yb24-7	*YB1*	KASP	closely-linked	co-dominant	Edwards *et al.* (2017)
EGY_2	*YB1*		gene specific	dominant	Edwards *et al.* (2017)
EGY_2kasp	*YB1*	KASP	gene and mutation specific	co-dominant	Edwards *et al.* (2017)
Yb5-1	*YB2*	KASP	closely-linked	co-dominant	Edwards *et al.* (2017)
Yb5-4	*YB2*	KASP	closely-linked	co-dominant	Edwards *et al.* (2017)

Table 2: QTLs and associated markers in *Nicotiana tabacum* L.

Trait	Marker	Linkage	Dominance	% Phenotypic variation	Publication
Bacterial wilt resistance	AFLP	closely-linked	dominant	0.3	Nishi *et al.* (2003)
Bacterial wilt resistance	SSR	LG 7, 6.6 cM	co-dominant	0.321	Drake-Stowe *et al.* (2017)
Black shank race 1 resistance	SSR	LG 4	co-dominant	0.25	Vontimitta and Lewis (2012)
Black shank resistance	SSR	LG 7, 6.6 cM	co-dominant	0.38	Drake-Stowe *et al.* (2017)
Increased leaf number	AFLP	3.1cM	dominant	increase 7-8 leaves	Lewis *et al.* (2007)
Delayed flowering time	AFLP	3.3cM	dominant	8 days	Lewis *et al.* (2007)
Black shank race 1 resistance	SSR	LG 8	co-dominant	0.2	Vontimitta and Lewis (2012)

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ANNEX A

Table S1: Molecular Markers in Tobacco – Marker Information for Major Genes

Trait	Gene	Marker	Linkage	Dominance	Developed By	Enzyme	Annealing Temp	Comments	Primer Sequence or Associated Sequence for KASP design	Publication
Alkaloid locus B	*Nic2*		gene-specific	dominant	Shoji, T.		55 °C		Forward: ATCCGAGCCCGAGGAGGGTGG Reverse: CGTGAATTATGACGTATCCCTG	Shoji, T., *et al.*, 2010, The Plant Cell, 22:3390-3409
Black root rot resistance *(ex N. debneyi) Thielaviopsis basicola*		RAPD	closely linked	dominant	Bai, D.		35 °C		UBC83: GGGCTCGTGG UBC418: GAGGAAGCTT	Bai, D., *et al.*, 1995, Theor Appl Genet 91:1184-1189
Black root rot resistance *(ex N. debneyi) Thielaviopsis basicola*		SCAR	1 cM	dominant	Julio, E.		62 °C	temperature critical - bands for all genotypes at 62 °C, better separation at 59 °C	Forward: TAACAGCCTAACCCTATTCC Reverse: AATTCAAGGGGTAAAGCTATTC	Julio, E., *et al.*, 2006, Theor Appl Genet 112:335-346
Black root rot resistance *(ex N. debneyi) Thielaviopsis basicola SS192650*	*BRR1*	CAPS	closely linked	co-dominant	Qin, Q.				Forward: GCATTAACTGATTAATGGGAAGGACCA Reverse: GTCCAACAAGTGAGTTCATCATTGGAT	Qin, Q., *et al.*, 2018, Mol Breed 38:76
Black shank race 0 resistance *(ex N. longiflora) Phytophthora nicotianae*	*Phl*	SCAR	closely linked	dominant	Li, D.		55 °C		Forward: TTGGAGGACTTCGGAGAGTG Reverse: GAACATTCTGGGCCTCTTCA	unpublished, personal communication

Trait	Gene	Marker	Linkage	Dominance	Developed By	Enzyme	Annealing Temp	Comments	Primer Sequence or Associated Sequence for KASP design	Publication
Black shank race 0 resistance *(ex N. plumbag) Phytophthora nicotianae*	*Php*	RAPD	closely linked	dominant	Johnson, E.S.		40 °C	linked in coupling phase	TCCCATGCTG	Johnson, E.S., *et al.*, 2002, Plant Disease, 86:13031309
Black shank race 0 resistance *(ex N. plumbag) Phytophthora nicotianae*	*Php*	RAPD	closely linked	dominant	Johnson, E.S.		40 °C	linked in repulsion phase	CCAGGAGGAC	Johnson, E.S., *et al.*, 2002, Plant Disease, 86:13031309
Black Shank Race 0 Coupling Phase		SCAR	closely linked		Kudithipudi, C., Adams, A.		65 °C		Forward: TCCCATGCTGTCCCAATCCCAC Reverse: TCCCATGCTGCGATGTTGGA	unpublished, personal communication
Black Shank Race 0 and bacterial wilt resistance	*Phn7.1*	CAPS	12.6 cM	co-dominant	Shi, R.	MluCI	62 °C		SAR8.2s Forward: GTTTTCCCAGTCACGACCTATCAAAATGTTTTCCAAAACTA Reverse: CGGATAACTATTTCACACAGCATCAGCTTGTGAGGATATTAC	Shi, R., *et al.,* 2021, Molecular Plant Pathology, 20(8): 1051-1066
Blank Shank Race 0 resistance	*Ph (LG20)*	SCAR	closely linked	co-dominant	Bao, Y., *et al.* (2019)		51 °C		BS-SCAR1 (coupling phase) F- TTGGAGGACTTCGGAGAGTG R-GAACATTCTGGGCCTCTTCA PT52961 (repulsion phase) F-CTGCCTGAATTCATTGAAGAAC R-AGTTTCGCAACTTTGCCAGT	Bao, Y., *et al.*, 2019, Molecular Breeding, 39(9)
Brown spot (Alternaria) resistance *Alternaria alternata*		RAPD	closely linked	dominant	Zhang, H.Y.		37 °C		S361: CATTCGAGCC	Zhang, H.Y., *et al.*, 2008, African J of Biotech, 7(15):2559-2561

Trait	Gene	Marker	Linkage	Dominance	Developed By	Enzyme	Annealing Temp	Comments	Primer Sequence or Associated Sequence for KASP design	Publication
Cis-abienol synthase; diterpene synthase	*abs cps2*		gene-specific	dominant	Sallaud, C.				abs: Forward: CCATGGCGTTTCGACTTTTACGGA Reverse: GCCATGGCACAATGAGCAACGTCTGA cps2: Forward: GCGAATTCGAGGTCTTAATATGTGTTCAAT CCAGA Reverse: CGGGATCCTTTCTAATTTAATTTTTGTTTTA TTCTTC	Sallaud, C. *et al.*,2012, Plant J 2012, 72(1):1-17
Demethylase *e10*	*CYP82e10*	CAPS	gene-specific	co-dominant	Li, D.	*HphI*	54 °C		Forward: TGAAATATACAAGTAAGGTATAAAACACTA Reverse: TACCACTTCTATAGGATTTACCT	Li, D., *et al.*, 2012, Molecular Breeding, 29:589-599
Demethylase *e4*	*CYP82e4*	dCAPS	gene-specific	co-dominant	Li, D.	*DdeI*	52 °C		Forward: AGCAGACACAGTTGCTCTTCACATAACTT Reverse: CAACACAATCTTCTACATTTTCGTG	Li, D., *et al.*, 2012, Molecular Breeding, 29:589-599
Demethylase *e5*	*CYP82e5*	dCAPS	gene-specific	co-dominant	Li, D.	*HaeIII*	54 °C		Forward: AGAAAGCACAAGAAGAGATCGATAA Reverse: TCTCTCTGGATCAAACTTATCAGGATTTGG	Li, D., *et al.*, 2012, Molecular Breeding, 29:589-599
Demethylase *e10*	*CYP82e10*	KASP	mutation specific	co-dominant	Pramod, S. & Adams, A.			KASP assays can be ordered from LGC genomics by providing the sequence	CTGGGAAAAATTATGAATCCGGTAAAGGA GATGAACAAGTGGAGAGATTTAGGAAAG CGTTTAAGGATTTTATAATTTTATCAATGG AGTTTGTGTTATG[G/A]GATGCTTTTCCAA TTCCATTGTTCAAATGGGTGGATTTTCAAG GCCATGTTAAGGCCATGAAAAGGACATTT AAGGATATAGATTCTGTTTTTCAGAATT	unpublished, personal communication

Trait	Gene	Marker	Linkage	Dominance	Developed By	Enzyme	Annealing Temp	Comments	Primer Sequence or Associated Sequence for KASP design	Publication
Demethylase *e4*	*CYP82e4*	KASP	mutation specific	co-dominant	Pramod, S. & Adams, A.			KASP assays can be ordered from LGC genomics by providing the sequence	AAACAAGGTATGTGAATAATTGATATTCCT TTTTTAATTATTCTTTTTTCCAGAGTTTGGT CTTGGATGCAGCAGACACAGTTGCTCTTCA CATAAATTG[G/A]GGAATGGCATTATTGAT AAACAATCAAAAGGCCTTGACGAAAGCAC AAGAAGAGATAGACACAAAAGTTGGTAA GGACAGATGGGTAGAAGAGAGTGATA	unpublished, personal communication
Demethylase *e5*	*CYP82e5*	KASP	mutation specific	co-dominant	Pramod, S. & Adams, A.			KASP assays can be ordered from LGC genomics by providing the sequence	ATTGTAGGACTAGTAACCCTTACACTTCTC TTCTACTTCCTATGGCCCAAAAAATTTCAA ATACCTTCAAAACCATTACCACCGAAAATT CCCGGAGGGT[G/A]GCCGGTAATCGGCCA TCTTTTCTACTTCGATGATGACGGCGACGA CCGTCCATTAGCTCGAAAACTCGGAGACTT AGCTGACAAATACGGCCCGGTTTTC	unpublished, personal communication
EGY_2	*NtEGY2*		gene specific	dominant	Edwards, K.D., *et al.*			PCR EGY_2 region and then use EGY_2kasp to track the indel	EGY2-nF - GCAATCGTTGTCCAGTGTCTA EGY2-nR - TTTCCGACCTCTGTTACATCA	Edwards, K.D., *et al.*, 2017, BMC Genomics, 18:448
EGY_2kasp	*NtEGY2*	KASP	gene and mutation specific	co-dominant	Edwards, K.D., *et al.*			KASP assays can be ordered from LGC genomics by providing the sequence	GCTTAGTGTTGCAAATTGTACAGTCACTTA AAACATTCTGTTTGTGCTGCTATCAGCTTG GTGGACCTCT[T/]GTCACTTCCTTGGGGAT TGTATGTGCTTATATGTCAGGTAATAAAAA TACATTGCTTTATGAAGTATTATGCTTGTG TAATAATATCGCCGTCCTATTTCATGGTAT A	Edwards, K.D., *et al.*, 2017, BMC Genomics, 18:448
Low Alkaloid Production	*MYC2a*	CAPS	gene specific	co-dominant	Burner, N., *et al.* (2022)	Hpy188I	63 °C		Forward- GTTTTGGCCCGGAACAACTA Reverse- CTGAATAGCACATGAGCCCGA	Burner, N., *et al.*, 2022, Molecular Breeding, 42(1)

Trait	Gene	Marker	Linkage	Dominance	Developed By	Enzyme	Annealing Temp	Comments	Primer Sequence or Associated Sequence for KASP design	Publication
Orobanche ramosa resistance	*Nt14*	KASP	closely linked	dominant	Julio, E., *et al.* (2019)			KASP assays can be ordered from LGC genomics by providing the sequence	GAAGCAGTCAAATATCATATGTAAAAACTTG ATATAGAAGCATATAATGAAAAGCAAAACA AAAATCATTCAATACAAAATCGTGTTAATTC GTATCTCCTACCCTACGAAAATTGCCAATGC CTGTATAGACAATTGAGCAAAGACGAGAACA ATTAGAATCCGTCGTTTCCTCTAGATCCACCA TAGCCGCCACCATATCCGCCACCACCAGAACC AAAACCACCGCTGCG[A/T]GGAGCTCTCTCTT GAGCAAGACTGACGCGGATGTTCCTCCCGTT GAGTTCCTGTCCATCCATTGCTGTCATAGCTTC	Julio, E., *et al.*, 2019, Euphytica, 216(1)
Potyvirus susceptibility	*eiF4E1.S*		gene specific	dominant	Dluge, K.L., *et al.*				eIF4E_C_F: GGATCCACGAAAATGGCAGAGGAAGC, eIF4E_C_R: GAGCTCGCTAATGTCTATAAACTTTCCAGT CCA	Dluge, K.L., *et al.*, 2018, BMC Genomics 19:484
Powdery Mildew Resistance	*NtMLO1* *NtMLO2*	CAPS	closely linked	co-dominant	Komatsu, T., *et al.* (2020)	BfaI for NtMLO1 BseRI for NtMLO2	60 °C		NtMLO1 Forward: TTCAGAATTATATTCTTCCCTCCC Reverse: TGACAGTATTGGTAAAAAGTTTCTG NtMLO2 Forward: CCAGAGAGGTTCAGGTTTGCAAGAG Reverse: 5'-TTTTGAACCCCCTTCGTGAAGATCC-3'	Komatsu, T., *et al.*, 2020, Breeding Science, 70(4): 502-507
PVY resistance Potato virus Y	*va*	SCAR	5.1 cM	dominant	Julio, E.		62 °C		Forward: TTAACAACAGCTTTTAGCAGACAC Reverse: ACAACTGGCAAGCTAAGCTCATT	Julio, E., *et al.*, 2006, Theor Appl Genet 112:335-346

Trait	Gene	Marker	Linkage	Dominance	Developed By	Enzyme	Annealing Temp	Comments	Primer Sequence or Associated Sequence for KASP design	Publication
PVY Resistance Potato virus Y	*EIF4E1.S*	Bi-PASA	gene specific	co-dominant	Lin, S., *et al.*		58 °C		Outer Primer Pair (Wild-type allele) P: CAAGTACCCTTTTCCTACTAAAATCTATAACTAAG Q: GCCGGACAGAATTAGTGTCACATAAAATTGAAG ATTTTAC Inner Primer Pair (mutant allele) A: CGGCACTTTTTCCACTGTCGAAGATTTTAG B: GAATATGAAATAACTTACCCCCAAAAACT	Lin, S., *et al.*, 2021, Plant Breeding, 140(4):693-702
PVY Resistance Potato virus Y	*EIF4E-2*	RT-qPCR	closely linked	co-dominant	Michel, V., *et al.* 2020		57 °C		Forward: GCAAGTTAGTTACGGGAGCA Reverse: CCTTACCTCGGACATTAACT	Michel, V., *et al.*, 2019, Molecular Plant Pathology, 20(8): 47-58
PVY resistance Potato virus Y	*va*	RAPD	closely linked	dominant	Lewis, R.		40 °C		a cluster of RAPD primers	Lewis, R., *et al.*, 2005, Theor Appl Genet 110:678-687
N-Gene (*N. Glutinosa* Virus Resistance)	*N-gene*		gene-specific	dominant	Adams, A., Kudithipudi, C.		60 °C		Forward: AACAAGTATAACTTTTTATGCTCAAATCAG Reverse: CAGTGTCATTGTTCTAGTTCTCAATATACA	Whitham, S., *et al.*, 1994, Cell 78 (6), 1101-1115
TMV resistance Tobacco mosaic virus	*N*	SCAR	gene-specific	dominant	Lewis, R.		55 °C		Forward: ACCAGAATGATATGTTCCAC Reverse: GGACTCAACGTTAATTCTCTG	Lewis, R., *et al.*, 2005, Crop Science, 45:23552362

Trait	Gene	Marker	Linkage	Dominance	Developed By	Enzyme	Annealing Temp	Comments	Primer Sequence or Associated Sequence for KASP design	Publication
TSWV resistance Tomato spotted wilt virus		AFLP SCAR	<5cM	dominant	Moon, H.		57 °C(172) 57 °C(268) 50 °C(169) 55 °C(228)		Four AFLP markers (AAC/CCC172,ACG/CCG169,AAG/CGA22 8, ACT/CTA268) SCAR markers: 172 Forward: AGCTTCTTTTCTCTTTCCATTTTT 172 Reverse: CAGAAGAAAAACTGCTGGAGCTAT Forward: CTGATCGTTCCAGCAGGTTCTTAT Reverse: GGAGCTATTTCCAGACACGAA 169 Forward: ACTTTTCACACCAAAAACTCACG 169 Reverse: GATGATGATAAAGATTGAAGAAAACAA 228 Forward: TAGATGTCATGAATGGAACTACGG 228 Reverse: TTTTGATCGAAAAACCCAACC	Moon, H. and Nicholson, J.S., 2007, Crop Science 47:1887-1894
Wildfire (race 0) resistance *Pseudomonas syringae* pv. t*abaci* tox-		RAPD	closely linked	dominant	Yi, H.Y.		36 °C		OPW-10: TCGCATCCCT UBC-575: GGAGATGTAC	Yi, H.Y., *et al.*, 1998, Tobacco Science, 42:5257
Yellow Burley (Yb) trait Yb24-2	*YB1*	KASP	closely linked	co-dominant	Edwards, K.D., *et al.*			KASP assays can be ordered from LGC genomics by providing the sequence	GAATCCTATGCTGGGATTAAGCCAAGCTG GTTTAGCTGCATCGGGATTTAATCCTTCGT T[T/C]GTGGGGATTGGTGCTGGTTATGATA TAAATAGCATTAATCCGGGCGTTTTAGGTA GTAGT	Edwards, K.D., *et al.*, 2017, BMC Genomics, 18:448
Yb24-4	*YB1*	KASP	closely linked	co-dominant	Edwards, K.D., *et al.*			KASP assays can be ordered from LGC genomics by providing the sequence	GTGGAATCTAAGGAGGGTAAAGTGTAAG CAAGTTTATCCTTGTCCTAGAAAGGTAGAG AT[A/G]TTGTTTCCGATAGATCCTTGGCTA AAAAAACGCAGAAAAGAAGCAGTGTCAAC AAGTAAT	Edwards, K.D., *et al.*, 2017, BMC Genomics, 18:448

Trait	Gene	Marker	Linkage	Dominance	Developed By	Enzyme	Annealing Temp	Comments	Primer Sequence or Associated Sequence for KASP design	Publication
Yb24-6	*YB1*	KASP	closely linked	co-dominant	Edwards, K.D., *et al.*			KASP assays can be ordered from LGC genomics by providing the sequence	GCAATATAAAAGTCAATACTAGTCTTAACG GTGTGAGGCCTTTTGGAAAAACTTGTACG G[T/G]CTTGGTCCAAAACAGACAATATCAC ACCATTTAAGAGTATCTTTAGGCCGTTTTA GTCCG	Edwards, K.D., *et al.*, 2017, BMC Genomics, 18:448
Yb24-7	*YB1*	KASP	closely linked	co-dominant	Edwards, K.D., *et al.*			KASP assays can be ordered from LGC genomics by providing the sequence	GACGGTATGGTTAAAGATGTATATACGGA AGACTTCTCGATCACCATCGGAGAGAAAT GC[T/G]TATTTTTATGTCTCTTTTGGCCCAA TGGAGAGCCCTTATTTATAAATGTAGAAG ATTGAA	Edwards, K.D., *et al.*, 2017, BMC Genomics, 18:448
Yb5-1	*YB2*	KASP	closely linked	co-dominant	Edwards, K.D., *et al.*			KASP assays can be ordered from LGC genomics by providing the sequence	TTAATAATAGTTCCTTTTTGACCGCGGGTA CGAGGGCTAGCGTGAAATTGGCCACGAA GT[T/C]TGCCTAGATTTGAGACTTAATAGA CGTTCGGGGTTGATACTCAATGTCGTACCT ACTAAT	Edwards, K.D., *et al.*, 2017, BMC Genomics, 18:448
Yb5-4	*YB2*	KASP	closely linked	co-dominant	Edwards, K.D., *et al.*			KASP assays can be ordered from LGC genomics by providing the sequence	GACTACTATCATTGCCAGTCTCAACCTGTT CTGGTTCAAGCTTCAGTTCCACGTCCTCTC[T/C]ATCAGTCTCTGCAGGCTCAACTGTTTC GATCTCCTTGTGTTCTGGAACTTCTTTAGA ATC	Edwards, K.D., *et al.*, 2017, BMC Genomics, 18:448

1. SNP or INDEL (**In**sertion **Del**etion) markers in the form of KASP™ and Taqman™ are co-dominant and reliable, and can be used for tracking trait specific mutations or as closely linked markers. They are easy to use and efficient, especially for breeding programs handling large volume of samples.
2. The CAPS and dCAPS markers are also useful markers, as they are reliable, and co-dominant markers that can be gene-specific. They can be used only when there are different PCR products from the two parents (e.g. they cannot be used where one allele results from a deletion). It is more time consuming to go from DNA to results, when compared to KASP™ or Taqman™, making it inefficient for breeding programs handing large volume of samples:
3. The next most useful markers are closely-linked SCAR markers; they are reliable, but they are dominant markers and so less useful to breeders because they cannot identify heterozygotes. They are used when the sequence is known, but there is PCR product from only one parent. RAPD and AFLP markers are not ideal markers, but they can be used when the sequence information is unknown.
4. RAPD markers are unreliable because they are not always repeatable. However, the procedure is simple and uses reagents and equipment routinely found in marker laboratories.
5. AFLP markers are more reliable and give more repeatable results than RAPD markers, but they are not as convenient. The procedure is more expensive, takes longer and uses specialised equipment not routinely used in most marker laboratories.

Table S2: Molecular Markers in Tobacco – QTLs

Trait	Marker	Linkage	Dominance	Author	% Phenotypic Variation	Annealing Temp	Primer Sequence	Publication
Bacterial wilt resistance	AFLP	closely linked	dominant	Nishi, T.	30%		adaptor PstI: CTCGTAGACTGCGTACATGCA, CATCTGACGCATGT MseI: GACGATGAGTCCTGAG, TACTCAGGACTCAT amplification primer pp: GACTGCGTACATGCAGNNN, GATGAGTCCTGAGTAANNNN selective extensionof the amplication primer M17: PstI (AAA) MseI(CAGG); M84: PstI(CAG) MseI(CCTA)	Nishi, T., *et al.*, 2003, Theor Appl Genet, 106:765-770
Black shank race 1 resistance	SSR	LG 4	co-dominant	Vontimitta, V.	25%	55 °C	PT61373: Forward- GCGGGATAAACATGGGTAAA; Reverse- ACCCAAATAACCGCTCACAT PT51164: Forward- CAAGGACCACTCCAATGCTT; Reverse- TTCCAATGTTGTTTCTGTGTCTG	Vontimitta, V., *et al.*, 2012, Mol Breeding, 29:89-98
Increased leaf number	AFLP	3.1cM		Lewis, R.	increase 7-8 leaves		M1 = AFLP marker EACT/MCCT-130	Lewis *et al.* 2007b, Theor Appl Genet,114:842-854
Delayed flowering time	AFLP	3.3cM		Lewis, R.	8 days		M1 = AFLP marker EACT/MCCT-130	Lewis *et al.* 2007b, Theor Appl Genet, 114:842-854
Black shank race 1 resistance	SSR	LG 8	co-dominant	Vontimitta, V.	20%	55 °C	PT61472: Forward- TCCAATACCTTTAATGCATCTCC; Reverse- GCATGACATGTTGAAGTGGG PT30174: Forward- TGGTCGATCAACATGACAAA; Reverse- TCTAAATCACGCTGCATTGG	Vontimitta, V., *et al.*, 2012, Mol Breeding, 29:89-98