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One-step, codominant detection of imidazolinone resistance mutations in weedy rice (*Oryza sativa* L.)

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ABSTRACT

Background: Weedy rice (*Oryza sativa* L.) is a noxious form of cultivated rice (*O. sativa* L.) associated with intensive rice production and dry seeding. A cost-efficient strategy to control this weed is the Clearfield rice production system, which combines imidazolinone herbicides with mutant imidazolinone-resistant rice varieties. However, imidazolinone resistance mutations can be introgressed in weedy rice populations by natural outcrossing, reducing the life span of the Clearfield technology. Timely and accurate detection of imidazolinone resistance mutations in weedy rice may contribute to avoiding the multiplication and dispersion of resistant weeds and to protect the Clearfield system. Thus, highly sensitive and specific methods with high throughput and low cost are needed. KBioscience's Allele Specific PCR (KASP) is a codominant, competitive allele-specific PCR-based genotyping method. KASP enables both alleles to be detected in a single reaction in a closed-tube format. The aim of this work is to assess the suitability and validity of the KASP method for detection in weedy rice of the three imidazolinone resistance mutations reported to date in rice.
Results: Validation was carried out by determining the analytical performance of the new method and comparing it with conventional allele-specific PCR, when genotyping sets of cultivated and weedy rice samples. The conventional technique had a specificity of 0.97 and a sensibility of 0.95, whereas for the KASP method, both parameters were 1.00.
Conclusions: The new method has equal accuracy while being more informative and saving time and resources compared with conventional methods, which make it suitable for monitoring imidazolinone-resistant weedy rice in Clearfield rice fields.

1. Introduction

Weedy rice (WR, *Oryza sativa* L, also known as "red rice") is a troublesome weed of paddy rice worldwide, compromising crop productivity and quality [1]. WR infestations are becoming increasingly severe in association with the adoption of dry seeding in Asia and Europe [2,3] and reduced rotation in the South Cone of South America [4]. Because WR belongs to the same species as cultivated rice, its control by non-selective herbicides is inefficient [5]. The Clearfield rice production system emerged in the 2000s as an efficient strategy to cope with WR. This system uses broad-spectrum, imidazolinone herbicides (IMI) together with mutant rice cultivars resistant to IMI (CL). Thus, Clearfield technology enables selective chemical control of WR in rice cultivation. CL cultivars have point mutations in the gene for the acetolactate synthase enzyme (ALS; EC 2.2.1.6), which is the target of IMI. These mutations determine substitutions of amino acids located on the herbicide binding site, thereby generating resistance to IMI [6,7]. Current CL cultivars carry the nucleotide substitution or single nucleotide polymorphism (SNP) $G_{336}A$, $G_{1883}A$ or $G_{1886}A$, which determine amino acid substitution $A_{122}T$, $S_{653}D$ or $G_{654}E$, respectively. Mutation $S_{653}D$ is present in, among others, the variety CL161 and the hybrid cultivar AvaxíCL, whereas mutations $A_{122}T$ and $G_{654}E$ are present in the varieties INTA Puitá CL and IRGA 422 CL, respectively [4].

The benefit of the Clearfield technology for rice is limited by the appearance of IMI-resistant WR (RWR). It could originate from either spontaneous mutations in the weed population [8] or by gene flow from CL cultivars to WR [3,9,10]. Some WR plants can escape to IMI control due to climatic, operational and biological factors [11]. Outcrossing between CL cultivars and escaped WR plants occurs at

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variable rates depending on flowering synchrony, environmental conditions, WR biotype and CL cultivar [12,13]. The continuous use of IMI herbicides for several seasons selects RWR individuals and may lead to the replacement of susceptible alleles by IMI resistance alleles in the WR population, that is, genetic assimilation [14]. Once assimilated, IMI resistance alleles severely limit the efficacy of the Clearfield system in rice. Gene flow events have been reported as the main source of RWR in the US [9,15], Brazil [10], Italy [16], Greece [17] and Uruguay [4]. Therefore, it is recommended that the Clearfield rice production system should not be used for more than two consecutive seasons, and should be complemented by exclusive use of certified seed, prevention of WR seed set by rouging, and rotation with non-ALS herbicide applications [18]. Economic and cultural factors may conspire against the effective implementation of these recommendations [11], leading to the appearance and spreading of RWR. Weed population monitoring is part of an integrated strategy for herbicide resistance prevention and control [19]. The sustainability of herbicide-resistant crop production relies on a proactive management of herbicide resistance in weed populations [20]. This approach should involve the monitoring of WR populations for early detection of RWR to identify risky situations and take timely actions that prolong the life span of the Clearfield rice production system.

IMI resistance reported to date in WR is due to point mutations in the ALS gene [9]. Hence, the methods of choice for RWR detection are those which enable the identification of SNP in the ALS gene determining IMI resistance [21]. An allele-specific PCR (AS-PCR) was used by Kadaru et al. [22] to detect mutations $S_{653}D$ ($G_{1883}A$) and $G_{654}E$ ($G_{1886}A$) in CL cultivars and RWR from Arkansas, USA. A variation of this technique, called single nucleotide-amplified polymorphism (SNAP), uses the reaction that recognizes the resistance allele. Roso et al. [10] applied SNAP to survey $A_{122}T$ ($G_{336}A$), $S_{653}D$ and $G_{654}E$ mutations in 481 accessions of RWR from Rio Grande do Sul (Brazil). AS-PCR and SNAP have limitations for high-throughput routine detection of RWR. They require gel electrophoresis for detection, and AS-PCR involves two reactions for each determination. Both methods lack internal amplification control; therefore, amplification failures may lead to misgenotyping heterozygous as homozygous in the case of AS-PCR, or resistant samples as susceptible in the case of SNAP. Furthermore, the SNAP method does not enable codominant genotyping, that is, it does not differentiate between homozygous and heterozygous mutant genotypes. More recent studies [3,16,17] analyzed RWR populations collected from CL fields in Italy and Greece by sequencing the gene region containing the target SNP. Although this approach is the most appropriate to capture all possible SNP in the studied gene region, its suitability for real-time monitoring of WR populations is limited by its low throughput and relatively high cost. KBioscience's Allele Specific PCR (KASP, http://www.lgcgenomics.com/genotyping/kasp-genotyping-reagents/) was developed by KBioscience Laboratories, currently LGC Genomics (Hoddesdon, United Kingdom). It is a competitive AS-PCR for closed-tube codominant SNP genotyping, based on endpoint detection of allele-specific Förster resonance energy transfer (FRET) fluorescence signals. KASP has low cost, high throughput, and high specificity and sensitivity as has been demonstrated in massive SNP genotyping studies in biomedical research [23], genome-wide SNP platforms for rice genotyping [24] and molecular markers–assisted wheat breeding [25].

In this study, the KASP method was used for the first time to detect IMI resistance mutations in rice. The objective of this work was to validate the KASP method for the detection of mutations $A_{122}T$, $S_{653}D$ and $G_{654}E$ in WR and rice cultivars. Validation was achieved through three approaches: I) determination of the sensitivity and specificity of the KASP method when analyzing three panels of 96 known genotypes, one for each mutation; II) comparison of KASP and AS-PCR results when analyzing for mutation $S_{653}D$, a set of 96 samples of known genotypes; and III) comparison of KASP and AS-PCR results when applying both methods for analyzing for mutation $S_{653}D$, a set of 270 Uruguayan WR accessions of unknown genotype.

2. Materials and methods

2.1. Panels of samples

Four panels of rice DNA samples were used in this work. Conformation of panels A, B and C is detailed in Table 1. These panels were made of 96 samples of known genotype: 23 mutant homozygous DNA samples, 23 wild-type homozygous, 23 heterozygous and 3 controls without a DNA template (no template content, NTC).

Panel D was made of DNA samples extracted from 270 Uruguayan WR accessions of unknown genotype.

2.2. Approaches for validation of molecular methods

Three validation approaches (Table 2) were used in this study. Approach I consisted of determining the specificity (SP) and sensibility (SE) of the KASP method for mutations $A_{122}T$, $S_{653}D$ and $G_{654}E$ in panels A, B and C, respectively. SP and SE were calculated using Equation (1) and Equation (2), respectively, where TP = true positives, TN = true negatives, FP = false positives and FN = false negatives. A confusion matrix for classification of TP, TN, FP and FN is given in Table 3.

$$SP = \frac{TN}{TN + FP} \quad \text{[Equation 1]}$$

$$SE = \frac{TP}{TP + FN} \quad \text{[Equation 2]}$$

Table 1
Panels A, B and C for validation of molecular methods.

	Panel A		Panel B		Panel C	
Genotype	$A_{122}T$	*n*	$S_{653}D$	*n*	$G_{654}E$	*n*
Homozygous, mutant (AA)	INTA PuitáCL	30	CL161	30	IRGA 422CL	30
Heterozygous (AG)	F_1 SWR/INTA PuitáCL	15	F_1 SWR/CL161	10	F_1 SWR/IRGA 422CL	15
	1:1 DNA mix (INTA PuitáCL:INIA Olimar)	15	1:1 DNA mix (CL161:INIA Olimar)	10	1:1 DNA mix (IRGA 422CL:INIA Olimar)	15
Homozygous, wild type (GG)	CL161	7	INTA PuitáCL	7	CL161	7
	INIA Olimar	7	INIA Olimar	7	INIA Olimar	7
	IRGA 422CL	6	IRGA 422CL	6	INTA PuitáCL	6
	SWR	10	SWR	10	SWR	10
NTC	Sample without DNA	6	Sample without DNA	6	Sample without DNA	6
Total		**96**		**96**		**96**

INTA PuitáCL: CL variety, homozygous for $A_{122}T$ mutation; CL161: CL variety, homozygous for $S_{653}D$ mutation; IRGA 422CL: CL variety, homozygous for $G_{564}E$ mutation; AvaxíCL: CL hybrid cultivar, heterozygous for $S_{653}D$ mutation; INIA Olimar: conventional cultivar (IMI susceptible); SWR: IMI susceptible WR; WR: WR of unknown genotype; 1:1 DNA mixes: 1:1 vol/vol mixes of 100 ng/μL DNA extracted from INIA Olimar and from CL cultivars homozygous for each interrogated mutation.

Table 2
Approaches for validation of molecular methods used in this study.

Approach	Purpose	Validated method(s)	Interrogated mutation(s)	Panel(s) of samples analyzed
I	Determination of KASP SP and SE	KASP	$A_{122}T$, $S_{653}D$, $G_{654}E$	A, B, C
II	Comparison between SP and SE of KASP and AS-PCR	KASP AS-PCR	$S_{653}D$	B
III	Determination of agreement between KASP and AS-PCR	KASP AS-PCR	$S_{653}D$	D

SP: specificity; SE: sensibility.

Approach II consisted of comparing the analytical performances of KASP and AS-PCR methods when genotyping panel B in parallel. To that end, SP and SE were calculated.

Approach III used Cohen's kappa coefficient (κ, [26]) to determine agreements not attributable to chance between AS-PCR and KASP when analyzing panel D, in order to assess comparability between the results obtained by both methods.

2.3. Plant material

DNA for panels A, B and C were obtained from the following sources: a) mutant homozygous DNA was obtained from CL varieties carrying the interrogated mutation (INTA PuitáCL for panel A, CL161 for panel B, and IRGA 422CL for panel C); b) wild-type homozygous DNA was extracted from conventional rice cultivar INIA Olimar, IMI susceptible WR (SWR) and CL cultivars without the interrogated mutation; c) heterozygous DNA was obtained from F_1 plants from WR/CL artificial crosses, the IMI-resistant hybrid cultivar AvaxíCL (for panel B), or by mixing equal quantities of DNA from CL and conventional rice cultivars. SWR accessions were selected from seeds of WR plants collected in CL-free fields whose progeny was confirmed to be 100% susceptible to IMI. Samples for panel D were obtained from 270 WR plants collected from Uruguayan rice fields with two or more seasons of continuous cropping of the cultivar CL161.

Plant response to IMI was determined by treating 40 seedlings from each cultivar or WR accession with two lethal doses (200 g ha^{-1}) of IMI herbicide Ki + Fix (Imazapic 17.50% w/w, Imazapyr 52.50% w/w, nonionic surfactant Plurafac 0.25%, BASF Argentina, Buenos Aires, Argentina), at 15 and 30 d post-emergence.

2.4. DNA isolation

Genomic DNA was isolated from rice leaves according to Dellaporta et al. [27] with modifications. In a 1.2 mL tube, 0.1 g of leaf tissue was placed with a 5/32-inch (3.97 mm) steel ball. Tubes were immersed in liquid nitrogen and tissue was finely ground by intense shaking for 1 min. Five hundred microliters of 10% CTAB solution containing 0.2% β-mercaptoethanol was added and incubated at 65°C in a water bath for 20 min, shaking every 5 min. Five hundred microliters of chloroform/isoamyl alcohol 24:1 v/v was added and mixed by inversion. Tubes were centrifuged 20 min at 2,100 *g* (Sigma 2–16 P Centrifuge, Sartorius Stedim Biotech, Germany). The aqueous phase was transferred to a new tube and mixed with 300 μL of chilled isopropanol. Tubes were incubated at 4°C for 30 min and centrifuged at 2,128 *g* for 5 min. Isolated DNA was resuspended in 100 μL of sterile MilliRO water (Direct-Q 3, Millipore, US). DNA concentration was estimated by agarose gel quantification and roughly adjusted to 100 ng μL^{-1}.

2.5. Conventional AS-PCR

AS-PCR, as described by Kadaru et al. [22], was used to detect $S_{653}D$ mutation. Briefly, two reactions per analysis were performed. One reaction to determine the presence of the mutant allele (resistant reaction) and another reaction for the wild type (susceptible reaction). Each reaction contained 20 ng of rice DNA, 1x PCR buffer, 1 U of Taq DNA polymerase (Invitrogen), 2 mM $MgCl_2$ (Invitrogen), 0.4 mM dNTPs (Invitrogen), 0.4 μM reverse primer 5′TGGGTCATTCAGGTCAAACA3′ in both reactions, and 0.4 μM forward primer 1 5′GTGCTGCCTATGATCC TAAA3′ to detect the mutant allele in the resistant reaction, or 0.4 μM forward primer 2 5′GTGCTGCCTATGATCCTAAG3′ to detect the wild-type allele in the susceptible reaction. The PCR profile was 27 cycles of 15 s at 95°C, 15 s at 60°C and 15 s at 72°C, with a final elongation of 5 min at 72°C. The reaction was performed in a Palm-Cycler thermal cycler (Corbett Research, United Kingdom). The PCR products were detected by 2% agarose gel electrophoresis.

2.6. KASP method

The KASP method was used to detect mutations $A_{122}T$, $S_{653}D$ and $G_{654}E$. The KASP reaction and its components are described at http://www.lgcgenomics.com/genotyping/kasp-genotyping-reagents/how-does-kasp-work, and a brief depiction is given in Fig. 1. Sequences of allele-specific and common primers are listed in Table 4. Sequences from the 50 nucleotides flanking SNP were used for primer design using Kraken LIMS system software (LGC Genomics, Hoddesdon, United Kingdom). Five nanograms of genomic DNA template, 12 μM allele-specific forward primers, and 30 μM reverse primers were incorporated into the KASP reaction. Amplification was carried out in a Palm-Cycler thermal cycler (Corbett Research, United Kingdom), starting with 15 min at 94°C, a touchdown phase of 10 at 94°C for 10 s and at 65°C for 60 s with a 0.8°C decrease in temperature per cycle, followed by 40 cycles of 94°C for 10 s and 57°C for 60 s. Endpoint detection of fluorescence was performed in a PikoReal 96 Real-time PCR System (Thermo Scientific, Vantaa, Finland). Fluorescence signal was acquired at 520 nm (green) and 556 nm (yellow) for 2 min at 25°C.

Table 3
Confusion matrix for classification of genotyping results in approaches I and II.

	Genotyped as AA	Genotyped as AG	Genotyped as GG	Genotyped as NTC
Actual AA	TP	FN	FN	FN
Actual AG	FP	TP	FN	FN
Actual GG	FP	FP	TN	FN
Actual NTC	FP	FP	FP	TN

AA: mutant homozygous; AG: heterozygous; GG: wild-type homozygous; NTC: sample without DNA template.

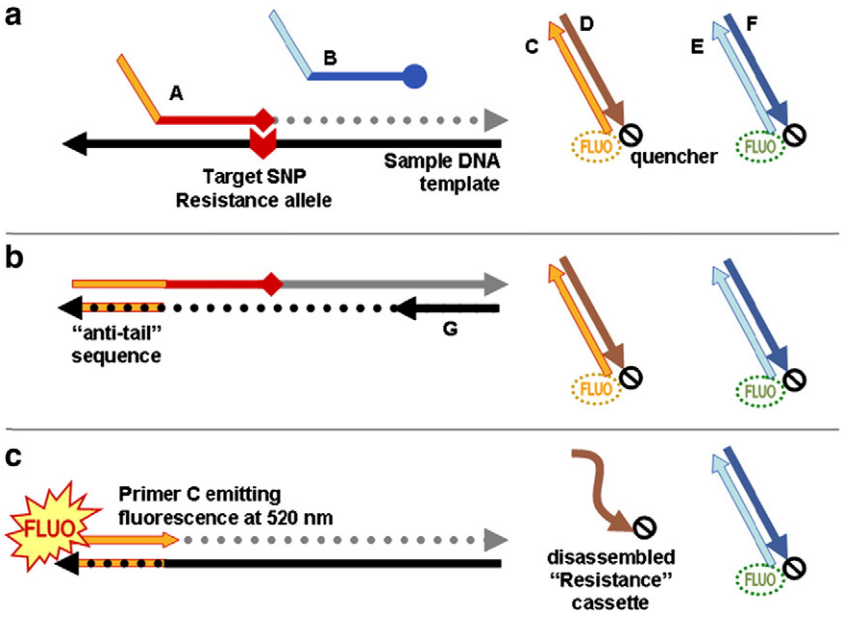

Fig. 1. Scheme of a KASP reaction for a mutant homozygous (resistant) sample. The following oligonucleotides are depicted (arrowheads correspond to 3′ ends): (A) 5′-tailed, unlabelled allele-specific primer that recognizes mutant (resistant) allele. (B) 5′-tailed, unlabelled allele-specific primer that recognizes wild-type (susceptible) allele. Allelic specificity of primers A and B are given by a single nucleotide substitution at 3′. Each primer has different 5′-tail sequence. (C) 5′ yellow fluorescence-labeled primer. Its sequence is identical to that of the 5′-tail of primer A. (D) Quenching oligonucleotide with a quencher molecule at 3′, complementary to C. Oligos C and D form "yellow" universal FRET cassette. (E) 5′ green fluorescence-labeled primer. Its sequence is identical to that of the 5′-tail of primer B. (F) Quenching oligonucleotide with a quencher molecule at 3′, complementary to E. Oligos E and F form "green" universal FRET cassette. (G) Reverse primer common to both alleles. The KASP mix is completed with usual PCR components, plus a third, orange fluorophore free in solution for normalization of fluorescence signals. KASP reaction proceeds as follows: (a) when no amplification product is present, FRET cassettes formed by oligos C and D and E and F are assembled and fluorescence stays quenched. (b) When specific amplification occurs, an anti-tail sequence is generated, complementary to the 5′-tail of primer A. (c) The anti-tail sequence anneals with primer C, disassembling the "yellow" FRET cassette. Then, yellow fluorescence corresponding to the resistant allele present in the sample is emitted, whereas "green" FRET cassette remains assembled and green fluorescence remains quenched. By contrast, wild-type homozygous (susceptible) samples would emit green fluorescence when amplified, whereas heterozygous samples would emit fluorescence at both wavelengths. Plotting of normalized fluorescence intensities at each fluorophore's emission wavelength enables allele calling and codominant genotyping.

3. Results and discussion

3.1. Approach I: KASP specificity and sensibility

KASP results for genotyping mutations $A_{122}T$, $S_{653}D$ and $G_{654}E$ in panels A, B and C, respectively, are shown in Fig. 2. Endpoint, normalized fluorescence intensities were plotted on X,Y graphs. The *x* axis corresponded to wild-type alleles with green fluorescence, and the *y* axis to mutant allele with yellow fluorescence. Samples were arranged in four clusters: wild-type homozygous genotypes were clustered near the *x* axis; mutant homozygous genotypes near the *y* axis; heterozygous genotypes in an intermediate area; and NTC samples fell near the origin of plot. All samples were correctly genotyped using the KASP method. Hence, its SE and SP were 1.00 for the three mutations. Two heterozygous samples from panel B (no. 40, F_1 SWR/CL61 and no. 53, AvaxíCL) were plotted slightly away from the heterozygous cluster. Also, heterozygous cluster in panel B is somewhat shifted toward the mutant homozygous group, presumably due to an imbalance in allele-specific primers concentration in the KASP mix. All this may lead to misgenotyping heterozygous samples as mutant homozygous. This can be solved by increasing the concentration of the primer specific to the wild-type allele, as suggested in the KASP troubleshooting guide at the manufacturer's web site.

Table 4
KASP primers for mutations $A_{122}T$, $S_{653}D$ and $G_{654}E$.

SNP	Tailed allele-specific primer (5′–3′)	Reverse primer
$A_{122}T$	(tail "A")-CCGTCCCGATGGTCGCC (tail "B")-CTCCGTCCCGATGGTCGCT	CGTCAGCGACGTGTTCGCCTA
$S_{653}D$	(tail "A")-CATGTGCTGCCTATGATCCCAAG (tail "B")-CATGTGCTGCCTATGATCCCAAA	GTCCTGCCATCACCATCCAGGAT
$G_{654}E$	(tail "A")-GCCTATGATCCCAAGTGG (tail "B")-GTGCTGCCTATGATCCCAAGTGA	GTCCTGCCATCACCATCCAGGAT
Tail "A"	GAAGGTGACCAAGTTCATGCT	
Tail "B"	GAAGGTCGGAGTCAACGGATT	

Nucleotide substitutions at 3′ in specific primers for mutant allele are underlined.

3.2. Approach II: analytical performances of KASP vs. AS-PCR

Analysis in parallel of panel B (96 samples of known genotype) for mutation $S_{635}D$ enabled comparison of KASP and AS-PCR. Analytical performances of both methods are shown in Table 5. Although no misgenotyping was observed in the results from KASP, AS-PCR had the following errors: sample no. 48 (1:1 DNA mix of CL161 with INIA Olimar) actually heterozygous, was genotyped as mutant homozygous; sample nos. 8 and 15 (CL161) actually mutant homozygous, as heterozygous; and sample no. 23 (CL161) actually mutant heterozygous, as no DNA template sample. Misgenotyping in sample nos. 48 and 23 may respond to amplification failures in the susceptible and resistant reaction, respectively, whereas errors in sample nos. 8 and 15 may be due to cross-contamination with susceptible DNA or, more likely, a slight lack of specificity in the allele recognition by the allele-specific primer of resistant reaction. However, errors found in AS-PCR genotyping do not have a significant effect on its SP and SE. Consequently, both methods showed high SP and SE.

3.3. Approach III: agreement between AS-PCR and KASP

Results for analysis of $S_{653}D$ mutation in panel D by AS-PCR and KASP were compared in a contingency matrix (Table 6). General agreement between both methods was $\kappa = 0.79$, with a standard error of 0.034 and a confidence interval of 0.73 to 0.86. Because the observed marginal frequencies limit the possible maximum agreement, the coefficient was

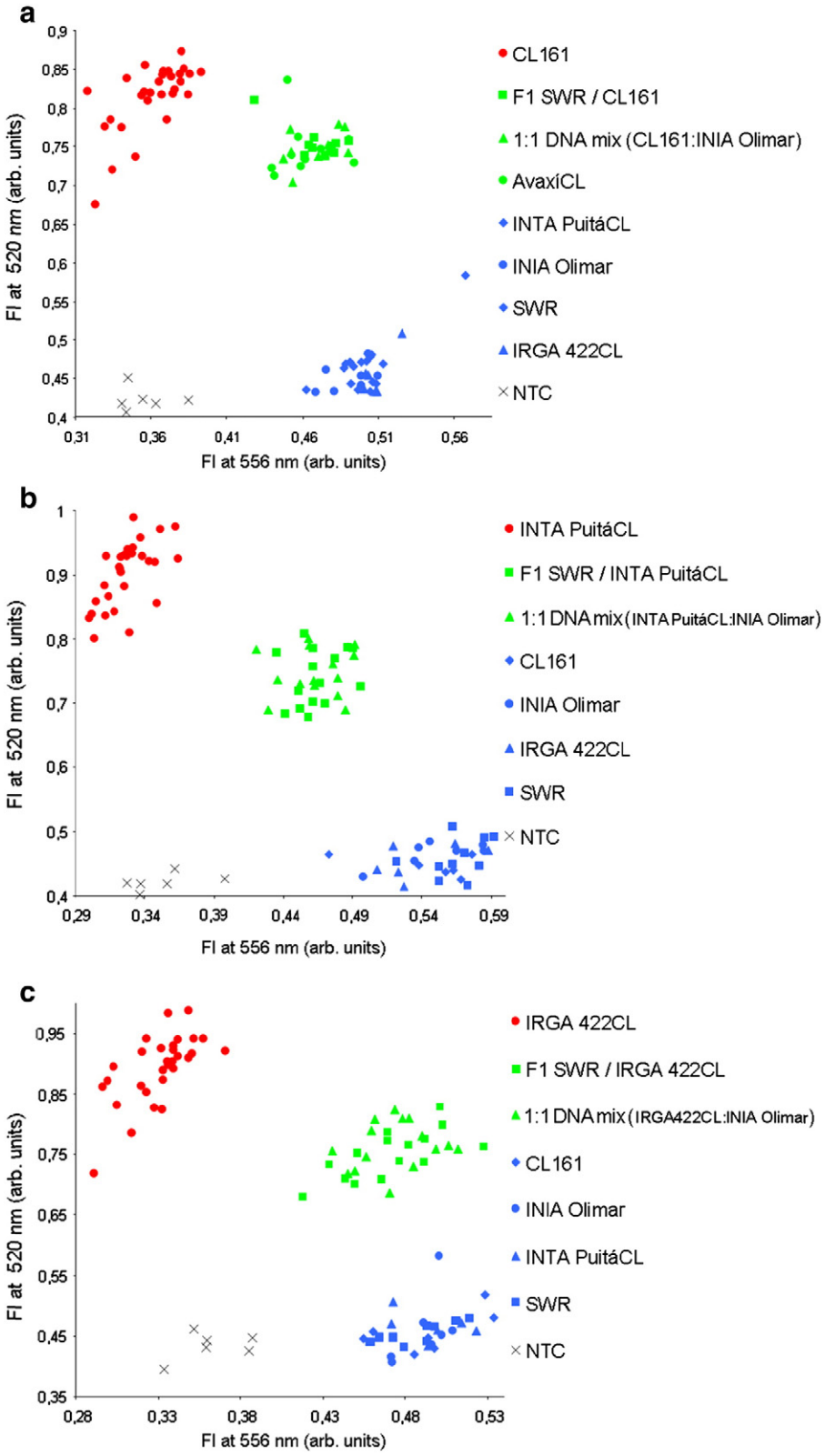

Fig. 2. Results of approach I: Genotyping by KASP. (a) Panel A for mutation $A_{122}T$; (b) panel B for mutation $S_{653}D$; and (c) panel C for mutation $G_{654}E$. Note: mutant homozygous samples are represented by red symbols, heterozygous by green symbols, and wild-type homozygous by blue symbols. Cultivars for each one of these categories may vary according to the interrogated mutation.

corrected based on this maximum [28]. Maximum agreement observed was 0.95; hence, the corrected agreement between the methods was $\kappa_M = 0.83$. This value can be considered according to Landis and Koch [29] as an "almost perfect" agreement.

In this work, molecular methods for detection of herbicide resistance mutations were validated for the first time by determining their SE and SP. The prevalence of resistant biotypes found when surveying post-herbicide treatment patches is usually low [30]. Thus, the ability

Table 5
Validation approach II: analytical performances of KASP and AS-PCR for mutation $S_{653}D$ when analyzing panel B made of 96 known genotypes.

	TP	TN	FP	FN	SP	SE
AS-PCR $S_{653}D$	56	36	1	3	0.97	0.95
KASP $S_{653}D$	60	36	0	0	1.00	1.00

to accurately identify rare mutant individuals is the overriding issue for the choice of technique when monitoring herbicide resistance in weed populations [31]. The high SE and SP found for KASP features it as a suitable method for RWR detection, particularly in populations with a low prevalence of mutants. As suggested by a possible cross-contamination for AS-PCR found in this study, open-tube formats are more prone to these kinds of analytical errors compared with homogeneous closed-tube methods [32]. This could lead to an operator- or laboratory-dependant rate of false-positive results. Hence, an inter-laboratory validation would be needed to compare methods and results from different countries or regions. However, the general good agreement found in this study between KASP and AS-PCR results may suggest an acceptable comparability of results obtained using both methods.

A limitation of the KASP method is that genotyping requires a minimum number of samples per run in order to get clear clusters (24 as suggested by the manufacturer). Nevertheless, due to the high number of samples expected in population surveillances, this may be a minor concern. Also, negative, positive and no-template controls (i.e. wild type, mutant and DNA-free samples, respectively) must be included in each run. In our experience, especially when working with low mutant allele frequency, at least three positive controls should be included. However, positive and negative controls should be used as well to validate each AS-PCR run.

The information provided by codominant genotyping methods is more informative and relevant than that by dominant markers. Because the Clearfield rice production system's usefulness is directly compromised by the genetic assimilation of IMI resistance alleles, WR population studies should comprise not only the distribution but also the zygosity of these alleles. Zhang et al. [15] reported IMI resistance as a dominant trait based on the analysis of ALS activity in F_1 CL/WR hybrids. On the contrary, Tan et al. [33] and Sala et al. [34] characterize this trait as semi-dominant. Likewise, Bond and Walker [35] found that hybrid CL cultivars were less tolerant to IMI than the homozygous variety CL161, and results from IMI dose–response studies in ALS extracted from homozygous and heterozygous CL cultivars also support semi-dominant IMI resistance. KASP codominant genotyping would be a useful complement tool for assessing the dependence of IMI resistance level on zygosity. Finally, knowing zygosity also enables us to infer the generation of the WR/CL progeny: mutant homozygous plants will indicate F_2 or more advanced generations. Analyses of WR/CL progenies showed low reproductive seed capacity for F_1 hybrids, but high fecundity levels in some F_2 individuals [36].

KASP was validated for the three currently reported mutations in CL rice thus far and found up to date in RWR accessions from field surveys [4]. With gene flow from CL cultivars being the primary cause of IMI resistance in WR [10], the vast majority of RWR can be identified by these validated assays. New assays can be readily developed and validated for the detection of other point mutations like $V_{669}M$ ($G_{1927}A$), associated with a decreased susceptibility to IMI in WR collected from Arkansas, United States [9], or new IMI resistance mutations being developed for CL.

Table 6
Validation approach III: contingency matrix comparing KASP with AS-PCR results for analysis of mutation $S_{653}D$ in panel D made of 270 unknown genotypes.

	KASP AA	KASP AG	KASP GG	Total
AS-PCR AA	**76**	7	3	*86*
AS-PCR AG	7	**127**	3	*137*
AS-PCR GG	4	10	**33**	*47*
Total	87	144	39	**270**

AA: mutant homozygous; AG: heterozygous; GG: wild-type homozygous. Concordant results are shown in bold characters, marginal values in italics.

This study demonstrated that KASP results are comparable with those obtained by conventional methods. In fact, KASP had SE and SP as high as those of AS-PCR, whereas enabling codominant genotyping in a single reaction. Equipment for KASP genotyping (a PCR machine with fluorescence detection capability) is on average about 2.5 times more expensive than for conventional PCR [37], but cost per determination is less than a half of that for conventional AS-PCR, and far below alternative methodologies like TaqMan or gene sequencing. Monitoring of RWR in paddy fields using Clearfield technology would require genotyping about 10 putative RWR plants per 100 ha^{-1}. By KASP genotyping, this could be done at 50 dollars ha^{-1}, 80% of which are for extra-laboratory logistics. Economical net returns from Clearfield rice production have been valued in 1,350 dollars ha^{-1} [38]. Thus, the method validated here for timely and accurate detection of RWR is a valuable and cost-effective tool for decision making in Clearfield rice management and regional surveillance of RWR in the framework of a sustainable use of this production system.

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