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Rapid in situ quantification of the strobilurin resistance mutation G143A in the wheat pathogen *Blumeria graminis* f. sp. *tritici*

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As the incidence of fungicide resistance in plant pathogens continues to increase, control of diseases and the management of resistance would be greatly aided by rapid diagnostic methods. Quantitative allele-specific PCR (ASqPCR) is an ideal technique for the in-field analysis of fungicide resistance as it can quantify the frequency of mutations in fungicide targets. We have applied this technique to the fungal pathogen *Blumeria graminis* f. sp. *tritici* (*Bgt*), the causal agent of wheat powdery mildew. In Australia, strobilurin-resistant *Bgt* was first discovered in 2016. Molecular analysis revealed a nucleotide transversion in the cytochrome *b* (*cytb*) gene in the cytochrome *bc1 enzyme complex*, resulting in a substitution of alanine for glycine at position 143 (G143A). We have developed an in-field ASqPCR assay that can quantify both the resistant (A143) and sensitive (G143) *cytb* alleles down to 1.67% in host and *Bgt* DNA mixtures, within 90 min of sample collection. The in situ analysis of samples collected during a survey in Tasmania revealed A143 frequencies ranging between 9–100%. Validation of the analysis with a newly developed laboratory based digital PCR assay found no significant differences between the two methods. We have successfully developed an in-field quantification method, for a strobilurin-resistant allele, by pairing the ASqPCR assay on a lightweight qPCR instrument with a quick DNA extraction method. The deployment of these type of methodologies in the field can contribute to the effective in-season management of fungicide resistance.

One of the major challenges that the agricultural industry faces is the control of fungal crop diseases during the emergence of fungicide resistance[1–4]. The presence of fungicide resistant pathogen populations in a crop, not only results in lower efficacy of chemical control methods but also, lower yields and quality due to increased disease pressure[5]. In addition, unnecessary treatment with inefficacious products would represent an additional cost to the grower and contribute to adverse environmental effects[6]. An optimal approach to combat this would be to determine what chemistries the populations are resistant to and use other, more efficacious chemicals instead[2].

Many cases of fungicide resistance have been functionally linked to specific genotype changes in target genes[4,7,8]. The detection of these genotypic changes is a valuable complement to the phenotypic screening of suspect pathogen isolates. Phenotypic screening requires the isolation of numerous pure cultures of the pathogen and testing for quantitative growth and/or germination inhibition. Such assays are very demanding of space, resources and time, taking many days or weeks. The potential of screening using genotypic methods has been recognised for more than 25 years[9,10]. A wide range of techniques have been used but all are limited by the need to isolate pure strains of the pathogen and to carry out the analyses in a laboratory environment. As such the minimum time for such an assay is still several days. Therefore, the time-frame within which to spray a fungicide can be lost by the time the samples are sent for analysis to a laboratory and results received. In addition, all the assays whether phenotypic or genotypic, which require the establishment of pure strains would induce a bias as this strain may not be representative of the entire population causing the infection[11,12].

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Other methodologies such as loop-mediated isothermal amplification (LAMP) are rapid and can be used with infected plant samples[13–15]. Based on this, LAMP has been used for the successful in-field and laboratory detection of different organisms including chemical resistance in fungi and weeds[16–21]. However, the results from this test are not quantitative and prone to false positives[22,23].

An ideal solution is an assay which can be performed rapidly, in situ, to quantify the population of fungal pathogens, that are resistant to a given fungicide, with high confidence results. This would prevent the spraying of a fungicide to which a fungus is resistant, as that would both fail to control the disease and increase the selection of fungicide resistant populations[24]. A rapid assay diagnosing the disease, its quantity and fungicide sensitivity status would allow farmers to select the optimum fungicide regime for on-going protection of the crop.

Allele-specific polymerase chain reaction (ASPCR)[25] is a specific and sensitive assay that was developed for detecting point mutations using PCR followed by gel electrophoresis. This method has been adapted for use with both intercalating dyes and probe-based assays to quantify fungi with genetic changes correlated with fungicide resistance[26–28]. If combined with crude DNA extraction and a 'fast' and robust polymerase that can tolerate field conditions, this assay could be conducted in the field with minimal laboratory equipment.

Blumeria graminis [DC.] E.O. Speer f. sp. *tritici* Em. Marchal (synonym *Erysiphe graminis* DC) (*Bgt*) is an obligate biotrophic pathogen of wheat (*Triticum aestivum* L.) causing wheat powdery mildew (WPM)[29]. *Bgt* can be controlled with strobilurins [quinone outside inhibitors (QoI)], which target the cytochrome bc_1 enzyme (*cytb*) complex, in regions where resistance is not present or at low levels[30,31]. The selection of genotypes resistant to this group of chemicals is facilitated by the combination of *Bgt*'s large population size and its intensive control using fungicides. Strobilurin-resistant genotypes of *Bgt* were first discovered in 1998 in Germany, just 2 years after their introduction for the control of various crop diseases, and have since been reported in wheat growing regions around the world[30,32,33]. Globally, strobilurins make up 20–25% of fungicide sales of which azoxystrobin represents a third and is the world's largest selling fungicide[34]. The development of resistance to strobilurins not only threatens effective crop protection, but also reduces the number of mode of actions (MOAs), available to growers, contributing to the increase of fungicide resistance risk on those systems[5].

Sequencing of the *cytb* gene from *Bgt* showed that a single nucleotide polymorphism (SNP) of a guanidine to cytosine at position 428 (*g428c*), was the only difference between the sensitive and resistant isolates[30]. This mutation results in the amino acid change of glycine to alanine at position 143 (G143A) in the Cytb protein, (homologous to the archetypal *G143A* mutation in the Cytb of *Zymoseptoria tritici*)[30,35]. However, due to *cytb* being a multi-copy mitochondrial gene, it can exhibit heteroplasmy whereby within any given fungal cell, the SNP maybe present only in a few copies within a mitochondrion and/or a few of these organelles[36]. Any fungal cultures with 100% abundance of the *cytb* G143 or A143 allele are referred to as homoplasmic cultures[26].

In Australia, over 10 Mha are sown with wheat annually, thus providing a large breeding ground for this pathogen[37]. However, due to the range of climatic zones within the Australian wheat belt, mildew infections are most common in areas with medium to high rainfall, as cool humid conditions are required for optimal disease development. In the regions of Australia where WPM is prevalent, it can cause up to 25% yield losses due to a reduction of photosynthetic leaf area and nutrients available to the crop[38]. The analysis of the cost of WPM to the Australian wheat industry, for production in the 1990s and 2000s, shows that in the absence of control losses of up to AU$18 million/year can be expected[38]. However, due to control measures these losses are currently at AU$3 million/year[38]. Of the AU$15 million in mitigated losses, use of chemical control attributes to one-third (AU$5 million) of the reduction in potential losses due to mildew[38]. The first report of strobilurin resistance in Australian *Bgt*, attributed to field failure observations, was from the states of Victoria and Tasmania in 2016[39].

Here we report the development of a quantitative allele-specific PCR (ASqPCR), performed on a portable thermal cycler, which can be powered by a battery, coupled with a simple 2-step DNA extraction protocol. The method successfully generated accurate genotype frequencies of the *Bgt* Cytb G143A fungicide resistance mutation, on-site, within 90 min of sample collection. To our knowledge, this is the first in situ 'closed tube' allele quantification in a plant pathogen.

Results

Identification of the G143A mutation in the CytB protein. A 465 bp PCR amplicon of the *cytb* gene was amplified from one barley powdery mildew and 10 WPM samples, and sequenced as described in the "Materials and methods" (Tables 1 and 2). The alignment of these sequences together with QoI-sensitive and resistant *Bgt cytb* reference sequences, revealed the presence of the mutation *g428c* (Cytb amino acid substitution G143A) only in the samples which had been collected from paddocks where the use of QoI fungicides had failed to control the disease i.e. two samples each from Tasmania and Victoria (Fig. 1). No mutations in the *cytb* gene were found in any of the remaining samples, which agreed with the successful control of WPM with QoI-containing fungicides in the paddocks where these samples had been collected. The analysis of the chromatograms also shows that for the Victorian samples Geelong 1 and 2 there was only a peak for a C base at the mutation site, whereas for the Tasmanian samples Launceston 1 and 2, there was an additional smaller peak for a G base. All samples analysed from Western Australia and South Australia were wild-type.

dPCR and ASqPCR successfully quantify *Bgt cytb g428c*.

The dPCR analysis successfully quantified the A143 allele in total DNA from field samples collected in 2016 corroborating the results from the sequencing of the *cytb* gene (Tables 1 and 3; Fig. 1). To evaluate the accuracy and sensitivity of the assay, genomic DNA from infected leaves with *Bgt* from Goomalling (100% G143; QoI sensitive) and Geelong 1 (100% A143; QoI resistant), were mixed in known allelic ratios and subjected to dPCR analysis. Scatter plot representation of the results showed well-defined, discrete groups of either wild type (G143) or mutant (A143) allele only, both alleles and neither allele present (Fig. 2a). The analysis of the dilution series indicated a high correlation between percentage of A143 allele detected and known G143/A143 allele

Year	Town/region, state[a]	No of samples	Cultivar[b]	A143 frequency (%)[c]
2016	Arthur River, WA	1	–	0
	Geraldton, WA	1	Wyalkatchem	0
	Goomalling, WA	1	Zen	0
	Moora, WA	1	Corrack	0
	Neuton, SA	2	–	0
	Launceston, TAS	1	Golden Promise[d]	0
	Launceston, TAS	2	Mackellar, Forrest	92.66, 93.05
	Geelong, VIC	2	Gazelle	99.96, 99.73
2018	Table Cape site 1, TAS	6	–	50.51 ± 22.30
	Table Cape site 2, TAS	6	–	44.14 ± 30.17
	Conara site 1, TAS	5	Manning	98.05 ± 2.29
	Conara site 2, TAS	5	Trojan	93.39 ± 6.78
2019	Northern Yorke Peninsula, SA	12	Chief	0
			Chief	0
			Trojan	0
			Sceptre	1.94
			Chief	2.34
			–	3.02
			–	6.67
			Chief	7.22
			Sceptre	27.79
			Sceptre	33.50
			Chief	34.01
			Chief	53.72

Table 1. Sample details and *cytb* A143 allele frequency of *Blumeria graminis* f. sp. *tritici* from infected wheat collected in this study. [a] *WA* Western Australia, *SA* South Australia, *TAS* Tasmania, *VIC* Victoria. [b] Unknown cultivars are denoted as –. [c] All values as per dPCR analysis except the 2019 samples which are from ASqPCR. For 2018 samples, the values represent averages (± s.d.) per site. [d] Barley sample.

Primer name[a]	Sequence (5′–3′)	Product size (bp)	Anneal temperature (°C)	Purpose	Source
BgCytB_G143A_F	CTGCATTCCTGG GTTATGTATTGC	86	See below	dPCR/qPCR primers	This study
BgCytB_G143A_R	TGGTATAGCGCT CATTAGGTTAGT GA				
BgCytB_G143A_dP1	*FAM*-CGGTTG CA**G**CCCAGT G-*NFQ*	-	58	dPCR probes	This study
BgCytB_G143A_dP2	*VIC*-ACGGTTGCA **C**CCCAGTG-*NFQ*				
BgCytB_G143A_mP1	*FAM*-CGGT̲TGCA **G**CCC̲AGTG-*BHQ1*	-	67	qPCR probes	This study
BgCytB_G143A_mP2	*HEX*-ACGGT̲TGCA **C**CCC̲AGTG-*BHQ1*				
WM-Cb-f	GCGGGATGTTAA TAACGGATGAT	465	55	Sequencing primers	Bäumler et al. (2003)
WM-Cb-R	GGAGCCATAGGT AATCTATCG				

Table 2. PCR, Digital PCR and qPCR primers and probes used in this study. [a] The probes dP1 and mP1 target the *cytb* A143 allele whereas probes dP2 and mP2 target the G143 wild-type allele. Italics—fluorophore/quencher; bold—single nucleotide polymorphism; underlined—locked nucleic acid.

ratios ($R^2 = 0.999$), with the lower level of quantification of the mutant allele determined to be 0.33%, and the allele detectable at 0.07% (Table 4; Supplementary Fig. S1). No cross-reaction was found when genomic DNA from other common wheat pathogens was tested, however, the closely related barley powdery mildew pathogen *Blumeria graminis* f. sp. *hordei* was positive for the G143 allele (Table 3).
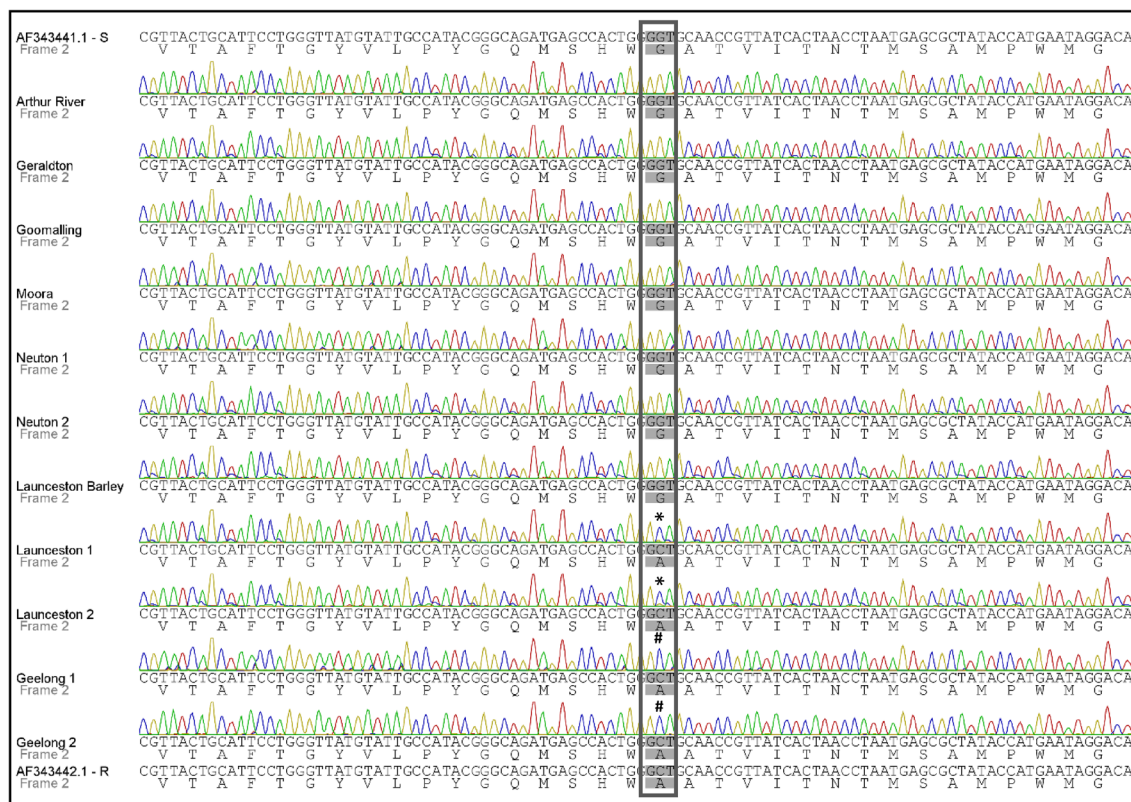
Figure 1. Multiple sequence alignment of the *cytb* gene sequence and its translation (yeast mitochondrial translation table_3) flanking the *g428c* mutation for samples collected in 2016 (Table 1). AF343441.1 and AF343442.1 correspond to strobilurin sensitive and resistant *Bgt cytb* reference sequences, respectively. The samples Launceston 1 and 2, and Geelong 1 and 2 contain the substitution G143A. Codon 143 is boxed; * denotes a mix of the G and C base; # shows C base only. Arthur River, Geraldton, Goomalling and Moora—Western Australia; Neuton—South Australia; Launceston—Tasmania; Geelong—Victoria.

DNA sample[a]	% A143	CI % A143[b]	Copies μL⁻¹ G143	CI copies μL⁻¹ G143	Copies μL⁻¹ A143	CI copies μL⁻¹ A143
Arthur river, WA	0	–	3231	3165.80–3297.60	0	–
Moora, WA	0.04	1.59E-2–7.75E-2	1275.50	1250.70–1300.80	0.45	0.20–1.01
Geraldton, WA	0.02	5.17E-3–7.08E-2	826.48	807.76–845.63	0.16	4.01E-2–0.64
Goomalling, WA	0	–	1524.60	1495.90–1553.70	0	–
Neuton 1, SA	0	–	401.88	389.95–414.18	0	–
Neuton 2, SA	0.01	2.50E-3–8.30E-2	530.47	516.65–544.67	7.77E−02	1.09E-2–0.55
Launceston 1, Tas	92.66	88.80–96.56	94.07	88.90–99.54	1187.30	1163.80–1211.40
Launceston 2, Tas	93.05	89.06–97.12	50.08	46.32–54.15	670.21	654.18–686.64
Geelong 1, Vic	99.96	95.74–104.24	0.42	0.17–1.00	1170.10	1145.60–1195.20
Geelong 2, Vic	99.73	94.51–105.16	0.80	0.43–1.49	300.65	290.60–311.05
Blumeria graminis f. sp. *hordei*	0	–	7547.60	7214.10–7896.60	0	–
Parastagonospora nodorum[c]	6.64	1.12–39.51	1.36	0.80–2.29	9.68E−02	1.36E-2–0.69
Wheat leaf rust [c]	23.02	10.77–48.40	2.14	1.47–3.13	0.64	0.32–1.27
Wheat leaf (uninfected) [c]	74.29	18.75–299.31	0.09	1.24E-2–0.62	0.26	8.51E-2–0.82
Water[c]	50	6.53–381.59	7.37E−02	1.04E-2–0.52	7.37E−02	1.04E-2–0.52

Table 3. Specificity of detection of the G143A dPCR assay on total DNA from *Blumeria graminis* f. sp. *tritici* infected wheat leaf field samples and controls. [a]*WA* Western Australia, *SA* South Australia, *Tas* Tasmania, *Vic* Victoria. [b]CI—95% confidence interval. [c]Total copies μL⁻¹ (copies μL⁻¹ G143 + Copies μL⁻¹ A143) must be over 200 for a valid result.
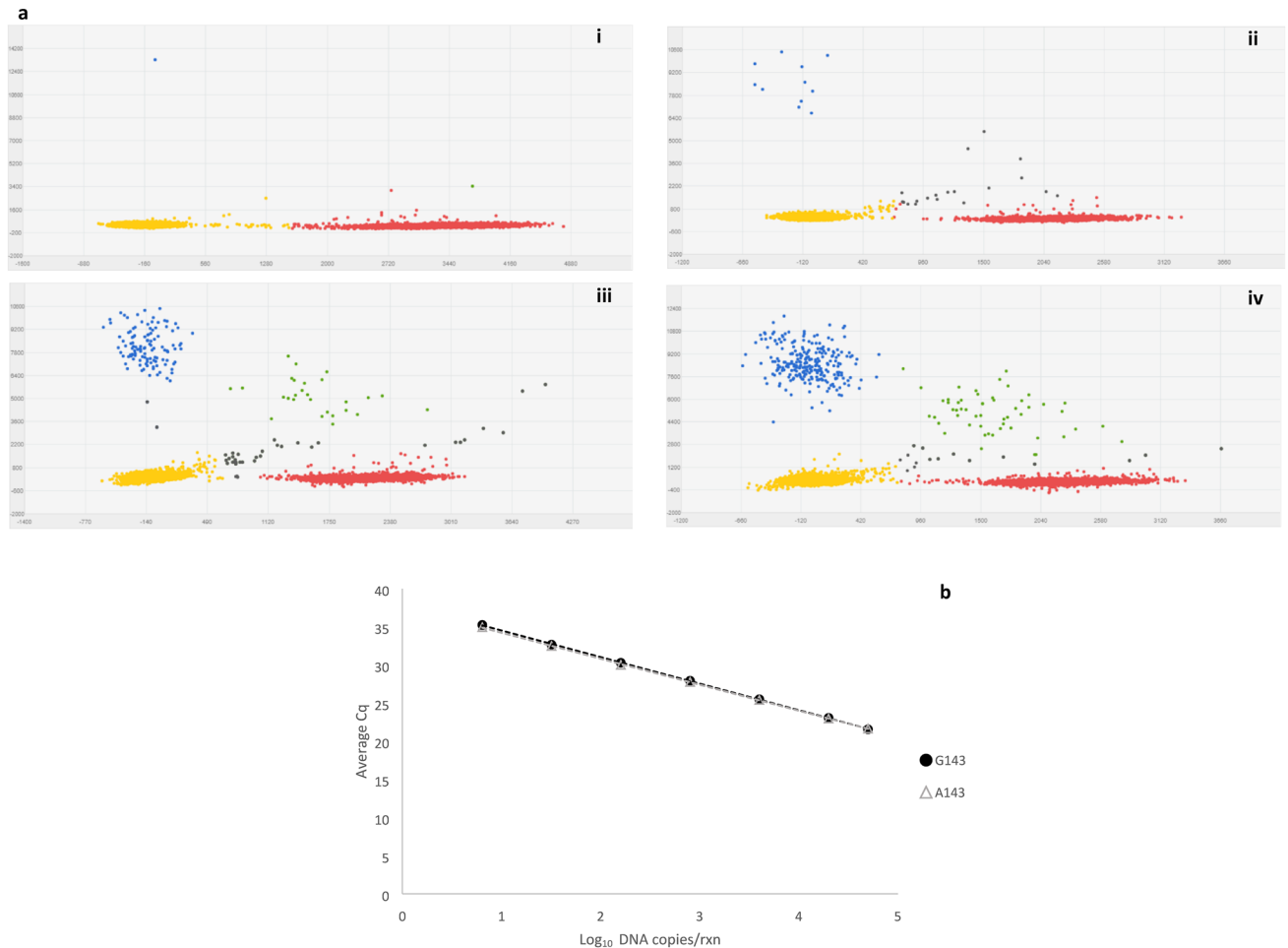
Figure 2. Sensitivity and specificity of the dPCR and ASqPCR assays targeting the *cytb* G143A. (**a**) Scatter plots for *Blumeria graminis* f. sp *tritici* (*Bgt*) G143A dPCR assay using known ratios of genomic DNA for the strobilurin resistant *Bgt* sample Geelong 1 (A143 allele) and sensitive *Bgt* sample Goomalling (G143 allele). Scatter plots were prepared with Quantstudio 3D AnalysisSuite. Wells with the A143 allele are represented by blue signals (FAM), G143 alleles are represented by red signals (VIC), detection of both alleles are represented by green signals, wells without any alleles (passive reference) are represented by yellow signals (ROX) and outliers represented by grey signals. (i) 100% Goomalling gDNA (G143 allele only); A143 frequency = 0.04%. (ii) 0.33% Geelong1 gDNA; A143 frequency = 0.23%. (iii) 3.39% Geelong 1 gDNA; A143 frequency = 3.10%. (iv) 6.90% Geelong 1 gDNA; A143 frequency = 7.87%. (**b**) Correlation of threshold cycle in ASqPCR and amount of DNA of either G143 ($R^2 = 0.9998$) or A143 ($R^2 = 0.9999$) alleles, respectively.

The assay identified very high frequencies of the A143 allele in locations where QoI control failure had previously been reported (Tables 1 and 3). Where QoIs controlled the disease satisfactorily, the frequency of the A143 allele remained below the assay detection level. The double peaks observed in the sequencing chromatograms of samples Launceston 1 and 2 were in agreement with the A143 allele frequencies determined by the dPCR assay (92.66 and 93.05%, respectively). In a similar manner, samples Geelong 1 and 2 showed A143 frequencies of virtually 100%, which correlated with the single peaks observed in the chromatograms of these two samples (Fig. 1; Table 3).

The SensiFast Probe No-Rox master mix was considered the most suitable for ASqPCR with crude DNA samples on a magnetic induction cycler (MIC) qPCR instrument (Bio Molecular Systems, Australia) under field conditions. In the reactions where the iQ multiplex Supermix was used, the G143 allele was detected in both the HEX and FAM channels. In the case of the samples analysed using the Immomix, no amplification was observed from any of the crude DNA extracts (Table 5).

The test for the interassay repeatability showed that the frequency of the A143 allele, for the 51% A143 sample, when calculated from the standard curves for both tests had a mean of 52.9% (n = 16; CV = 1.2%; variation from theoretical value = 3.7%). Subsequent analysis of the dilution series, with ASqPCR showed that the lower level of quantification of the mutant allele in mixture with the wild type was in this case 1.67% (Table 4; Supplementary Fig. S1). In addition, in homoplasmic reactions, the assay quantified down to seven copies per reaction for both G143 and the A143 alleles (Fig. 2b). As with the dPCR assay, wheat pathogens *Parastagonospora nodorum* and *Pyrenophora tritici-repentis* were not detected. The assay however detected the closely related barley mildew *B. graminis* f. sp. *hordei*, but not the grape mildew *Erysiphe necator* (data not shown).

DNA sample[a]	dPCR		ASqPCR	
	% A143	CI % A143[b]	% A143	CI % A143
100% Goo	0.04	1.19E-2–0.16	0	–
0.07% Gee 1	0.30	0.18–0.51	0	–
0.17% Gee 1	0.10	4.41E-2–0.25	0	–
0.33% Gee 1	0.23	0.13–0.42	0	–
0.67% Gee 1	0.57	0.37–0.88	0	–
1.68% Gee 1	2.01	1.63–2.48	1.48	1.32–1.64
3.39% Gee 1	3.10	2.61–3.68	4.06	3.82–4.30
6.90% Gee 1	7.87	6.98–8.87	8.00	7.22–8.77
18.18% Gee 1	21.72	19.98–23.59	20.24	19.96–20.51
40% Gee 1	38.45	36.00–41.04	NA[c]	–
66.67% Gee 1	65.15	61.41–69.05	68.40	67.90–68.89
100% Gee 1	99.67	94.43–105.11	100	–

Table 4. Sensitivity of the G143A dPCR and ASqPCR assays on a mixture of total DNA of leaves infected with mutant (A143) and wild type (G143) *Blumeria graminis* f. sp. *tritici*. [a]*Goo* Goomalling (G143), *Gee1* Geelong 1 (A143). [b]*CI* 95% confidence interval. [c]This concentration was not tested with ASqPCR.

|	G143 target (HEX channel)			A143 target (FAM channel)		
	Mix 1	Mix 2	Mix 3	Mix 1	Mix 2	Mix 3
100% G143 (L)[a]	19.02 ± 0.50[d]	19.22 ± 0.50	34.25 ± 1.21	**25.65 ± 0.34**	–	–
100% G143 (Q)[b]	23.79 ± 0.56	24.49 ± 0.44	–	–	–	–
100% A143 (L)	–	–	–	18.39 ± 0.40	18.64 ± 0.35	32.52 ± 0.22
100% A143 (Q)	–	–	–	23.43 ± 0.25	23.89 ± 0.22	–
51% A143 (L)[c]	23.53 ± 0.63	23.78 ± 0.65	38.24 ± 0.09	21.60 ± 0.09	21.99 ± 0.15	36.73 ± 0.27
NTC	–	–	–	–	–	–

Table 5. Cq values obtained from the assessment of three qPCR mastermixes. Mix 1 (iQ Multiplex Supermix), Mix 2 (Sensifast Probe No-Rox mix) and Mix 3 (Immomix), with total DNA of leaves infected with wild type (G143) and mutant (A143) wheat powdery mildew. Non-target amplification is indicated in bold. [a]DNA extracted using a standard laboratory method. [b]DNA extracted using the quick field extraction method. [c]49% G143. [d]s.d. (5 replicates).

Optimisation of the in-field allele frequency quantification pipeline. The suitability of the in-field *Bgt cytb* A143 allele quantification method was assessed as part of a two-day field trip across the Northern wheat-growing region of Tasmania (Fig. 3). The ASqPCR assay conducted on the MIC qPCR instrument (Bio Molecular Systems), with crude DNA extracted in the field as described in the "Materials and methods", successfully distinguished between G143 and A143 alleles under field conditions. The resistant genotype could be detected in all of the 22 samples tested across the four collection sites, albeit at varying levels (Fig. 4a).

The largest variation in the frequency of the *cytb* mutant allele was observed in the samples collected on day 1. Frequencies of the A143 allele in the Table Cape region ranged from 27.3–86.0% (average = 52.3% ± 19.7) in the first site (Fig. 4a, 1A–1F) and from 9.0–94.0% (average = 40.6 ± 30.7) in the second site (Fig. 4a, 2A–2F). The analysis of the samples collected at the two sites in the Midlands region revealed a higher frequency of the A143 allele, with the majority of the samples showing values above 99% (average = 98.7 ± 3.6) (Fig. 4a, 3A–3E and 4A–4E).

In-lab analysis of samples. The second set of powdery mildew infected wheat disc halves was sent by overnight courier and analysed in our laboratory as described earlier. The frequency of the A143 allele in both the dPCR and ASqPCR analyses was comparable to the results from the field analysis (Fig. 4a). Samples collected on day 1 had, in general, lower and more variable A143 allele frequencies than those from day 2. The frequency of the mutant allele in the samples collected from site one was 36.8% to 85.9% and 21.8% to 78.0% for the dPCR and ASqPCR assays, respectively (Fig. 4a, 1A–1F). For these analyses as well, the largest variation in the A143 frequency was found in the samples from the second site, which had frequencies ranging from 16.9 to 98.6% for dPCR and 18.9 to 96.8% for ASqPCR (Fig. 4a, 2A–2F).

As with the in-field analysis, the mutant allele was found at frequencies close to 100% in the majority of samples collected on day 2. In this case, the frequency of the A143 allele observed ranged from 82.0 to 99.9% and 84.1 to 100% for dPCR and ASqPCR, respectively (Fig. 4a, 3A–4E). No results were obtained when the crude extract, which had been shipped with the set 2 of infected material, was analysed using the dPCR assay (data not shown).
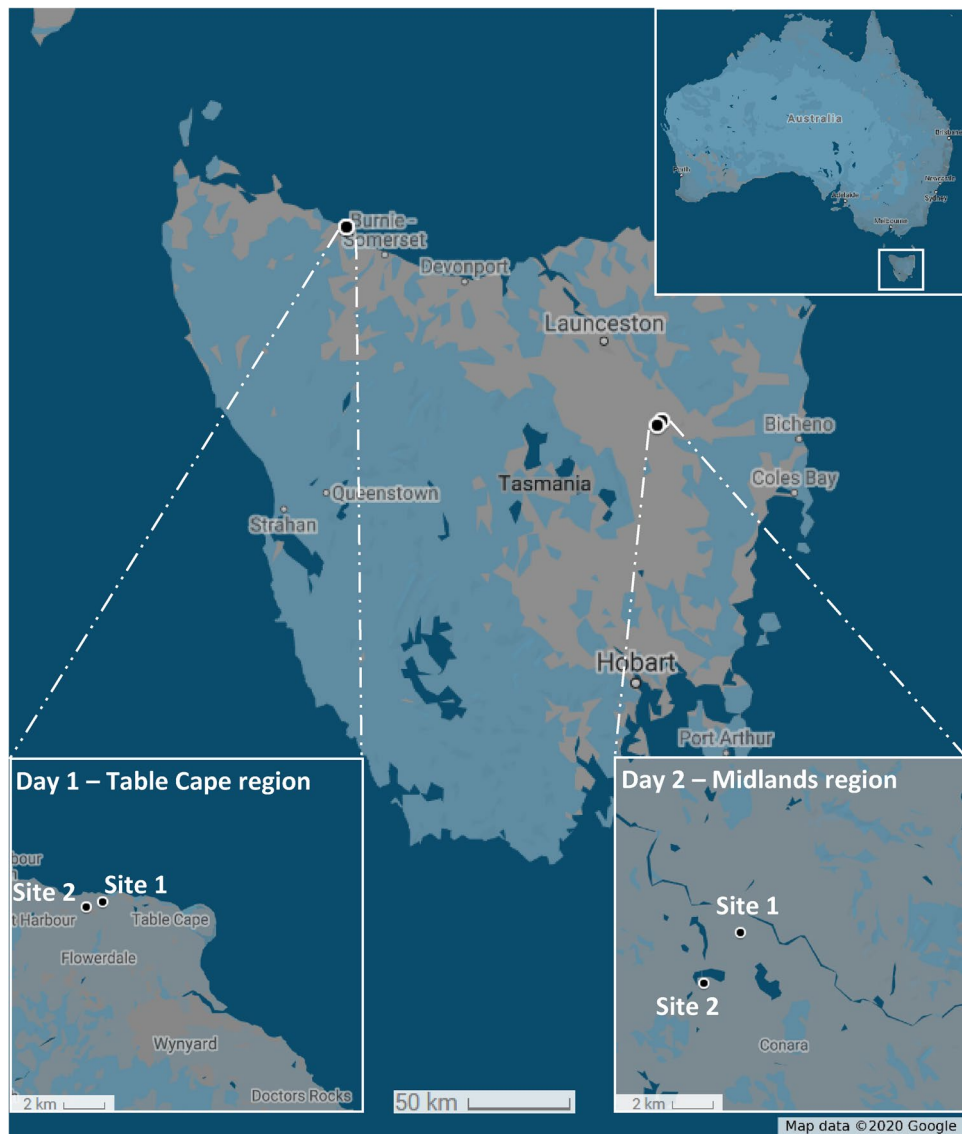
Figure 3. Map showing sampling locations in Tasmania (Australia).

Comparison of test methods. To determine the accuracy of the in-field ASqPCR method, A143 allele frequency data was compared to that from ASqPCR and dPCR experiments performed in our laboratory. Paired sample *t* test analysis showed that the frequency results obtained in the field were not significantly different from those of the same experiment performed in the laboratory using a standard method to extract DNA from the original samples (*p* value = 0.190). The same was observed when the A143 allele frequencies from the field analysis were compared to the results obtained with dPCR using DNA extracted in the laboratory (*p* value = 0.677). The box plots (Fig. 4b) also showed that the mean and median values of the results for each method were within the interquartile range (IQR) of the other methods, which reinforces the observation that the results are not dissimilar.

The Bland–Altmann analysis also showed a good correlation between the field ASqPCR and the laboratory dPCR methods, with data points distributed about the line of bias (Fig. 5). On average, the A143 allele frequencies calculated using the field ASqPCR were overestimated by 0.86% (bias), compared to the dPCR results. Twenty out of 22 samples (90.91%) were within the upper and lower limits of agreement of 19.21 and -17.48, respectively (Fig. 5).

Deployment of the field *Bgt cytb* G143A ASqPCR. Towards the end of the 2019 growing season, South Australian agronomists were reporting poor control of wheat powdery mildew after treatment with QoI fungicides. The A143 ASqPCR method was showcased as part of an extension activity organised within an industry field day in Bute, Yorke Peninsula (South Australia). A total of 12 symptomatic samples were brought by participants from surrounding wheat fields and analysed on the spot. The frequency of the *cytb* A143 allele ranged from 1.9 to 53.7% (average = 14.2 ± 17.4; n = 9 samples which showed the presence of the *cytb* A143 allele)
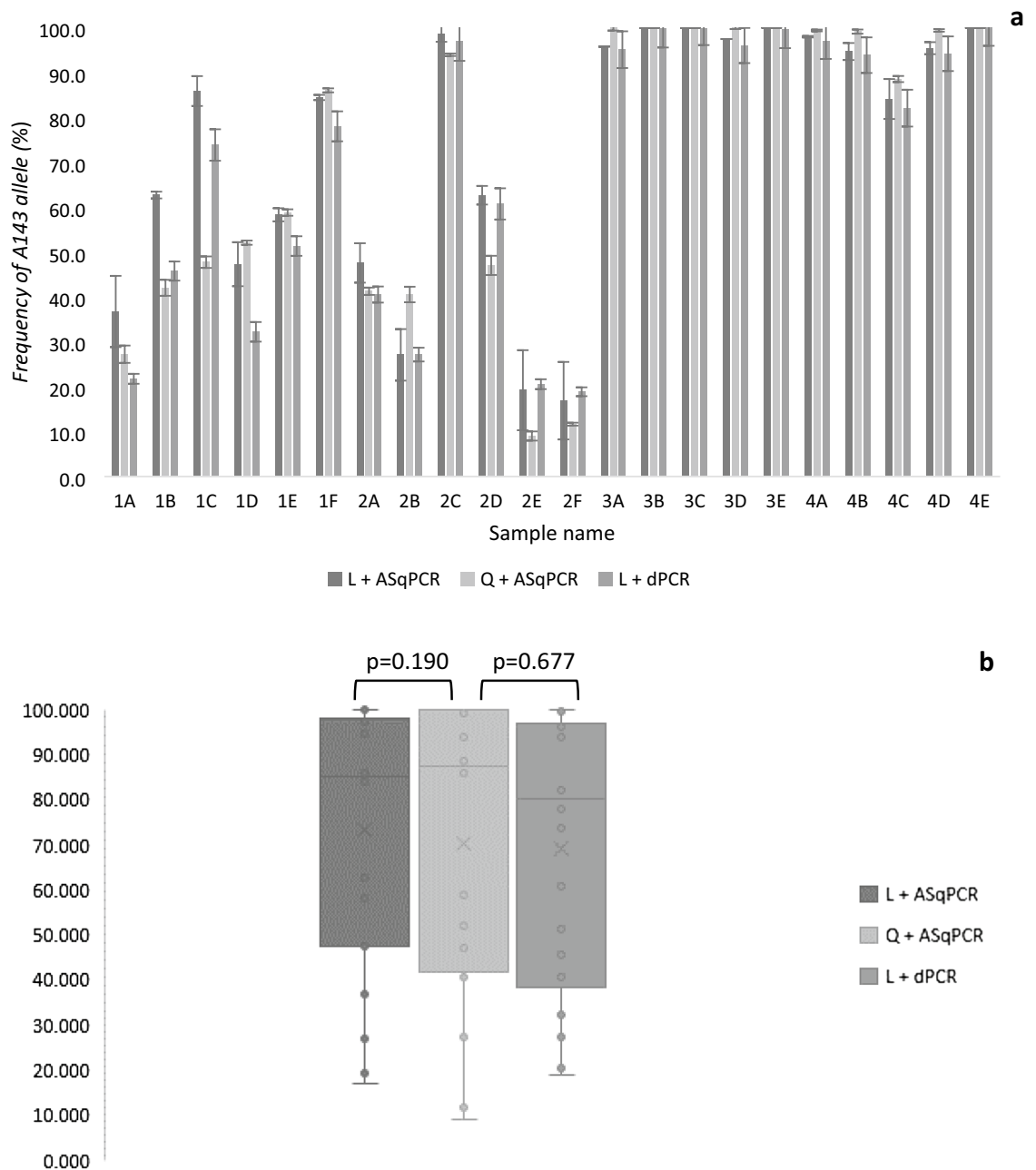
Figure 4. Comparison of *cytb* A143 frequency results of samples from Tasmania. Q + ASqPCR—crude DNA + ASqPCR, L + ASqPCR—Lab DNA + ASqPCR, L + dPCR—Lab DNA + dPCR. (**a**) Individual sample results 1A-1F and 2A-2F—samples collected from sites 1 and 2 in the Table Cape region; 3A-3E and 4A-4E—samples collected from sites 1 and 2 in the Midlands region. Error bars show standard error of mean of triplicates. (**b**) Box plots of average results for each analysis type and paired *t* test. Boxed area represents the inter-quartile range, horizontal line within box represents the median, x represents the mean.

suggesting a relatively high spread of the mutation in the area (Table 1). Three samples had no detectable levels of the A143 allele.

Discussion and conclusion

Failure of QoI fungicides to control *Bgt* in wheat paddocks in the Australian states of Tasmania and Victoria was first reported in 2016[39]. Sequencing analysis of the *cytb* gene revealed that the well characterised mutation G143A was present in all samples collected from wheat fields in these two Australian states where adequate control of the disease could not be achieved with the use of QoI fungicides (Fig. 1)[30]. As the G143A genotype continues to spread, and possibly emerge, across other wheat growing regions in the world, due to the overexposure to this group of fungicides, the availability of a rapid and robust field-based allele-specific detection method could provide a key advantage for the in-season management of QoI resistance.
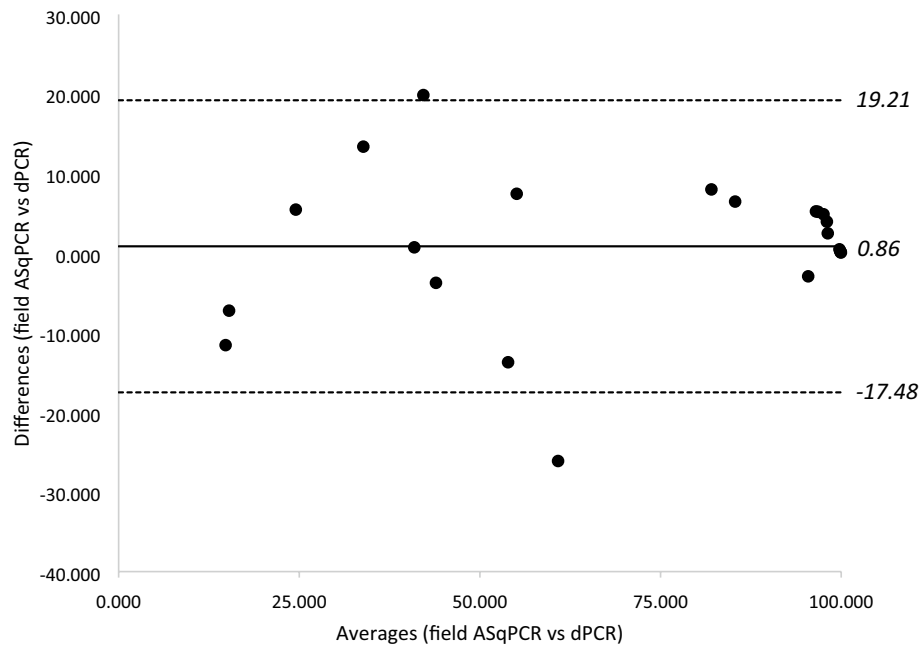
Figure 5. Bland–Altman analysis to check agreement between the results from the field qPCR and lab dPCR (n = 22). The bias of 0.86 represented the average difference between the results from the methods and shows that the results from the ASqPCR are overestimated by 0.86 compared to the latter. The upper and lower limits of agreements at 19.21 and -17.48 respectively represent the "bias ± 1.96 s.d." (s.d. of differences is 9.36) 90.1% of samples are within the limits of agreement.

The detection of fungicide resistance in crop pathogens, previously mostly conducted using bioassays, now largely relies on the use of molecular methods when the genetic mechanism associated with the resistance is known[7]. For novel field failures, where the target gene is known, capillary sequencing methods are used to determine if any known mutations are the cause[3,40]. Once a mutation has been confirmed, PCR and LAMP are commonly used for its detection, whereas for quantification qPCR and more recently digital PCR are used[18,26,41–43]. Except for LAMP, none of the other methods have been used in the field for the detection of fungicide resistance, therefore currently the quantification of mutations associated with fungicide resistance is exclusively laboratory based[20]. We have demonstrated the successful development of a TaqMan probe-based ASqPCR assay, which allows for both the mutant and wild-type alleles to be detected within the same reaction. This has been coupled with a quick, field-friendly DNA extraction method and a robust DNA polymerase to enable the in-field quantification of the QoI resistant mutation G143A in *Bgt* on a portable and fast qPCR instrument.

The quantification of the CytB G143A mutation in *Bgt* using ASqPCR was first conducted by Fraaije et al.[26] using the intercalating dye SYBR 1, with the assay detecting as low as 1 in 10,000 copies of the mutant A143 allele in a sample extraction that contained a mix of fungal and wheat DNA. A qPCR assay developed for the analysis of mutation Y136F in the Cyp51 target site of *E. necator* had a limit of detection and a limit of quantification of 0.85 and 2.85%, respectively[44]. More recently, Zulak et al.[43] developed a chip dPCR assay able to quantify mutations S509T and Y136F in the Cyp51 of *B. graminis* f. sp. *hordei* infected samples down to 0.2%. In our study, the quantification limit of the field-based G143A ASqPCR assay, when using a DNA extract containing a mixture of both A143 and G143 alleles, and wheat DNA, was 1.67% (Table 4). However, in samples with homogenous (homoplasmic) genotypes, in a background of wheat DNA, the assay successfully detected seven copies of the G143 or A143 alleles per reaction (Fig. 2b). The latter could be considered as the equivalent to a single spore detection, as each spore has multiple mitochondria and each mitochondrion may have multiple copies of the *cytb* gene within it[36]. A laboratory based G143A chip dPCR assay was also developed for comparative purposes. The G143A dPCR assay successfully quantified the *cytb* A143 allele in a background of G143 and wheat DNA down to a level of 0.33%, whereas its detection limit was estimated at 0.07% (Fig. 2a; Table 4). In general, dPCR has lower target detection limits and higher reproducibility, than qPCR, the latter due to the use of absolute quantification rather than relying on standard curves[43,45,46]. Due to these properties, dPCR could be considered the gold-standard in molecular detection of fungicide resistant populations.

The assessment of the ASqPCR analysis pipeline under field conditions revealed a marked A143 allele frequency difference between two wheat growing regions of Tasmania (Fig. 4). While the average frequencies of the A143 allele in samples collected at two sites in the Table Cape region remained between 40–50%, the values found in the sites sampled in the Midlands region showed levels above 99% in nine out of ten analysed samples. The accuracy of the in-field G143A ASqPCR method was validated by comparing the A143 allele frequency results obtained in the field to those from the laboratory using the same method with DNA extracted from the original samples following standard laboratory procedures. The lack of significant differences between the two datasets

indicated that neither the quick in-field DNA extraction nor the lack of a controlled laboratory environment had an important effect on the outcome of the in-field analysis (p value = 0.190).

The ideal way to directly compare the results from the ASqPCR with dPCR would have been using the same crude extract on both methods. However, when the crude extract was tested on dPCR, no results could be obtained. This could be attributed to the incompatibility of potential inhibitors in the crude extract with the dPCR reagents or with the degradation of the DNA. Therefore, the results from the DNA extracted in the laboratory were used for all further comparison of the analysis methods. To assess the level of sensitivity of the in-field G143A ASqPCR method, we compared the A143 allele frequency results to those obtained from dPCR using Bland–Altman analysis. When this analysis is applied to two testing methods and the results are identical, the average of the differences between paired results should be zero[47]. In this study, the comparison of the two tests indicated that ASqPCR is, on average, overestimating the mutant allele in a sample by just 0.86 units (line of bias; Fig. 5). This is quite remarkable considering that the dPCR method used DNA extracted in the laboratory as opposed to the crude extractions analysed in the field. In our analysis, the limits of agreement, which ranged from 19.21 to -17.48 units, represents 95% of the range of the differences between the measurements, and eight of the 12 differences greater than 5% are due to the field analysis showing higher frequencies than the dPCR analysis. The biggest differences were observed in the samples from the Table Cape region where the more moderate A143 levels seemed to display better the overestimating effect (Fig. 4a, 1A–2F). We believe a few factors to be causing this effect, the most likely to be a consequence of the higher sensitivity of the dPCR assay. The DNA analysed by the two methods was extracted from different areas of the same leaf disc and when one allele is very rare, the ratio will have high inherent variability due to stochastic sampling issues. This could also be reflected in a higher variability in the G143A frequency in WPM pustules from samples collected in paddocks where strobilurin resistance is emerging.

Over the past 5 years, the Northern region of Tasmania (Midlands region) had on average greater than six times the area dedicated to growing wheat compared to the North-western region (Table Cape; Cradle Coast) (Supplementary table S2)[48–52]. This could suggest that larger pathogen populations and more frequent use of fungicides from the QoI group could be responsible for the higher A143 allele frequency observed in the Midlands region, as the *Bgt* population would be saturated with strobilurin-resistant mutants. Recently, the G143A mutation has been found at high frequencies in the Yorke Peninsula region of South Australia following new QoI control failure episodes in 2019. The deployment of the in-field G143A ASqPCR method during a field day allowed to correlate higher frequencies of the A143 allele in fields displaying high disease levels after QoI treatment. The results of the analysis were provided on site and the fact that many participants decided to review their chemical control strategies highlights the power of this approach not only as an in-season tool for the management of fungicide resistance, but also as part of more broader extension efforts.

Although there is a lack of specific knowledge about the threshold abundance of the A143 allele which would translate into QoI control failure for the wheat powdery mildew system, studies of this mutation in other organisms have shown that its frequency would have to exceed 60% before resistance to QoIs becomes apparent[53,54]. This can be used as a guide to demark the threshold for control failure of QoIs in *Bgt* containing the A143 allele. Future research in this space should assess the level of A143 required for control failure of WPM.

We believe that the ASqPCR conducted in the field is a reliable tool that can be used for rapid and on-site quantification of *Bgt* mutants carrying the G143A Cytb mutation, and provide valuable information to chemically tackle the disease. An additional advantage of this method is that it can be adapted to detect changes in other genes encoding different fungicide targets. To that effect, a similar assay for the detection of pyrimethanil resistance in the grape pathogen *Botrytis cinerea* has already been trialled in the field (Harper et al., unpublished).

The technology we describe here substantially increases the speed with which quantitative pathogen diagnosis and fungicide resistance evolution can be measured and delivered to growers. These techniques promise to markedly increase the flexibility of growers to modify fungicide application timing, dose and product choices within a few hours of sampling.

The methodology could be enacted using portable instruments housed in the cab of a vehicle. However, we recognise that the true value of rapid diagnosis will be realised only when it is coupled with reliable advice and product choices. As such, the technology is perhaps best suited to implementation via testing stations located within cropping zones where information from the tests can be coupled with local pathology experience and weather forecasts. This would mean that the treatments used would be tailored to the disease and resistance problems within the crop, which in turn would eliminate the use of unnecessary treatments and reduce the selection pressure that occurs when using unsuitable treatments.

Materials and methods

Blumeria graminis f. sp. *tritici* (*Bgt*) field sample PCR and *cytb* sequencing. Ten wheat and one barley powdery mildew samples were collected from the states of Western Australia, South Australia, Victoria and Tasmania in 2016 (Table 1). The leaf samples were placed in 15 ml polypropylene tubes filled with 2 ml of 50 mg L^{-1} benzimidazole water agar[55], sealed and placed in polystyrene containers with ice packs for express shipment to the laboratory for DNA extraction. For each sample, approximately 3 cm length of the infected tissue was cut into small pieces and DNA was extracted using a BioSprint 15 instrument and BioSprint 15 DNA plant kit (Qiagen, Australia) according to the manufacturer's protocol. The resulting mix of fungal and wheat DNA was eluted in 1 × TE buffer pH 8.0 and stored at − 20 °C. The remaining sample was stored at − 20 °C until further analysis.

The *cytb* gene from *Bgt* was amplified and sequenced using primers WM-Cb-f and WM-Cb-R[56] (Table 2). Each 100 µl PCR reaction contained 5 U of MyTaq DNA polymerase (Bioline, Australia), 20 µl 5 × MyTaq reaction buffer, 0.4 µM each primers, 50–100 ng DNA template and water. The thermal-cycling was conducted with

initial denaturation for 5 min at 95 °C, followed by 34 cycles of 30 s at 95 °C, 1 min at 55 °C and 1 min at 72 °C, followed by final extension for 10 min at 72 °C. The PCR products were sequenced by Macrogen (Seoul, South Korea) and the results aligned on Geneious (Biomatters Ltd) with existing *Bgt cytb* gene reference sequences (GenBank accessions: AF343441.1, AF343442.1) to determine if mutation *g428c,* responsible for amino acid substitution G143A, was present.

Design of digital PCR and ASqPCR for the analysis of *g428c*.　　The digital PCR (dPCR) primers and TaqMan probes used to quantify the presence of mutation *g428c* in this study were designed as per Zulak et al.[43] with some modifications (Table 2). The sequence of *cytb* flanking 100 bp of the mutation was added to the Custom TaqMan Assay Design Tool, with the species/scale set at 'Non-Human: small scale'. Resulting *Bgt cytb* wild type (G143 allele; strobilurin-sensitive) and mutant (A143 allele; strobilurin-resistant) primers and probe combinations were ordered as a single-tube mix containing 36 μM of each primer and 8 μM of each probe (Thermo Fisher Scientific, Australia). The probe targeting A143 allele was labelled with the fluorophore FAM on its 5′ end and the probe targeting the G143 allele labelled with VIC. The 3′ ends of both probes were labelled with a non-fluorescing quencher (NFQ).

Each 17 μl dPCR reaction consisted of 8.5 μl QuantStudio 3D Digital PCR 2× Master Mix (Applied Biosystems, Australia), 0.3 μl primer probe pre-mix, and 3 μl of 10–20 ng DNA template and made up to 17 μl with water. Fifteen μl of this reaction was loaded onto a QuantStudio 3D Digital PCR Chip v2 (Applied Biosystems) and cycled on a Geneamp 9700 flat block thermal cycler (Applied Biosystems) under the following conditions: 10 min at 96 °C, then 40 cycles of 2 min at 58 °C and 30 s at 98 °C, followed by 2 min at 58 °C, and 10 min at 22 °C. Chips were read on a QuantStudio 3D Digital PCR Instrument (Applied Biosystems). The copy ratio of mutant to wild type allele in each sample was determined using QuantStudio 3D AnalysisSuite software (Applied Biosystems). The DNA from the sequenced samples was tested along with DNA from uninfected wheat leaf, wheat leaf infected with *Puccinia triticina* and pure *P. nodorum* DNA. In addition, a dilution series of the DNA from the Goomalling sample (G143 allele, Table 1) and Geelong 1 sample (A143 allele, Table 1) was tested with their respective target probes to determine the detection limits of the assay (A143 abundance: 100%, 66.67%, 40.50%, 18.18%, 6.90%, 3.39%, 1.68%, 0.67%, 0.33%, 0.17%, 0.07% and 0%; DNA concentration 5000–6000 copies μl^{-1} template). Each test was conducted in triplicate.

The dPCR primers and modified dPCR probes (Sigma-Aldrich, Australia) were used to detect mutation *g428c* on a magnetic induction cycler (MIC) qPCR instrument (Bio Molecular Systems, Australia). In order to increase probe T_m and specificity, three bases in each probe were modified to Locked Nucleic Acids (LNA) (Table 2). In addition, the fluorophore on the probe targeting the G143 allele was changed from VIC to HEX.

Three different qPCR mixes were tested using the Goomalling and Geelong 1 DNA and a DNA sample adjusted to 51% A143 (Table 1). Each 20 μl ASqPCR reaction consisted of 10 μl of 2× mastermix [iQ Multilplex Powermix (Bio-Rad, Australia), Sensifast Probe No-Rox mastermix (Bioline) or ImmoMix™ (Bioline)], 0.5 μl each of 10 μM forward and reverse primers, 0.3 μl each of 5 μM FAM and HEX probes, 5 μl of up to 20 ng DNA template and 3.4 μl water. The assay was conducted under field conditions using the following parameters: initial denaturation for 5 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, and 30 s at 67 °C for annealing and extension. This was tested twice with samples run in triplicate in the first experiment and in duplicate in the second. The Sensifast Probe No-Rox mastermix (Bioline) was chosen for future testing. The repeatability of the quantification of the ASqPCR assay, was tested as above with the 51% A143 DNA sample and its frequency calculated based on standard curves generated using the Goomalling and Geelong 1 DNA. This was tested twice with samples replicated ten times in the first experiment, and six times in the second.

A dilution series similar to that described for dPCR including single and mixture of known allele proportions was tested to determine the detection limits of the assay (A143 abundance: 100%, 66.67%, 18.18%, 6.90%, 3.39%, 1.68%, 0.67%, 0.33%, 0.17%, 0.07% and 0%; DNA concentration 5000–6000 copies μl^{-1} template). A standard curve was also constructed using a serial dilution of the Goomalling and Geelong 1 DNA to calculate the amount of fungal DNA in the mixtures. The A143 allele frequency was calculated as percentage value using micPCR software v2.8.0 (Bio Molecular Systems). The species specificity of the assay was tested using DNA from plant material infected with closely related pathogens *B. graminis* f. sp. *hordei* and *E. necator*, as well as pure DNA from pathogens sharing the same host; *P. nodorum* and *P. tritici-repentis*.

Development of an in-field allele frequency quantification pipeline.　　To enable the testing to be conducted in a field setting, a quick DNA extraction protocol was developed as a part of this study. Preliminary testing of this extraction method was conducted using approximately 100 mg of infected plant tissue (Goomaling and Geelong 1) that had been kept at -20 °C. The material was crushed with a plastic micro pestle in 400 μl of the lysis buffer (0.5% w/v SDS, 1.5% w/v NaCl) until the plant tissue fragmented and turned the solution green. This lysate was then diluted 100-fold in 1× TE buffer pH 8.0 and 5 μl used as DNA template in ASqPCR reactions. DNA extraction and its thermal cycling was conducted under field conditions on a lightweight qPCR instrument (Magnetic induction cycler, Bio Molecular Systems) powered using a ITECH200 12 V 200AH lithium ion battery (iTechWorld, Australia) connected to a 2000 W 12 V pure sine wave inverter (iTechWorld). The cycling run (56 min) was initiated and analysed using the micPCR software on a laptop connected to the qPCR instrument.

Twenty-two wheat powdery mildew samples were collected in 2018 from four locations in the Northern region of Tasmania during a two-day collection trip (Fig. 3; Table 1). On day 1, six samples were collected from each of two fields; each sample, consisting of up to 2 leaf fragments, came from an individual plant chosen at random within a 200 m² area in their respective fields. The sampling on day 2 was conducted in the same manner, however only 5 samples per field were collected. The samples were maintained in polypropylene tubes
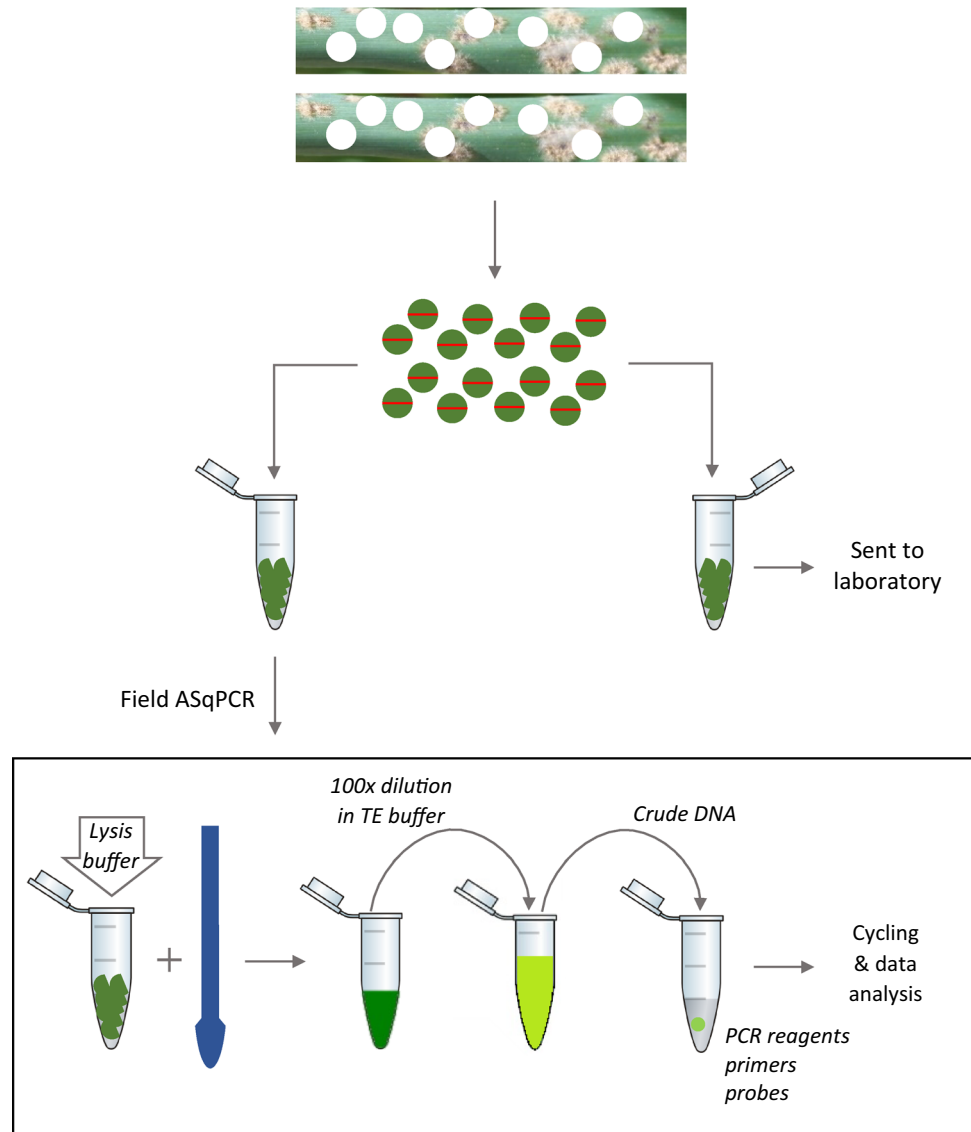
Figure 6. Schematic representation of the procedure followed in the field for the extraction of DNA.

containing benzimidazole agar, as previously described, and stored in a polystyrene box with ice bricks to prevent deterioration prior to processing.

On each day, once sampling was concluded, sixteen 7 mm discs per infected leaf sample were excised using a WellTech Rapid-Core-7.0 (RapidCore, Australia) disinfected with 70% v/v ethanol between samples. The discs were halved and separated into two 1.5 ml microfuge tubes to obtain two sets of tubes (1 and 2) with 16 halves each, per sample. The material in set 1 was used for immediate processing and that of set 2 shipped on ice by overnight courier to our laboratory in Perth for dPCR analysis (Fig. 6). Samples in set 1 were processed using the quick DNA extraction method described earlier. The ASqPCR reactions were set-up as described above and 5 µl of the DNA extract was used directly in the reaction mix. Each sample was tested in triplicate and the amount of fungal DNA was measured using a standard curve constructed from Goomalling and Geelong 1 DNA of known concentrations amplified alongside the samples. The amount of mutant allele in each sample was reported as a percent value. Remaining crude DNA was shipped with the tubes from set 2 for subsequent dPCR analysis in the laboratory.

In-lab sample processing. Upon receiving set 2 in the laboratory, tissue was frozen in liquid nitrogen then crushed to a fine powder using a 2 mm stainless steel ball bearing in a Retsch mixer mill MM400 (Retsch, Germany), followed by DNA extraction with a Biosprint Plant DNA 15 kit (Qiagen), as per manufacturer's instructions. The DNA was eluted in 200 µl of 1× TE buffer and tested using both dPCR and ASqPCR in the same manner as above. Any sample that fell outside the 200–2000 copy range on the dPCR was diluted/concentrated and re-tested as required.

Statistical analysis of dPCR and ASqPCR results. The results from all three methods were simultaneously compared using a box-plot as well as comparing paired results using a *two tailed t test* on Microsoft Excel. Additionally, the results of the dPCR and field ASqPCR analyses were compared using SigmaPlot v13 (Systat Software, San Jose, CA). A Bland–Altman analysis, also known as the Tukey's mean difference test was conducted. The Bland–Altman plot was constructed by plotting the difference between the paired data points (method 1 *minus* method 2) on the y-axis and the mean of the paired data points on the x-axis[47]. The mean of the difference between the pairs represents the bias i.e. whether the method 1 is overestimating (positive bias) or underestimating (negative bias) compared to method 2. The upper and lower limits of confidence are calculated as the mean (bias) ± 1.96 standard deviation of the differences.

In-field application of the ASqPCR pipeline for the detection of *cytb* mutation *g428c*. In October 2019, suspected strobilurin-resistant wheat powdery mildew samples (n = 12) collected from different farms and brought by growers attending an industry field day at Northern Yorke Peninsula (South Australia), were processed in-field (Table 1). The tissue was not excised using the coring tool, rather cut into small fragments using scissors and forceps disinfected with 70% v/v ethanol between samples. Each sample was added up to the 100 μl graduation in 1.5 ml microfuge tubes. This was followed by a quick DNA extraction and ASqPCR assay on the MIC qPCR instrument (Bio Molecular Systems) as detailed above, and mutant frequency results communicated to the growers upon assay completion.

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Author contributions

F.L.-R. designed the project. K.D. and R.O. contributed to the conception of the work. K.D. and B.C. performed the analysis and interpretation of data. K.D. and F.L.-R. wrote the main manuscript and KD prepared Fig. 6. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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