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Rapid LAMP Assays to Detect *MgCYP51* and/or *MgMFS1* Overexpressing Strains of *Zymoseptoria tritici* in Leaf Samples

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FINDINGS

Robust molecular-based diagnostics, targeted to specific pathogen species or genotypes, are valuable tools that can be used to help ensure appropriate plant disease management strategies. PCR assays have been developed, but these require time consuming laboratory-based processing and are thus not readily applicable to point of care (POC) field-based use. An alternative option is the use of the more recently introduced isothermal DNA amplification assays operating at a single constant temperature. A range of different technologies/platforms have been developed (Table 1). These assays open up the future prospect of POC application due to (1) faster reaction times (< 30 mins); (2) improved sensitivity and specificity; (3) increased tolerance to inhibitors present in environmental samples and (4) no requirement for temperature cycling and, therefore, low energy consumption.

Loop-mediated isothermal amplification (LAMP) assays have already been developed for the rapid detection of a diverse range of bacterial, fungal, oomycete and viral plant pathogens (e.g. Table 2). Many of these pathogens are of considerable economic importance, and some have had major impacts on human history (e.g. the potato late blight pathogen *Phytophthora infestans*). However, LAMP technology offers a much broader potential than pathogen species detection alone, for instance monitoring of fungicide resistance in pathogen populations. Resistance mechanisms in phytopathogenic fungi include (1) changes in the target site encoding sequence (reducing binding affinity between a fungicide and its target), (2) target site gene overexpression and (3) increased fungicide efflux. To date only a few LAMP assays for fungicide resistance detection have been described for a small number of pathogens, e.g. *Fusarium asiaticum* (Duan et *al.* 2016), *F. graminearum* (Duan et *al.* 2014a) and *Sclerotinia sclerotiorum* (Duan et *al.* 2015). These LAMP assays all target changes in target site coding sequence; no assays have yet been fully described for detection of resistance mediated by target gene overexpression or increased fungicide efflux mechanisms.

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Table 1 Overview of some isothermal technologies used for phytopathogen detection.

Technology	Brief methodology	Target pathogen	References
Helicase Dependent Amplification (HDA)	DNA helicase used to obtain single strand DNA template. Primer hybridizes and extended by DNA polymerase.	The oomycete *Phytophthora ramorum* (sudden oak death)	Vincent et *al.* (2004); Schwenkbier et *al.* (2015)
Loop-mediated isothermal amplification (LAMP)	Typically, four primers target six regions of target sequence via DNA polymerase enzyme. Optional loop primers reduce reaction times.	The fungus *Sclerotinia sclerotiorum* (Sclerotinia stem rot)	Duan et *al.* (2014b)
Nucleic Acid sequence-based amplification (NASBA)	Amplification only of RNA template using a cocktail of enzymes.	The bacteria *Xanthomonas citri* subsp. *citri* (citrus bacterial canker)	Scuderi et *al.* (2010)
Recombinase Polymerase Amplification (RPA)	DNA binding proteins bind to primers. Upon binding to target sequence, polymerase enzyme extends the primer with opposite strand acting as template.	*Bean golden yellow mosaic virus*	Londoño et *al.* (2016)
Rolling Circle Amplification (RCA)	Exploitation by a DNA polymerase of continuously amplifying circular DNA sequences. Enzyme displaces newly synthesized strand; thus DNA synthesis ‘rolls on’.	*Banana streak virus*	James et *al.* (2011)

Septoria tritici blotch (STB) caused by the ascomycete fungus *Zymoseptoria tritici* (previously *Mycosphaerella graminicola*) is an important disease of wheat (*Triticum aestivum*) crops. It is especially important in Europe where it poses a serious and consistent challenge to production (O’Driscoll et *al.* 2014). The disease is controlled primarily using demethylation inhibitors (DMIs, commonly referred to as azoles) in combination with newer succinate dehydrogenase inhibitors (SDHIs) (Cools & Fraaije 2013; O’Driscoll et *al.* 2014) and/or multisites. However, a reduction in sensitivity to azole fungicides has been reported in recent years (Cools & Fraaije 2013) and, worryingly, a small number of strains have now been identified in Ireland and the UK that appear insensitive to most SDHIs tested (Dooley et *al.* 2016; B. Fraaije, unpublished).

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Table 2 LAMP assays available for rapid species-specific detection of important phytopathogens.

Target species	Pathogen type	Disease caused	Reference
Phytoplasma (16SrXI and 16SrIII)	Bacteria	Napier stunt phytoplasma	Obura et *al.* (2011)
Pseudomonas syringae pv. *actinidiae*	Bacteria	Bacterial canker of kiwifruit	Ruinelli et *al.* (2016)
Botrytis cinerea	Fungus	Grey mould	Tomlinson et *al.* (2010a)
Sclerotinia sclerotiorum	Fungus	Sclerotinia stem rot	Duan et *al.* (2014b)
Phytophthora infestans	Oomycete	Late blight of potato and tomato	Hansen et *al.* (2016)
Phytophthora ramorum	Oomycete	Sudden oak death	Tomlinson et *al.* (2010b)
Potato virus X	Virus	Potato virus x	Jeong et *al.* (2015)
Wheat streak mosaic virus	Virus	Wheat streak mosaic virus	Lee et *al.* (2015)

Until 2009, resistance in *Z. tritici* was only known to be mediated by changes in target site coding sequences, although additional mechanisms have since been identified in field strains. The first involves *MgCYP51* target gene overexpression, and is associated with a 120 bp insertion in the gene promoter region (Cools et *al.* 2012). The second involves increased fungicide efflux by the major facilitator superfamily (MFS) transporter, MgMFS1, and is associated with multi drug resistance (MDR) including reduced sensitivity to strobilurins, azoles and SDHIs in both lab mutants (Roohparvar et *al.* 2007) and field strains (Omrane et *al.* 2015). A 519 bp insert in the *MgMFS1* promoter region was linked to gene overexpression in MDR strains, and was present in half the MDR *Z. tritici* field isolates examined, suggesting a correlation between insert and MDR phenotype (Omrane et *al.* 2015).

In this study we designed novel LAMP assays for specific detection of these 120 and 519 bp promoter inserts linked with overexpression of *MgCYP51* and *MgMFS1*, respectively, and associated with fungicide insensitivity. These LAMP assays were then validated by screening against a panel of *Z. tritici* isolates, for which presence or absence of the correctly sized insert was also confirmed by PCR (Figure 1). We then demonstrate their direct applicability to infected STB leaf samples (data not shown), bypassing the requirement for time consuming isolation procedures. The new assays represent the first use of LAMP for fast fungicide resistance detection in the economically important wheat pathogen *Z. tritici* and, given the potential advantages of LAMP technology, open up the future prospect of POC use. They will also allow rapid detection of any future increase or geographical spread of these promoter inserts, and therefore could help inform STB disease management and fungicide anti-resistance strategies.

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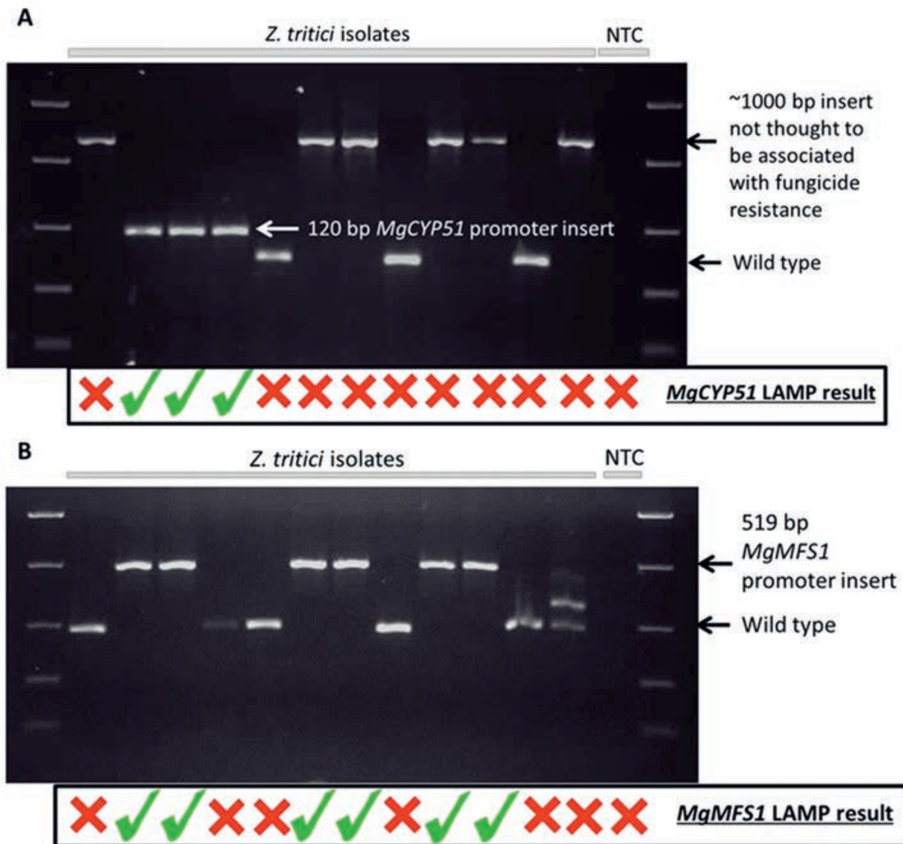

Figure 1 PCR validation of new LAMP assays targeted to 120 bp *MgCYP51* and 519 bp *MgMFS1* promoter inserts in *Zymoseptoria tritici*. (A) The 120 bp *MgCYP51* insert LAMP assay detects only *Z. tritici* isolates with that sized insert (as confirmed by PCR gel results). (B) The 519 bp *MgCYP51* insert LAMP assay detects only *Z. tritici* isolates with that sized insert (as confirmed by PCR gel results). Note that the same panel of *Z. tritici* isolates are shown in (A) and (B). First lane contains EasyLadder1 (Bioline); NTC is a no-template control.

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