THESIS

PHYLOGENETIC ANALYSIS OF *XANTHOMONAS TRANSLUCENS* & GENOME-ENABLED DIAGNOSTIC TOOLS FOR PATHOGENS OF CEREALS AND NON-CEREALS

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ABSTRACT

PHYLOGENETIC ANALYSIS OF *XANTHOMONAS TRANSLUCENS* & GENOME-ENABLED DIAGNOSTIC TOOLS FOR PATHOGENS OF CEREALS AND NON-CEREALS

Prevalence of *Xanthomonas translucens*, which causes bacterial leaf streak (BLS) in cereal crops and bacterial wilt in forage and turfgrass species, has increased in many regions in recent years. Because the pathogen is seedborne in economically important cereals, it is a concern for international and interstate germplasm exchange, and thus, reliable and robust protocols for its detection in seed are needed. However, historical confusion in the taxonomy within the species has complicated the development of accurate and reliable diagnostic tools for *X. translucens*. The goal of this study was to clarify the genetic relationships of *X. translucens* pathovars, and to use that information to develop useful and robust diagnostic tools. We sequenced genomes of 15 *X. translucens* isolates representing six different pathovars. Based on Multilocus Sequence Typing (MLST), wheat isolates designated as *X. translucens* pv. *undulosa* are in the same phylogenetic clade as barley isolates identified as *X. translucens* pv. *translucens*. The wheat and barley pathovars, *undulosa* and *translucens*, are genetically distinct from the *cerealis* pathovar isolated from either cereals or non-cereals, as well as pathovars isolated from other non-cereals, including *arrhenatheri*, *graminis*, and *poae*. Using unique genomic regions, Loop Mediated Isothermal Amplification (LAMP) primer sets were designed that selectively amplified *X. translucens* (species-specific), or that selectively amplified strains belonging to *cerealis* and *poae* pathovars. In addition, LAMP PCR assays were developed that distinguished *X. translucens* strains

associated with cereal leaf streak (CLS), such as *undulosa*, *translucens*, *hordei*, and *secalis*, from the other cereal or non-cereal pathovars.

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CHAPTER ONE

INTRODUCTION: *XANTHOMONAS* PATHOGENS OF CEREALS AND NON-CEREALS

Xanthomonas **pathogens of interest, diseases associated, and agronomic importance**

Xanthomonas is a large genus of rod shaped, gram negative bacteria, consisting of many species that cause different diseases on plants and have differing host ranges. *Xanthomonas* causes diseases on broad and diverse plants, including cereals, fruits, grasses, trees, and vegetables. Within each species of *Xanthomonas* there is often a pathovar or multiple pathovars, each of which demonstrates a different disease symptom phenotype or has a different host or host range than other pathovars in the species. Currently the genus of *Xanthomonas* has over 20 plant associated species that contain more than 140 different pathovars (Vauterin, Rademaker, and Swings 2000). My research has been focused on two species of *Xanthomonas*; *X. oryzae* and *X. translucens*.

X. oryzae consists of two pathovars that are both found on and cause disease on rice (*Oryza sativa* L.) in all parts of the world, with each pathovar causing a different disease. The major difference in these pathovars is that one infects the plant systemically in the vascular tissue and the other infects the host intercellularly. *X. oryzae* pv. *oryzae* (*Xoo*) is the causal agent of bacterial blight (BB) that infects rice by colonizing the xylem tissue causing limited water and nutrient flow, causing entire fields to become blighted. This results in wilting, necrosis, and yield losses often around 20-50%. The pathovar *X. oryzae* pv. *oryzicola* (*Xoc*) is the causal agent of bacterial leaf streak (BLS), a disease in rice that results from intercellular spread of the pathogen.

This pathovar does not colonize the vascular system but rather takes refuge and spreads between the mesophyll cells in the leaves. Both pathogens control efflux of important plant nutrients and carbon, resulting in reduced yields. Typically, yield losses from *Xoc* are not as severe or as widespread as *Xoo*, however 10-20% losses are common. *X. oryzae* has a huge impact on the global rice industry, and has been responsible for huge economic losses. Both pathogens are considered quarantine organisms by the United States and are highly monitored and regulated in all parts of the world. *Xoc* is known to be a seed transmitted bacterium, and seed that tests positive for identification of *Xoc* is often destroyed. Misdiagnosis can be costly; anecdotal reports of destruction of seed lots due to false detection of *Xanthomonas oryzae* are common. Therefore, with rice being regarded as one of the largest imported and exported crops, the need for accurate and reliable means of detection is essential for global food security and economic stability. As part of my graduate work, I co-published on the development of genome-based molecular diagnostics for *Xanthomonas oryzae* pathovars (Lang et al. 2014). In that work, we developed Loop Mediated Isothermal Amplification (LAMP) assays that distinguished *X. oryzae* pv. *oryzae* from *X. oryzae* pv. *oryzicola*. My role was to help in the development of four LAMP assays that detected the two pathogens in rice, and that distinguished between the two pathovars. In this work, two other LAMP assays were developed that identified whether the pathogen *X. oryzae* pv. *oryzae* was from Africa or Asia, based on unique geographic dependent sequences. The primers/assays are now being adopted in international seed testing labs.

In this document, I will describe my major research efforts, which were centered on the group of pathogens in the *Xanthomonas translucens* complex. *X. translucens* is the causal agent of bacterial leaf streak (BLS), also known as cereal leaf streak (CLS), black chaff (if seed heads are

infected), and bacterial wilt of grasses. Yield losses due to CLS and black chaff can range from negligible to up to 40% (Mcmullen and Adhikari 2011). In the cases of wheat and barley, *X. translucens* are transmitted and found in the seed (Smith, Jones, and Reddy 1919). Due to the pathogen being seed transmitted, planting of clean seed is recommended to avoid the disease, but the pathogen can also be found in plant residue and in the soil (Mcmullen and Adhikari 2011). Seed with obvious signs of *X. translucens* or that has been shown to be contaminated, is often not sold for commercial or agricultural use. With low tolerances to contamination and limited ability to detect and distinguish the pathogens in seed and plant tissues, there is a need for accurate and reliable detection for this pathogenic bacteria. Compared to *X. oryzae*, *X. translucens* causes disease or is isolated from a much larger diverse set of hosts, and thus, the host range for *X. translucens* is larger and less specific than *X. oryzae*. *X. translucens* is important to many industries such as the beer production industry, flour industry (Duveiller, Ginkel, and Thijssen 1993), golf course industry (Mitkowski et al. 2005; Wichmann 2011), and forage grass industry (Wichmann et al. 2013). Therefore, detection of *X. translucens*, is needed not only for these many industries, but from both seed and leaf tissue is very important. Currently, *X. translucens* is comprised of nine accepted pathovars, three of which are found on and associated with cereal hosts including rye, barley and wheat. These *X. translucens* pathovars can infect the leaves or the seed heads/glumes of cereals. When signs of the disease are in the leaves of cereals it is referred to as cereal leaf streak (CLS), and when in the glumes, the disease is referred to as black chaff. The pathovars associated with rye, barley and wheat are s*ecalis*, *translucens*, and *undulosa*, respectively. The *cerealis* pathovar is also associated with wheat and barley but is often found on wild bromegrass and quackgrass. In this work I demonstrate that the *cerealis* pathovar is more closely related to, but genetically distinct from the CLS causing

pathovars (CHAPTER 2). Another pathovar named because of its association with barley, *X. translucens* pv. *hordei*, is now accepted to be a true *X. translucens* pv. *translucens* (Bragard et al. 1997) and will be treated as such in this study.

Five not yet mentioned pathovars of *X. translucens* are found on various non-cereal grasses and have differing host ranges on which they cause disease or are isolated. Some of the classified non-cereal pathovars include *X. translucens* pv. *arrhenatheri*, *phlei*, and *phleipratensis*; this group infects various types of forage grasses. The pathovar *arrhenatheri* has been isolated from false oatgrass (*Arrhenatherum elatius*) and rough bluegrass (*Poa trivialis*), while the pvs. *phlei* and *phleipratensis* are both frequently isolated from timothy-grass (*Phleum pratense*) (Rademaker et al. 2006). Pathovar *graminis* has been isolated from golf course fairway grasses and forage grasses ranges and is virulent on some orchard grasses (*Dactylis* spp.), tufted grasses (*Lolium* spp.), and some species in the genera *Phleum* and *Poa*, but strains of this pathovar are not pathogenic to *Arrhenatherum* spp. (Rademaker et al. 2006; Wichmann 2011). Lastly, *X. translucens* pv. *poae* is a non-cereal pathovar that is widely associated with bacterial wilt of turfgrass, and infects annual bluegrass (*Poa annua*) and rough bluegrass (*Poa trivialis*). These five pathovars make up the *X. translucens* that are not found on cereal hosts. Recently, a newly proposed pathovar, *X. translucens* pv. *pistaciae*, has been identified as being the causal agent of pistachio dieback in Australia (Facelli et al. 2009). This pathogen has only been reported in and isolated from orchards in Australia from stained xylem tissue of two-year-old stems, and has been isolated less commonly from lesions in the bark. The work reported here will describe phylogenetic comparisons using genomic data, with the intention of resolving the complex taxonomy of the *Xanthomonas translucens* complex.

Historical background of *Xanthomonas*; from classification to advances in diagnostics

Over the years, our means of classifying and identifying plant pathogenic bacteria has moved away from time consuming methods such as morphological trait characterization, pathogenicity testing, and culturing methods, and has improved greatly due to rapid advances in molecular technologies (Vauterin et al. 1995). Although many of these classic methods are still very important and will never be replaced, new genome based methods have revolutionized the field of diagnostics because of their accuracy, rapidity, and reliability. The advances and declining costs in genome sequencing have enabled previously difficult and cost prohibitive genomic studies to become more feasible, including sequencing and assembly of many closely related species or pathovars, and genes with repetitive nucleotide regions, such as those found in transcription activator-like (TAL) effectors in the *Xanthomonas* genus (Bart et al. 2012; Sebra et al. 2015). Methods for detection and identification of *Xanthomonas* and other plant pathogenic bacteria began through methods of microscopy, dilution plating, and host plant inoculations. Besides host plant pathogenicity testing, there were other methods used in the field of pathogen diagnostics that helped classify and identify microorganisms. Pathogenicity testing along with serological studies including protein and fatty acid analysis, contributed to understanding pathogen relationships. However, these methods had major limitations and lack of detailed information that could be derived from the studies. In the case of gram staining, bacteria can be divided into two main subgroups, gram negative and gram positive. This was and still may be the most important stain in microbiology but has little usefulness for identifying a specific bacterium, and if that particular organism was the cause of the disease. Serological studies allowed for a more detailed analysis that could help differentiate some genera or species of bacteria from each other based on shared characteristics such as cell wall membranes, proteins,

or the polysaccharides excreted by many bacteria (Elrod and Braun 1947). The first report using serology as a means for both identifying and differentiating a plant pathogenic bacterium was performed using an agglutination test (Jensen 1918, as cited by Schaad 1979). Over the years, many different antisera were developed and used that aided in pathogen, species, or pathovar differentiation, and these antisera could detect their targets in killed or lysed bacterial cells, or in isolated glycoproteins, membrane protein extracts, as well as purified enzymes (Fierz 2004; Shukla, Lauricella, and Ward 1992; Schaad 1979; Elrod and Braun 1947). Unfortunately many studies showed unreliable results due to cross-reactions, and had low resolving power between species or pathovars due to the high homologies of some of the organisms (Schaad 1979). This inconsistency was not good for reliable means of detection, and it was a problem in which results were sometimes dependent on what material was used, reaction conditions, or sometimes just chance. Therefore, serology was not looked upon highly as the scientific standard for differentiating most bacteria.

The most important techniques and methods in plant pathogen diagnostics were nucleotide based and protein or fatty-acid based methods. Methods, such as SDS-PAGE, Restriction Fragment Length Polymorphism (RFLP), DNA-DNA hybridization, protein electrophoretic patterns, isoenzyme studies, and fatty acid composition, added to the understanding, diagnostics, and taxonomy of the genus *Xanthomonas* (Elrod and Braun 1947). But, methods such as those relying on protein or fatty acid composition are dependent on the physiological state of the organism, and thus problematic. Because DNA does not change with physiological state, reliance on methods that detected DNA differences emerged in importance. RFLP for instance, was one of the first DNA fingerprinting techniques ever used for crime scene investigations and was later

used in many applications, including plant pathology diagnostics (Roewer 2013; Nybom, Weising, and Rotter 2014). This method uses nucleotide patterns made from fragmenting DNA using restriction enzymes to observe a 'genetic fingerprint', that could in turn be potentially unique to an individual, bacterial genera, species, pathovar, or strain, in some cases. The drawbacks of RFLP are that it is a very time and resource intensive process, and does not always have the desired resolving power (Nybom, Weising, and Rotter 2014). Now methods such as polymerase chain reaction (PCR) and gene or genome sequence comparisons are providing a much higher resolution for taxonomic classification and development of diagnostics, and are leading to revisions in nomenclature (Vauterin et al. 1995; Blom et al. 2009). These technologies enable a much better understanding of the genetic relatedness that organisms share, rather than just grouping pathogenic strains solely based on host range studies. In the *Xanthomonas*, along with many species and pathovars, lots of confusion and uncertainty has resulted due to the multiple re-orderings of the phylogenetic structure (Vauterin, Rademaker, and Swings 2000). As stated by Vauterin et al the term ‘pathovar’ was designed for special-purpose nomenclature to fulfill the needs of plant pathologists, who had to assign names to organisms that were specific to certain hosts or caused a certain disease (Vauterin et al. 1990). Vauterin contends that bacterial taxonomists should be concerned with classifying organisms based on true genealogical relationships, rather than based on phenotypic traits, i.e. phytopathogenicity. Fortunately, with recent advances and improvements in both genome sequencing and analysis, true phylogenetic relationships can now be uncovered, and resolution of the relationships of xanthomonads is becoming a reality.

Much renaming has occurred in many of the xanthomonads. Development of genome-based molecular diagnostics and taxonomic clarification has been particularly successful for the rice pathogens *Xanthomonas oryzae* pv. *oryzae* and pv. *oryzicola* (Rodriguez-R et al. 2012; Vauterin et al. 1995; Lang et al. 2014; Lang et al. 2010). *X. oryzae* for instance, was first classified as *Bacillus oryzae* by Bokura in 1911, and was later renamed as *Pseudomonas oryzae* then again reclassified later as *Xanthomonas oryzae* (Ishiyama 1922). In China, a study differentiated the two causal agents of the two diseases on rice, bacterial blight (BB) and bacterial leaf streak (BLS), and gave the pathogen of BLS the name *Xanthomonas oryzicola* (Fang et al. 1957). However, later the bacteria was reclassified as *X. translucens* f. sp. *oryzae* (Goto 1964). *X. oryzae* pv. *oryzicola* had also previously been referred to as *X. translucens* f. sp. *oryzicola* and also *X. campestris* pv. *oryzicola* (Ou 1985; Niño-Liu, Ronald, and Bogdanove 2006). Finally, in 1990 both pathogens were assigned to *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, two separate pathovars in the same species that cause different diseases, BB and BLS, respectively (Goto 1992; Vauterin et al. 1990). With all of this back and forth naming, the species *X. oryzae* has been a particularly good model for resolution of phylogenetic relationships through genomic studies; these, in turn, have helped with the understanding of virulence mechanisms, host range specialization, as well as the development of genome-based diagnostics (Lang et al. 2010; Lang et al. 2014; Wilkins et al. 2015; Triplett et al. 2011).

Techniques such as gene and genome sequencing and polymerase chain reaction (PCR) changed the face of diagnostics yet again, because they enabled accuracy in predicting taxonomic relationships and diagnostics unmatched by other techniques. In *Xanthomonas,* information derived from DNA sequences from conserved genes, such as 16S and other ribosomal DNA

sequences, resulted in a reassessment of taxonomy (Vauterin et al. 1995). These and other nucleotide based methods provide undisputable answers for pathogen classification and diagnostics. PCR is a very accurate and reliable tool for pathogen detection and has been heavily used in diagnostics of *Xanthomonas*. With two specific and specialized primers binding only to appropriate nucleotide sequences, amplification of a gene or genomic region can provide an answer to what genus, species, pathovar, or even geographic origin a plant pathogen is from. A relatively recent innovation, the PCR method called Loop Mediated Isothermal Amplification, has added new sensitivity to the detection process (Notomi et al. 2000). This technique uses a completely different DNA polymerase enzyme that is from *Geobacillus stearothermophilus*, rather than from *Thermus aquaticus*. The importance of this enzyme is that it has the ability to displace DNA strands while amplifying. Therefore, the need for thermal cycling and denaturing the DNA into two strands before primer annealing, is eliminated. This enables the reaction to occur at a constant temperature typically around 65°C. Additionally LAMP utilizes four to six different primers for added sensitivity and specificity.

As I began my research, there were no published methods that distinguished among the pathovars of *X. translucens*, although studies had shown there were distinct groups within the species (Bragard, Verdier, and Maraite 1995; Bragard et al. 1997). Few studies had applied PCR as a means of detection or for identifying the species *X. translucens* using conserved ribosomal DNA sequences, but none were able to distinguish single pathovars or a group of pathovars associated with a certain host range.

Scope of the thesis

The scope of this thesis is to help resolve the complex taxonomy and classification of the *X. translucens* complex, and to use comparative genomics to develop genome-based diagnostics that is associated with *X. translucens*. In Chapter 2 I report the draft genome sequences of 15 strains from six *X. translucens* pathovars. These draft genomes were analyzed to reveal phylogenetic relationships of organisms in the *X. translucens* complex. I identified and used unique regions found in the genomes of pathovars or groups of pathovars, to develop LAMP PCR primers to improve molecular detection and differentiation of *X. translucens.* I report five LAMP assays that detect the pathovars *cerealis* and *poae* specifically, pathovars associated with cereal leaf steak (*translucens*/ *hordei*, *undulosa*, *secalis*), and additionally two species specific assays, one of which does not amplify most *graminis* pathovars. This work was a collaboration with Jacob Snelling, John Hamilton, Claude Bragard, Ralf Koebnik, Valérie Verdier, Lindsay R. Triplett, Ned A. Tisserat, and Jan E. Leach. The release of these genomes and molecular diagnostic assays will be important tools to provide valuable insights for improving the understanding of *X. translucens* pathogenicity and epidemiology.

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CHAPTER TWO

CHARATERIZATION OF THE *XANTHOMONAS TRANSLUCENS* COMPLEX USING DRAFT GENOMES, COMPARATIVE GENOMICS, PHYLOGENETIC ANALYSIS, AND DIAGNOSTIC LAMP ASSAYS

Introduction

The *X. translucens* complex causes diseases on a broad host range of cereals and non-cereal grasses. Classification of *X. translucens* pathovars has been based on the host from which they were first isolated, or sometimes, based on different symptoms caused on the same host or different hosts (Bragard et al. 1997; Vauterin, Rademaker, and Swings 2000). Diseases were assigned names for the different hosts infected or symptoms associated with the presence of *X. translucens*. When *X. translucens* infects the glumes of cereals, the disease is referred to as black chaff, and when infection results in blighting of cereal leaves it is referred to as cereal leaf streak (CLS) (Dye et al 1980). In forage or turf grasses, disease caused by *X. translucens* is referred to as bacterial wilt (Mitkowski et al. 2005; Dernoeden et al.; Egli and Schmidt 1982).

Attempts to assign pathovar names to *X. translucens* is further complicated because the pathovar designation frequently does not reflect overlapping host ranges among pathovars and variation in host ranges within pathovars (Maes 1996). For example, CLS-associated strains, while often most virulent on the host of isolation (Rademaker et al. 2006), also can infect a variety of additional hosts. For example strains isolated from barley are still able to cause disease on wheat and rye and in some cases, other non-cereal hosts, including bromegrass (*Bromus* spp.) and quack grass (*Elymus repens*) in inoculation studies (Bragard et al. 1997; Boosalis 1952; Azad

and Schaad 1988; Rademaker et al. 2006). Some *X. translucens* strains colonize and cause disease on plants outside of the cereal and grass host range. For example, *X. translucens* pv. *undulosa* has been isolated from ornamental asparagus, and there is a newly proposed pathovar, *X. translucens* pv. *pistachiae,* which is virulent on a number of hosts outside of its natural host range (Rademaker et al. 2006; Facelli et al. 2009; Adhikari et al. 2012; Convener et al. 2012; Marefat et al. 2006).

As a result of the above history, there are ten named pathovars of *X. translucens*, three of which are commonly isolated from cereals (pvs. *secalis, translucens, undulosa,* and, in some cases, *cerealis*). *X. translucens* pv. *secalis* is associated with rye, while *X. translucens* pv. *undulosa* is found on wheat and triticale (Smith 2006). *X. translucens* pv. *translucens* includes strains from barley, which were previously named pv. *hordei*, but are now grouped with pathovar *translucens* (Bragard et al. 1997; Smith 2006). In addition to the three cereal pathovars, there are six *X. translucens* pathovars pathogenic to non-cereals (pvs. *arhennatheri*, *phlei*, *phleipratensis*, *poae*, *graminis*, and, in some cases *cerealis*) (Maes 1996).

A consequence of the overlapping in host range is the complication of pathovar-level diagnosis for *X. translucens*. This is particularly important for the cereal pathovars, which are seed disseminated, and thus, have implications for controlling of disease spread and quarantine regulations (Majumder et al. 2013; Tubajika et al. 1998). Attempts to distinguish pathovars using DNA-DNA hybridization, membrane protein assays, and biochemical, physiological, and serological tests provided insights into variation within the species, but did not resolve at the pathovar level (Vauterin et al. 1990; Rademaker et al. 2006; Bragard et al. 1997; Schaad 1979;

Azad and Schaad 1988; Elrod and Braun 1947). Methods with improved resolving power, such as genomic fingerprinting techniques (Amplified Fragment Length Polymorphism (AFLP) and Restriction Fragment Length Polymorphisms (RFLP), still did not resolve pathovars (Vauterin, Rademaker, and Swings 2000; Maes 1996; Rademaker et al. 2006). Of the methods in practice today for identification of *X. translucens*, the most commonly used techniques include enrichment using semi-selective media (Duveiller 1990; Schaad and Forster 1984) followed by serological tests such as ELISA (Frommel and Pazos 1994; Azad and Schaad 1988), immunofluorescence assays (Duveiller and Bragard 1992; Bragard, Verdier, and Maraite 1995), or rDNA based PCR amplification with primers to 16S, 23S, and ITS regions (Maes 1996; Marefat et al. 2006; Mitkowski et al. 2005). While these methods, particularly the PCR-based methods, have improved detection of *X. translucens*, they still do not differentiate among pathovars, nor do they reflect the relatedness within the *X. translucens* complex (Maes 1996; Marefat et al. 2006; Mitkowski et al. 2005).

Comprehensive analysis of *Xanthomonas* spp has shown that phytopathogenic specializations are frequently not correlated to phylogenetic relationships (Vauterin and Swings 1997). However, using comparative genomic analyses, enabled by whole or partial genome sequencing, relationships and distinctions among pathovars, geographical groups, or phylogenetic clades for some *Xanthomonas* species have been resolved (Francis et al. 2013; Rasko et al. 2008; Lang et al. 2014; Triplett et al. 2014). These comparative genomic approaches identified unique genomic regions that allowed development of pathovar-specific diagnostic tools, including PCR-based assays that distinguish *X. arbicola* pv. *pruni* from other *X. aribolara* pathovars (Pothier et al. 2011), *X. oryzae* pv. *oryzae* from *X. oryzae* pv. *oryzicola* (Lang et al. 2014), and *X. oryzae* pvs.

oryzae and *oryzicola* from a US-derived group of *X. oryzae* with no pathovar designation (Triplett et al. 2011).

The use of comparative genomics information for development of pathovar-specific diagnostics for the *X. translucens* complex has been limited not only by the fuzzy taxonomy, but also by the small amount of genomic data available for *X. translucens*. The first three *X. translucens* draft genomes were made public in 2012 [*X. translucens* DAR61454 (Gardiner et al. 2014) ; *X. translucens* pv. *graminis* ART-Xtg29 (Wichmann et al. 2013); *X. translucens* pv. *translucens* DSM 18974 (Vorhoelter et al direct submission, 2015)]. During the preparation of this manuscript, genomes for *X. translucens* pv. *cerealis* CFBP2541 (Pesce, Bolot, Cunnac, et al. 2015), *X. translucens* pv. *graminis* 2053 (Pesce, Bolot, Berthelot, et al. 2015), *X. translucens* pv. *poae* LMG728 (Wibberg direct submission, 2015), were announced. These genome sequences are being used to better understand the pathogenic specializations and virulence mechanisms of this important *Xanthomonas* species (Pesce, Bolot, Cunnac, et al. 2015; Wichmann et al. 2013).

In this study, we report the draft genomes for 15 *X. translucens* strains, representing six different pathovars. Our objectives were to use comparative genomic approaches to clarify the phylogenetic relationships among pathovars of *X. translucens* and to enable the development of robust diagnostic tools for related groups. Loop Mediated Isothermal Amplification (LAMP) assays were developed that distinguish *X. translucens* from other species of *Xanthomonas* and that distinguish *X. translucens* isolated from cereals from those isolated from non-cereal grasses.

Materials and Methods

Genome sequencing and comparative genomics

Genomic DNA from 15 *X. translucens* strains representing six pathovars were extracted using the Invitrogen, Easy-DNA gDNA Purification Kit, and sequenced using either the Illumina GAIIX or HiSeq2000 platforms for paired-end 75 bp or 100 bp reads, respectively. Sequencing was performed at the Research Technology Support Facility at Michigan State University, the Epigenome Center Data Production Facility at the University of Southern California, the Genome Center Core Facilities at the University of California - Davis, and DNA Vision in Belgium (Avenue George Lemaitre 25 - B-6041 Charleroi). Genomes were assembled with the Velvet short read sequence assembler V1.1.06. Gene models were first predicted using GLIMMER (http://www.cs.jhu.edu/~genomics/Glimmer/), then annotated using the MAKER genome annotation pipeline. Later assemblies were machine annotated using the Rapid Annotation using Subsystem Technology (RAST) server (Aziz et al. 2008). Genome comparisons were made by virtual DNA-DNA hybridization of the assembled draft genome sequences by using JspeciesV1.2.1. Average nucleotide identity (ANI) values were determined by MUMmer (ANIm); ANI values greater than 95% are considered to correlate to DNA-DNA hybridization values that infer species boundaries (Goris et al. 2007; Richter and Rosselló-Móra 2009). Draft genome assembly details and MUMer Average Nucleotide Identity (ANIm) comparisons were performed against CFBP 2054 (pv. *translucens*), ATCC-33804 (pv. *poae*), NCPPB1944 (pv. *cerealis*), and CFBP 2053 (pv. *graminis*) for our sequenced and publicly available *X. translucens* genomes. The open reading frames of twelve housekeeping genes (*atpD, dnaK, fusA , fyaA, glnA, gltA, groEL, gyrB, kup, lepA, recA,*and *rpoD*) were identified in the *X. translucens* genome assembled contigs. Sequences were concatenated, aligned, and gaps

removed using CLC Genomics Workbench, to yield 20 KB of sequence for multilocus sequence typing (MLST) analysis. The concatenated alignment was examined for the best fit phylogenic model using MEGA 6. Bayesian phylogenetic analyses were performed using the MrBayes program (http://mrbayes.sourceforge.net/), using the general time-reversible model with inverse-gamma rates of evolution for 1,000,000 generations. TreeGraph (http://treegraph.bioinfweb.info/) was used to construct a phylogenetic tree.

The EDGAR platform (Blom et al. 2009) was used to generate phylogenetic trees based on the complete core genomes of 41 *X. translucens* genomes. Strains *X. translucens* pv. *phleipratensis* PDDCC5744 and *X. translucens* pv. *graminis* UPB1175 were left out due to contamination and poor assembly quality.

The core genome of the remaining 41 strains consisted of 1,092 CDS per genome, summing up to 44,772 CDS with 13,881,345 amino acid residues in total. The 1,092 core gene sets were aligned individually using the MUSCLE software (Edgar 2004) and subsequently concatenated. The resulting huge multiple alignment was used to generate a phylogenetic tree. A distance matrix was calculated using the Kimura method and a rooted tree was inferred using the *Unweighted Pair Group Method with Arithmetic mean* (UPGMA) method as implemented in the PHYLIP package (Felsenstein 1995).

Primer design, optimization, and screening

To develop diagnostic primers that differentiate between the cereal and non-cereal pathovars of *X. translucens*, unique sequences were identified using in-house primer pipeline developed by Lindsay Triplett (unpublished) called Uniqprimer version 0.5.0 that was scripted to complement the speed and utility of the MUMmer 3.0 sequence aligner (Kurtz et al. 2004). Regions highly

specific to individual *X. translucens* pathovars or groups of pathovars were identified by first comparing the assembled contigs of all available *X. translucens*. These sequences were compared to all publically available plant pathogenic bacterial genomes in the NCBI database in 2012 to ensure they would amplify the target only in a mixed population sample. Primer conditions were selected for a constant 60°C annealing temperature and a 100-800 bp range of amplicon size. The hundreds of primer pairs identified in the first pass were subjected to a second pass of *in silico* screening that involved evaluation of primers for non-specific binding using the primer search algorithm in the EMBOSS software suite (Rice, Longden, and Bleasby 2000). Only primer pairs with perfect matches to the target *X. translucens* pathovars were advanced to PCR screening. Primers were synthesized by Integrated DNA Technologies (Coralville, IA).

Once specificity for the targeted regions was confirmed by conventional PCR, four LAMP-PCR primer sets were designed for each locus, each set consisting of a forward inner primer (FIP), a backward inner primer (BIP), a forward outer primer (F3), and a backward outer primer (B3), using Primer Explorer V4 (http://primerexplorer.jp/e/). No loop primers were used. All LAMP primer sets were evaluated *in silico* for specificity to all available nucleotide sequences in the NCBI database in 2013. Specificity of LAMP primers was assessed first by screening target and non-target bacterial isolates for amplification with the outer primers (F3 and B3) with conventional PCR followed by separation in agarose gels. One specific LAMP primer set was selected per locus for subsequent screening with other diverse bacteria. Once specificity was confirmed, the primers were tested for sensitivity of detection using serial dilutions of bacterial cells (below).

Bacterial isolates and plant samples

Purified bacterial DNA, bacterial cells, *X. translucens* infected leaf tissue, and *X. translucens* contaminated seed lots were used in LAMP detection assays. Bacterial DNA was purified using the Invitrogen Easy-DNA^{TM} Kit, and was adjusted to 20 ng μl^{-1} for assays. For direct use in PCR assays, bacterial cells were streaked for single colonies from glycerol stocks onto nutrient agar (NA), and incubated at 28° C for 2 days. Bacteria were suspended in sterilized distilled water to OD_{600} of 0.2, and the suspensions were heated to 95° C for 15 min in a thermocycler prior to use as templates for PCR reactions. *X. translucens* strains, seed lot samples, leaf samples, bacterial cultures received as unknowns, and all bacterial strains used in this study are listed in Table 3-4 and Table S.2-S.3. Purified bacterial isolates were stored long term in 25% glycerol at -80° C. Seed lot and leaf samples were tested for presence of *X. translucens* by culture methods as well as LAMP assays as described below. To isolate bacteria from leaf tissue exhibiting disease symptoms, approximately 3 cm^2 of wheat or barley leaf tissue, including the edge of necrotic lesions, was excised. Leaf tissue pieces were soaked in 1 ml distilled water for 5 min and crushed with a pipette tip. A 100 µl aliquot of each extract was treated at 95° C for 15 min and used as template for PCR and LAMP reactions. The remaining extracts were diluted serially to 10^{-4} or 10^{-5}, and 100 µl of each dilution was spread on modified Wilbrink's boric acid-cephalexin (WBC) semi-selective medium containing 10 mg $liter^{-1}$ Cephalexin, 5 mg $liter^{-1}$ Neomycin, and 200 mg $liter^{-1}$ Cyclohexamide (Duveiller 1990; Spradlin 1990).

Bacterial isolation from contaminated barley seed lots was performed using a protocol developed by C. Bragard (personal communication). Samples of 40 g seed were mixed with 40 ml (w:v = 1:1) of cold saline solution (0.85% NaCl containing 0.02% v/v Tween 20) and shaken vigorously

for 3-5 min. The seed saline mixture was allowed to settle for 1 min. Then 100 µl of seed supernatant was heat treated at 95° C for 15 min in a thermocycler, and used as template for LAMP reactions to detect *X. translucens* directly from field samples. With the remaining supernatant, one ml was serially diluted to 10^{-4} or 10^{-5}, and 100 µl was spread evenly across three plates of modified WBC media as mentioned above. Suspected *X. translucens* colonies appearing on modified WBC were sub-cultured for further testing by LAMP-PCR (below).

Reaction conditions and Reaction mixtures for LAMP

Real-time LAMP reactions were performed on a Bio-Rad CFX Connect Real-Time (Bio-Rad, Hercules, CA) system at 65° C. The reaction mixture contained 7.2 µl Isothermal Master Mix (Optigene, Sussex, United Kingdom) and 640 nM FIP/BIP and 64 nM F3/B3, respectively, for *ina*-Xt, *gyrB*-Xt and Xt-CLS primers. The Xt-Poae and the Xt-Cerealis primers were added at half the concentrations listed above. One µl of template (bacterial genomic DNA at 20 ng μl^{-1}, heat-killed bacterial cells, seed wash supernatant, or excised leaf tissue extract) was added per 12 µl reaction and incubated in a real time thermal cycler. Samples were incubated for 70 min at 65° C for isothermal amplification followed by a melt curve analysis from 60°C to 95°C in 0.5°C increments to observe LAMP product melt temperature ranges. Consistent melt temperature ranges indicated amplification of the same single region and demonstrated target specificities. All LAMP assays were replicated at least twice per sample, and all experiments included positive controls and no-template controls.

We tested visual detection protocols for LAMP PCR assays to facilitate field diagnoses .Visual detection LAMP assays were performed by heating the reaction mixture in a thermal cycler or a

water bath at 65°C for 70 min. The 25 µl reaction mixtures were as follows: 0.8 M Betaine (Sigma Aldrich, St. Louis, MO, B0300-1VL), 1.4 mM dNTPs, 6 mM additional $MgSO_4$ for a final concentration of 8 mM (New England Biolabs, Ipswich, MA), 10x Isothermal Amplification Buffer (New England Biolabs, Ipswich, MA #B0537S), the respective LAMP primers at the concentrations mentioned above, 4 U *Bst* DNA Polymerase 2.0 (New England Biolabs, Ipswich, MA), and 2 µl of template to be amplified. Mineral Oil (EMD Millipore, Darmstadt, Germany) was added on top of the reaction mixture (20 µl) to minimize introduction of aerosolized product in workspaces. Reactions were performed in individual 0.2 ml PCR tubes to prevent cross contamination during pipetting. One µl of the intercalating dye Quant-IT™ PicoGreen® Reagent (Invitrogen, Carlsbad, CA, USA, P11495) was added post-reaction to the samples and briefly centrifuged and vortexed to mix the PicoGreen with the sample. A positive amplification resulted in a color change from orange to green. In later assays, to prevent the release of aerosols, we used a complete closed tube system in which 2 µl of a 1:1 mix of PicoGreen and glycerol was added to the inside of reaction tube lids. After completion of reactions, the tubes were centrifuged briefly, vortexed, and examined under normal and ultraviolet light to determine amplification.

LAMP primer specificities were determined using a pooling strategy to screen collections of bacterial species (Lang et al. 2014). Non-target bacterial DNAs (40 ng each) or dead bacterial cells adjusted to 0.2 OD_{600} (2 µl each) were pooled in equal concentrations of 10 different isolates in each pool. A duplicate of each pooled sample was spiked with 1 µl of positive-control genomic DNA or *X. translucens* cells to validate detection in a mixed sample. *X. translucens* strains from diverse geographic regions and different hosts were screened individually using the

ina-Xt and *gyrB*-Xt primers, as well as the pathovar-specific primers Xt-Poae, Xt-Cerealis, and the cereal-specific Xt-CLS primers. Positive controls included known *X. translucens* strains whose genomes were used in primer design and others that had been tested previously via pathogenicity tests and confirmed to be *X. translucens*. All bacterial isolates and pooled bacterial samples were screened a minimum of two times with each assay. Assay sensitivities were determined using serial dilutions (1:10) of heat-killed cells adjusted to an OD_{600} of 0.2 (approximately 1 x 10^8 CFU ml^{-1}). Each dilution (from 10^8 to 10^1 CFU ml^{-1}) was tested a minimum of three times, averaged, and standard deviations were calculated to correlate bacterial number with cycle of amplification.

Results

Draft genome sequence and assembly.

Assembly details for the 15 draft genome sequences generated for *X. translucens* strains representing six pathovars are shown in Table 1. Assemblies ranged between 4.21 and 4.96 Mb, with GC content ranging from 65.6-68%. The sequence data from this study is currently being deposited NCBI under the strain names: UPB455, NCPPB1943, UPB437, ATCC-33804, CNC2-P4, Utah5-P1, B99, SIMT-07, SLV-2, BLSB3, UPB458, UPB787, NARK-1, BLSW16, and UPB513. The raw sequence reads will be deposited into the NCBI sequence read archive and given individual accession numbers.

Comparative genomics and phylogeny

To examine relationships among the species, assembled *X. translucens* genomic contigs, including those we generated and the publicly available genomes, were compared in a pairwise

manner using JSpecies and MUMer. All individual comparisons examined within the species complex fell at or above the accepted 95% ANIm threshold value for species delineation (Table 1).

A multilocus sequence typing (MLST) approach using 12 housekeeping genes (*atpD* 1280 bp, *dnaK* 1908 bp, *fusA* 2006 bp, *fyaA* 2135 bp, *glnA* 1396 bp, *gltA* 1046 bp, *groEL* 1586 bp, *gyrB* 2344 bp, *kup* 1807 bp, *lepA* 1783 bp, *recA* 999 bp, and *rpoD* 1810 bp for a total of 20 kb for the concatenated data set) was performed to examine phylogenetic relationships among the 21 strains of *X. translucens* representing six pathovars (Figure 1). Using the best fit phylogenic model in MEGA 6, a maximum likelihood tree was constructed from the MLST data. The phylogenetic reconstruction distinguished cereal and non-cereal *X. translucens* pathovars into two distinct clades. The non-cereal isolates grouped into smaller sub-clades, in general reflecting their pathovar designation. Within the cereal clade, pathovar *cerealis* strains formed a subclade that was distinct from the larger grouping of cereal pathogens (including pv. *translucens* and *undulosa* strains). These results agree with previous phylogenies for the species (Bragard et al. 1997).

The second phylogenetic tree was generated using the EDGAR platform with MUSCLE and PHYLIP packages (Fig S.1). This tree was based on a core genome of 1092 CDS between 41 strains and demonstrated the same grouping of cereal and non-cereal infecting strains, consistant with our MLST

We further addressed the relationships within the *X. translucens* complex by examining the homologous protein coding sequences (CDS) using EDGAR (Blom et al. 2009). A Venn diagram (Figure 2), based on strains representing five pathovars, shows that the strains share a core genome of 2,504 highly homologous genes. The variable genome includes hundreds of unique genes per individual pathovar and discrete sets of genes specific to either cereal or non-cereal pathovars. For these selected strains, 126 CDS were common to members of the cereal clade (*translucens, undulosa,* and *cerealis*), and 143 CDS were common to members of the grass clade (*graminis* and *poae*). Pathovar *cerealis*, which can cause disease on both non-cereals and cereals, shares 126 CDS with the cereal clade and only 50 CDS with the non-cereal clade, consistent with the MLST groupings.

LAMP assay sensitivities and specificities

Using genomic regions that were unique to (a) the species *X. translucens* relative to other *Xanthomonas* species, (b) the pathovar *cerealis*, (c) the pathovar *poae*, (d) and the group of pathovars *translucens*/*hordei*, *undulosa*, and *secalis*, we designed five sets of LAMP primers (Table 2). Alignment of the primers to draft genome sequences confirmed specificity for amplification of targets (Figure S.5). The primers and LAMP assays were tested on eight other bacterial genera and 15 other species and pathovars of *Xanthomonas*, for a total of over 150 bacteria from around 18 countries. All primers amplified only their intended targets (Table 3). All five sets of LAMP primers detected their targets in dilutions of heat-killed bacteria over the range of 10^3-10^4 CFU ml^{-1} when assays were performed in a Real-Time thermal cycler (Figure 3 and 4). Assay sensitivity thresholds were consistent with previous reports for other plant-pathogenic bacteria, ranging from 10^3 and 10^4 CFU ml^{-1} (Lang et al. 2014; Ash et al. 2014;

Bühlmann et al. 2013). The *gyrB*-Xt primers amplified all *X. translucens* pathovars. The *ina*-Xt primer set, developed from the conserved ice nucleation gene *inaX*, did not amplify *X. translucens* pv. *graminis* strains or the single *X. translucens* pv. *phlei* strain tested, but did amplify all strains in the remaining eight *X. translucens* pathovars. LAMP assays using with the Xt-Cerealis, Xt-Poae, and Xt-CLS primers amplified only their target pathovars (Table 3 and Table S.2).

We tested visual detection protocols for LAMP PCR assays to facilitate field diagnoses. Reactions included SYBR green, and were performed in 0.5ml closed tubes, by adding mineral oil to the top of the reactants, and placing a 1:1 picogreen-glycerol mix to the inside of the tube cap before amplification. Spinning down tubes post-reaction enables no need to open the tubes. Reactions were performed in thermal cyclers and laboratory water baths for 70 min and 65°C. Due to the very high efficiency of amplification, a critical warning for visual detection assays with LAMP PCR is to not open tubes post reaction, because the amplification products are aerosolized and will contaminate the laboratory environment. Opening the caps can be avoided by adding a 20 µl of mineral oil to the top of the reactants, and placing 2 µl of a 1:1 picogreen-glycerol mix to the inside of the tube cap before amplification. For all primer sets, specific targets were amplified and detected from heat-killed bacterial cells and crude preparations from barley seed or wheat and barley leaf tissue. An example showing detection of SYBR green-stained products using the Xt-Poae LAMP assay is shown in Figure S.4.

Detection from Leaf and Seed field samples

Barley leaves and seed lots and wheat leaves that exhibited varying degrees of symptoms, and that were suspected of being infected with *X. translucens,* were tested for presence of *X. translucens* using the LAMP PCR assays (Table 4 and S.3). Of the 13 barley seed lots, only two did not test positive in LAMP assays for *X. translucens,* and did not yield *X. translucens*-like colonies on modified WBC media. The isolates did not amplify with the Xt-Cerealis or Xt-Poae primers, but did amplify with the Xt-CLS primers. Similarly, the four barley leaf samples that were symptomatic for CLS yielded *X. translucens*-like colonies on modified WBC media, and amplified with the *ina*-Xt, *gyrB*-Xt, and Xt-CLS primers. A single wheat leaf sample yielded *X. translucens* colonies on modified WBC media as well as tested positive under the *ina*-Xt, *gyrB*-Xt, and Xt-CLS assays but did not amplify under the Xt-Cerealis and Xt-Poae LAMP assays. Based on the specificities of the primers, our results show that the field isolates from barley and wheat are members of the cereal clade of *X. translucens,* which includes *X. translucens* pvs. *undulosa, translucens,* or *secalis.* The turf grass sample tested amplified with the Xt-Poae primers, but not with the cereal-specific primers or the Xt-Cerealis primers, consistent with the sample being infected with *X. translucens* pv. *poae.*

Discussion

Resolving relationships among pathovars and strains in the *X. translucens* complex has been the topic of a number of studies, each using approaches with different levels of resolution (Bragard et al. 1997; Vauterin, Rademaker, and Swings 2000; Rademaker et al. 2006). Our comparative genome and phylogenetic analyses, which were based on draft genome sequences that we and others generated (Table 1) and MLST comparisons, confirmed that all tested 21 *X. translucens*

strains representing six pathovars are within the species *translucens* (ANI > 95%). Phylogenetic analyses, based on MLST using 12 housekeeping genes and EDGAR using CDS derived from the draft genome sequences, allowed separation of the *X. translucens* strains into two distinct clades. One clade distinguished strains associated with cereals, particularly barley and wheat (*translucens* and *undulosa*) from those associated with non-cereals (*arrhenatheri*, *poae*, and *graminis*). Strains representing pathovar *cerealis* grouped within this cereal clade, but more distant from other pathovars; strains of pathovar *cerealis* are known to infect cereals (wheat, barley, and rye) as well as non-cereals (bromegrass and quack grass) (Bragard et al. 1997; Rademaker et al. 2006). The revelation of two major clades confirms previous phylogenetic groupings developed using other technologies (Rademaker et al. 2006; Bragard et al. 1997). Based on the EDGAR analysis of homologous protein coding sequences (Figure 2), we observe a core genome of 2504 genes for five different pathovar strains of *X. translucens*: CFBP2541 (pv. *cerealis*), DAR61454 (pv. *translucens*), ART-Xtg29 (pv. *graminis*), ATCC-33804 (pv. *poae*), and Xtu 4699 (pv. *undulosa*). This analysis shows the relative amount of all genes that are shared between and among the strains compared, as well the number of genes unique to each isolate representing one of the five above mentioned pathovars. The distinction of the *cerealis* pathovar in our venn diagram comparison can be demonstrated by observing the CDS it shares with the cereal host strains as well as the non-cereal host strains. When looking at the two non-cereal strains ATCC-33804 and ART-Xtg29 which are the pathovars *poae* and *graminis* respectively, they uniquely share 143 coding sequences (CDS). When looking at how many CDS these two non-cereal strains only share with the *cerealis* strain NCPPB1944, we see that they only share 50 CDS. In the case of the cereal host strains, pv. *translucens* DAR61454 and pv. *undulosa* 4669, these two share 124 CDS with only each other. The number of CDS shared between both of

these cereal host strains and the *cerealis* strain NCPPB1944 is 126 CDS. This shows in another light, aside from the *cerealis* subclade in Figure 1, that the *cerealis* pathovar is more closely related to the cereal host strains due sharing 126 unique CDS, rather than only sharing 50 unique CDS with the non-cereal host strains. By focusing on regions of the *X. translucens* genomes that overlapped among pathovars or that were unique to specific pathovars, primers that enabled identification of the species *X. translucens,* and at the pathovar level for *X. translucens* pvs. *poae* and *cerealis*. Selection of genomic regions that were found only in the cereal-infecting group allowed development of primers that distinguished cereal from non-cereal-infecting pathovars. Using field-grown seed and leaves, LAMP assays with the developed primers detected and correctly group cereal-infecting *X. translucens* pathovars, and a non-cereal-infecting pathovar. LAMP protocols offer highly sensitive and simple tests that are adaptable to field conditions (Lang et al. 2014; Fischbach et al. 2015). Some reports (Lang et al. 2014; Ash et al. 2014; Bühlmann et al. 2013; Hong et al. 2012; Li and Ling 2014) include two additional primers called loop primers are sometimes used for added specificity and sensitivity. However it is not necessary for the reaction and in fact many LAMP primer designing softwares such as the one we used for this study, Primer Explorer V4 (http://primerexplorer.jp/e/), do not include them in the design. Others reports include visual detection assays that simplify even further the use of LAMP assays without the need for detection equipment (Fischbach et al. 2015; Lang et al. 2014). A major problem in developing and testing diagnostic tools for plant pathogenic bacteria is the historical misclassification of strains or mix-ups within culture collections. Of the large number of bacterial strains tested in this study, we observed only two cases where results of LAMP assays with our primers disagreed with prior classification or suggested strains may have been historically misnamed or mixed up. *X. translucens* pv. *phleipratensis* strain UPB441, was the

only of three pv. *phleipratensis* strains that amplified with the Xt-CLS primers. *X. translucens* pv. *graminis* strain UPB437, which has a 99.9% match to *X. translucens* pv. *graminis* strain ART-Xtg29, was the only of 13 *X. translucens* pv. *graminis* isolates to amplify with the *ina*-Xt and Xt-CLS primers, even if sequence alignments indicated the gene should not amplify. Without backtracking or re-inoculation of various hosts, it is difficult to resolve whether these discrepancies are due to misclassification, culture mix-ups, or due to genuine genetic variation in the genomes.

Due to the very high efficiency of amplification, a critical warning for visual detection assays with LAMP PCR is to not open tubes post-reaction, which aerosolizes the amplification product and contaminates the laboratory environment. Detection of *X. translucens* especially to the pathovar level, can take days or weeks to obtain results, due to the need of obtaining pure cultures and inoculate putative host plants. The LAMP assays described in this study allow detection and diagnosis of *X. translucens* in plant tissues, and supports distinction to cereal or non-cereal groups that may guide regulatory officials concerned with movement of the cereal-infecting pathovars to new regions.

In conclusion, the comparative genomic analysis of the *X. translucens* complex, enabled by the draft sequencing of 15 new genomes, confirms that all tested strains belong in the species *X. translucens*, and that there are two distinct clades within the species. The release of these *X. translucens* genomes will contribute to our understanding of *X. translucens* phylogeny and will provide a platform for mining traits that contribute to pathogenic specialization and virulence. The LAMP assays that distinguish the clades as well as some pathovars within the species will

be a valuable resource for detection, diagnosis and monitoring of this group of important pathogens.

Table 1. Genome assemblies of *X. translucens* strains sequenced in this study, and those available at the time of this research. Average Nucleotide Identity (ANI) values confirm *X. translucens* species designation for our genomes and those previously published.

Pathovar	Strain	Sequence Length (bp)	Coding Sequences	GC %	MUMmer Average Nucleotide Identity (ANIm)				Source
					CFBP 2054^T	ATCC-33804^T	NCPPB 1944	CFBP 2053^T	
					translucens	*poae*	*cerealis*	*graminis*	
arrhenatheri	UPB455	4773032	4038	67.2	95.7	96.5	95.1	97.7	*Arrhenatherum elatius*: Switzerland
cerealis	NCPPB1943/ LMG7393	4424722	3745	65.6	95.1	95.0	99.8	95.1	*Hordeum vulgare*: United States
graminis	UPB437	4223356	3662	66.0	95.8	96.5	95.0	99.9	*Lolium multiflorum*: Switzerland
poae (NZ_CXOK01000001)	ATCC-33804/ LMG728[a]/ CFBP2057^T	4520765	3777	67.4	95.6	---	95.0	96.6	*Poa trivialis*: Switzerland
poae	CNC2-P4	4589301	3840	66.8	95.6	99.9	95.0	96.5	Turfgrass: North Carolina
poae	Utah5-P1	4612646	3849	67.6	95.8	96.5	95.1	97.5	Turfgrass: Utah
poae	B99	4592840	3942	65.4	95.5	99.8	94.9	96.5	Turfgrass: Illinois
translucens	SIMT-07	4527808	3871	67.3	97.5	95.5	95.2	95.7	*Hordeum vulgare*: Montana
translucens	SLV-2	4594304	3605	66.7	97.7	95.6	95.3	95.8	*Hordeum vulgare*: Colorado
translucens	BLSB3	4560169	3774	67.7	97.6	95.5	95.3	95.7	*Hordeum vulgare*: North Dakota
translucens	UPB458/ NCPPB2389	4546735	3784	67.0	98.5	95.4	94.8	95.5	*Hordeum vulgare*: India
translucens	UPB787	4964258	3616	66.9	98.8	95.7	95.2	96.0	*Hordeum vulgare*: Paraguay
Undulosa	NARK-1	4494696	3861	66.3	97.6	95.5	95.2	95.7	*Triticum aestivum*: Arkansas
Undulosa	BLSW16	4596175	3811	67.3	97.5	95.5	95.2	95.7	*Triticum aestivum*: North Dakota
Undulosa	UPB513	4677671	3752	68.0	97.6	95.5	95.2	95.7	*Triticum aestivum*: Mexico
Previously published or announced genomes (accession number)									
Cerealis (JWHD01000000)	NCPPB1944 / UPB945/ CFBP 2541^T	4489185	3887	63.5	95.1	95.0	---	95.0	*Bromus inermis*: United States
Graminis (NZ_LHSI00000000)	UPB1156/ CFBP2053$^{T\,a}$	4216996	3649	67.2	95.7	96.5	95.0	---	*Dactylis glomerata:* Switzerland
graminis (NZ_ANGG00000000)	ART-Xtg29	4100864	3319	69.0	95.8	96.5	95.1	99.9	*Lolium multiflorum*: Switzerland
translucens (NZ_AMXY01000000)	DAR61454	4452091	3846	68.3	97.5	95.5	95.2	95.6	*Triticum aestivum*: Australia
translucens (NZ_CAPJ01000155)	DSM18974/ CFBP 2054^T	4463577	3709	68.2	---	95.6	95.1	95.8	*Hordeum vulgare*: Minnesota
Undulosa (CP008714)	Xtu 4669	4561144	3585	68.1	97.5	95.5	95.2	95.6	*Triticum spp.*: Kansas

T= pathoype strain [a]= genome also from this study but announced previously

Table 2. LAMP primers that amplify *Xanthomonas translucens* pathovars. Abbreviations are FIP (Forward Inner Primer), BIP (Backward Inner Primer), F3 (Forward outer primer), and B3 (Backward outer primer). Primers were designed using PrimerExplorer V4 software based on unique genomic regions found by genome comparisons.

Primer set	Single Primer	Nucleotide Sequence	Target	Gene
ina-Xt	FIP	5'-TATCGTAGCCGGCGGTCTGGGACGGCGGGTCACGACA-3'	*X. translucens* pathovars except *graminis*	Ice nucleation protein
	BIP	5'-GCCCAACAAGACAGTTCGCTCAGCCGGCGATCAGCGTA-3'		
	F3	5'- GGCTACGGCAGTACCTCG-3'		
	B3	5'-GCCAGCGGTCTGCGTA-3'		
gyrB-Xt	FIP	5'- CGGGCATCTTCAACAGCGCGTATCGACCAGTTCGATGCCCA-3'	All *X. translucens* pathovars	DNA gyrase subunit B
	BIP	5'- GGTCAGCAACGCGATCACCGATCCGGCGCAGCTGTAC -3'		
	F3	5'- ACGAACAGGTCGTCGCC-3'		
	B3	5'-CTGCCGGTGGACGAGT-3'		
Xt-CLS	FIP	5'-AGCCAGATTGGCTTGCCTGCGATGAGGTGGCGCATTGG-3'	*X. translucens* pvs. *undulosa, translucens (hordei), secalis,*	Hypothetical protein
	BIP	5'-TGCAAGACAAATCTTCGTGCGCGTAGACAACTGCGCTTCCG -3'		
	F3	5'-AACGAGCGAAGCCGTATG -3'		
	B3	5'-GCATCCAACTTGGCTACAGT-3'		
Xt-Poae	FIP	5'- TTCCGAGCGCTGCTTGGAATTGTCAGGCTACCGAGGCTTTC-3'	*X. translucens* pv. *poae*	DNA-binding HN-S protein
	BIP	5'- TCGCGGGCTTTGTGATCAATGGAGTATTTGGGGGCCACTTCT-3'		
	F3	5'- AAGCCCAACTTCGGCAAG-3'		
	B3	5'- CTCTTGGGTGTGCGGAAG-3'		
Xt-Cerealis	FIP	5'-TAGATCGCTTCTGCTTCGGCTGTTTACGTCGAATCGTTCCGG-3'	*X. translucens* pv. *cerealis*	Spans the EF Hand Domain/ Calcium binding protein and pteridine-dependant deoxygenase
	BIP	5'- CTATCAGTTCGTGCGCTCGCCCTACTGGACGGTCGTGTCT-3'		
	F3	5'- GGCAAGCCGAACCAAAGT -3'		
	B3	5'-CGTTGAATAGCGACTGCGG-3'		

Table 3. Summary showing the specificity of *Xanthomonas translucens* primers in LAMP assays. Cells shaded in gray expected to amplify with the primer set in the same row. Sample sources and details are in Table S.2.

	Specificity (number of strains that amplified/number tested)										
Primer set	*arrenatheri*	*cerealis*	*Graminis*	*phlei*	*phleipratensis*	*poae*	*translucens*	*undulosa*	*secalis*	Other genera	Other *Xantho-monas*
gyrB-Xt	1/1	5/5	13/13	1/1	3/3	10/10	14/14	78/78	2/2	0/8	0/15
ina-Xt	1/1	5/5	1/13[a]	0/1	3/3	10/10	14/14	79/79	2/2	0/8	0/15
Xt-Cerealis	0/1	5/5	0/13	0/1	0/3	0/10	0/15	0/78	0/2	0/8	0/15
Xt-Poae	0/1	0/5	0/13	0/1	0/3	10/10	0/15	0/78	0/2	0/8	0/15
Xt-CLS	0/1	0/5	1/13[a]	0/1	1/3[a]	0/10	14/14	78/78	2/2	0/8	0/15

[a]Uncertain historical classification of *X. translucens* pv. *graminis* UPB437 and pv. *phleipratensis* UPB441.

Table 4. Validation of LAMP assays and isolation of *Xanthomonas translucens* with field-derived tissues. Each sample was tested at least two times per primer set. Cells shaded in gray expected to amplify with the primer set in the same row. All samples were isolated using modified WBC media and stored in 25% glycerol at -80°C . Sample sources and details are in Table S.3.

				Xt Lamp Primer Assays				
				(number of samples that amplified/number tested)				
Sample Type	Origin	Symptoms	*X. translucens* isolated	*ina*-Xt	*gyrB*-Xt	Xt-Cerealis	Xt-Poae	Xt-CLS
Barley Leaf	Montana (2), Idaho (2)	4/4	4/4	4/4	4/4	0/4	0/4	4/4
Wheat Leaf	North Dakota	1/1	1/1	1/1	1/1	0/1	0/1	1/1
Barley Seed	Colorado (5), Montana (4), North Dakota (4)	11/13	11/13	11/13	11/13	0/13	0/13	11/13

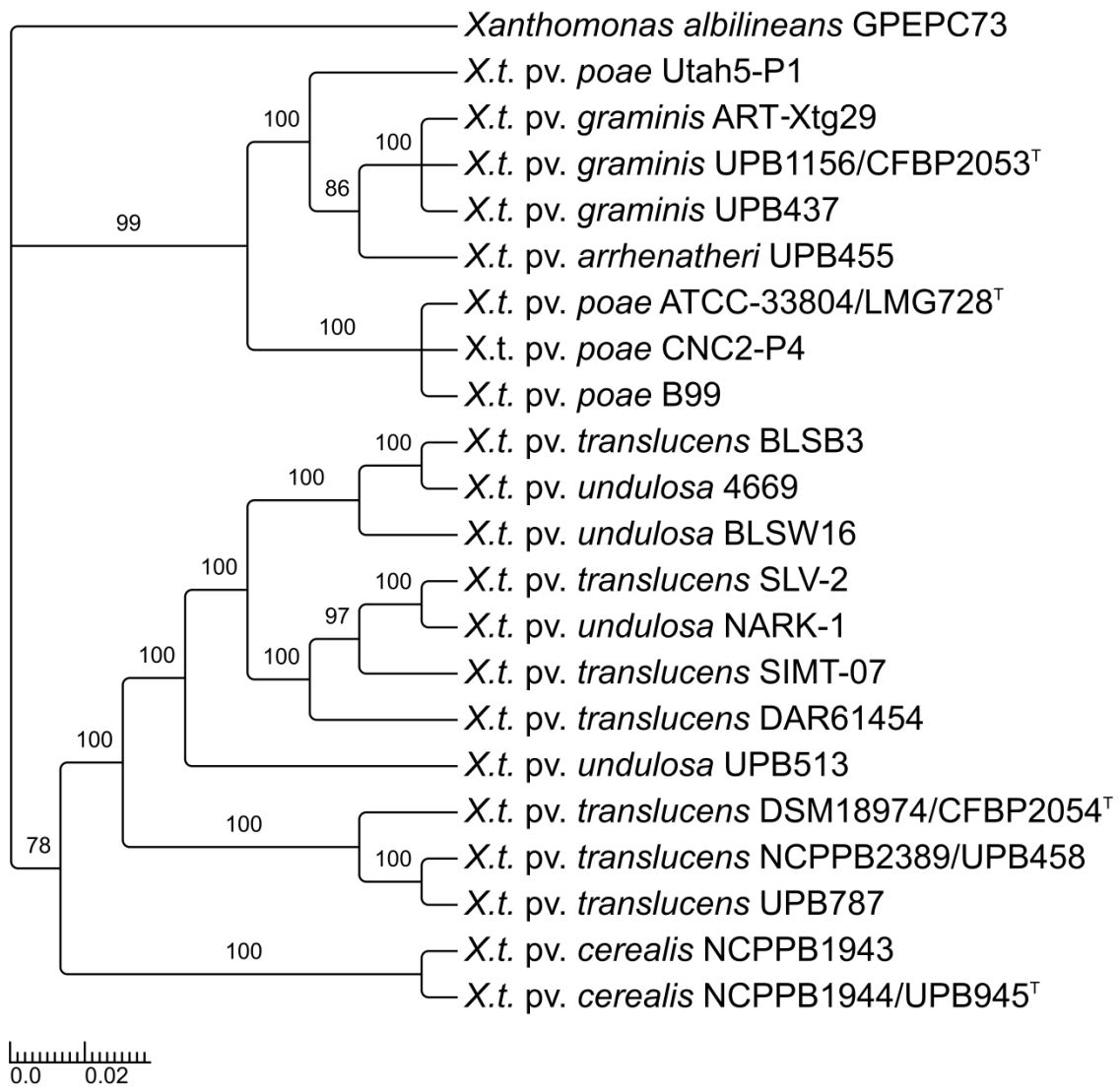


Figure 1. Phylogenetic tree of *X. translucens* pathovars (21 strains) based on 20 kb of concatenated sequences of 12 housekeeping genes (*atpD, dnaK, fusA , fyaA, glnA, gltA, groEL, gyrB, kup, lepA, recA,* and *rpoD*) that were identified from available *X. translucens* genome assembled contigs. Cereal and non-cereal *X. translucens* pathovars are distinguished into two clades by MLST using the best fit phylogenic model in MEGA 6. Bayesian phylogenetic analyses were performed using the MrBayes program, using the general time-reversible model with inverse-gamma rates of evolution for 1,000,000 generations. The phylogenetic tree was constructed using TreeGraph 2. *Xanthomonas albilineans* was included as an outgroup.

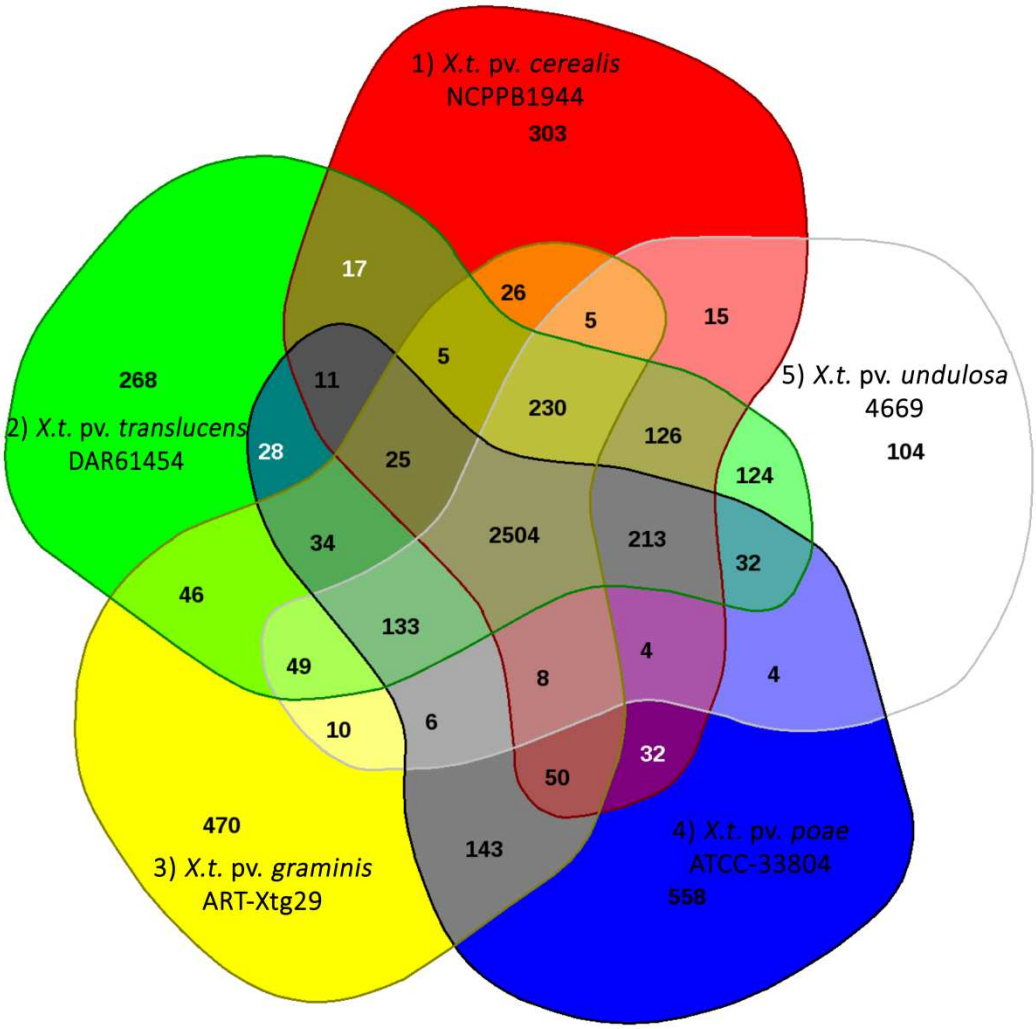


Figure 2. Venn diagram demonstrating the core and dispensible genomes for five different *Xanthomonas translucens* pathovars. This diagram shows unique and shared homologous protein coding sequences among of the pathotype strains pv. *cerealis* CFBP 2541; pv. *translucens* DAR61454; pv. *graminis* ART-Xtg29; pv. *poae* ATCC-33804; and pv. *undulosa* Xtu 4699.

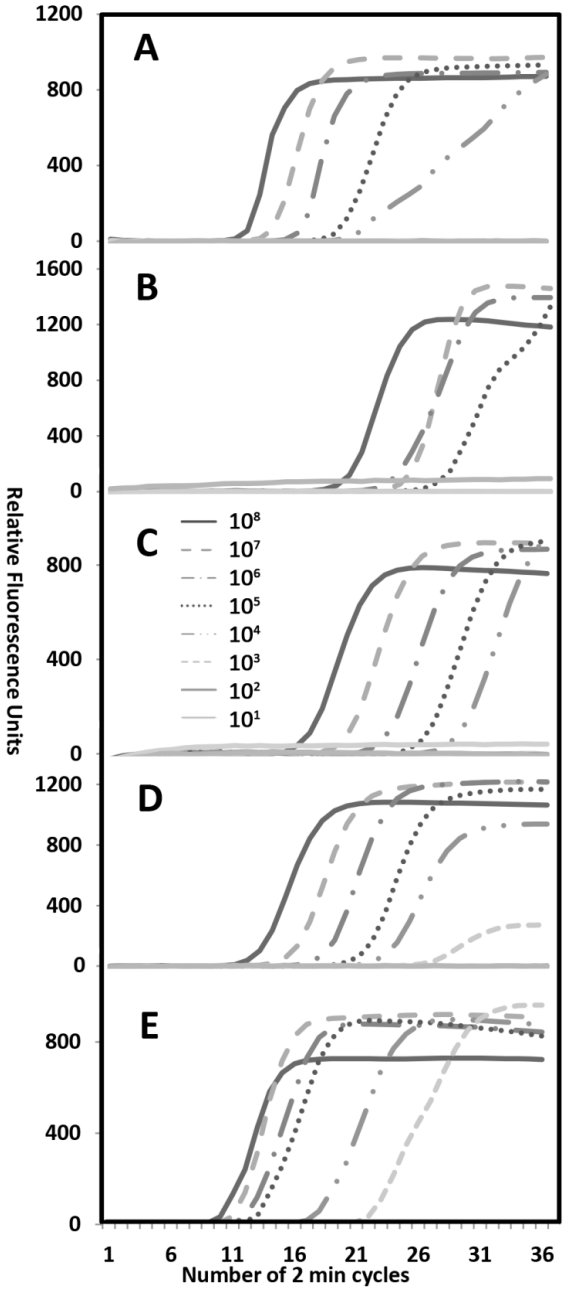


Figure 3. Sensitivity of Real-Time thermal cycler- LAMP primers using serial dilutions starting from 10^8 CFU mL^{-1} of *Xanthomonas translucens* heat-killed cells. (A) *ina*-Xt, showing amplification using barley seed isolated *X. t.* pv. *translucens*, (B) *gyrB*-Xt, showing amplification using barley seed isolated *X. t.* pv. *translucens*, (C) Xt-Cerealis, showing amplification of *X. t.* pv. *cerealis* strain B50; (D) Xt-Poae, showing amplification of *X. translucens* pv. *poae* strain ATCC-33804; (E) Xt-CLS, showing amplification using barley seed isolated *X. t.* pv. *translucens*. Each dilution was tested three times.

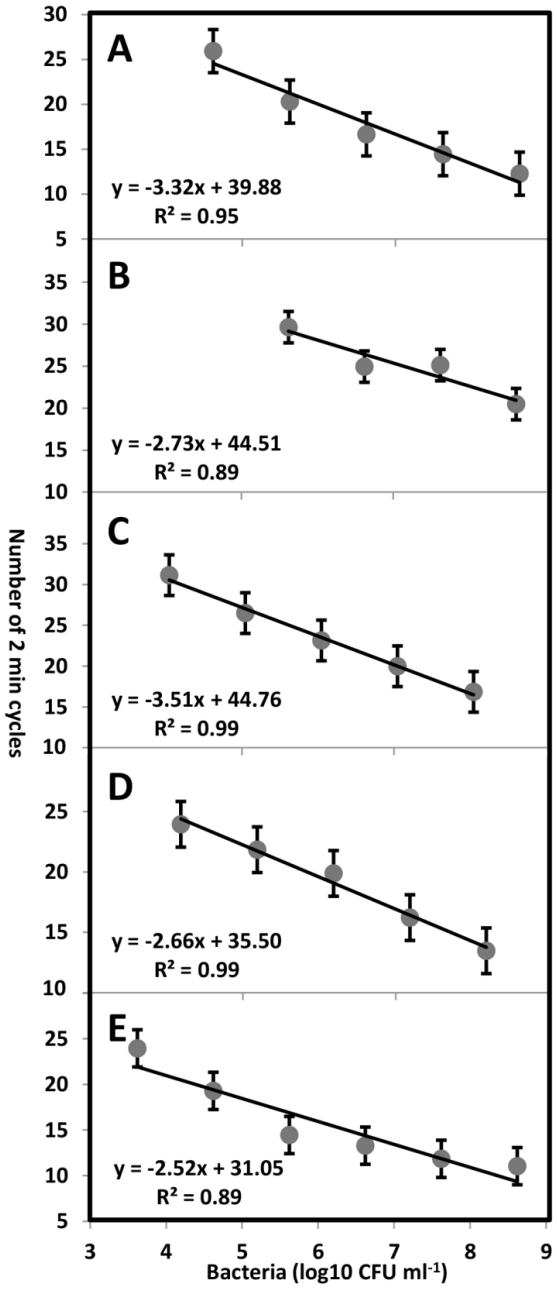
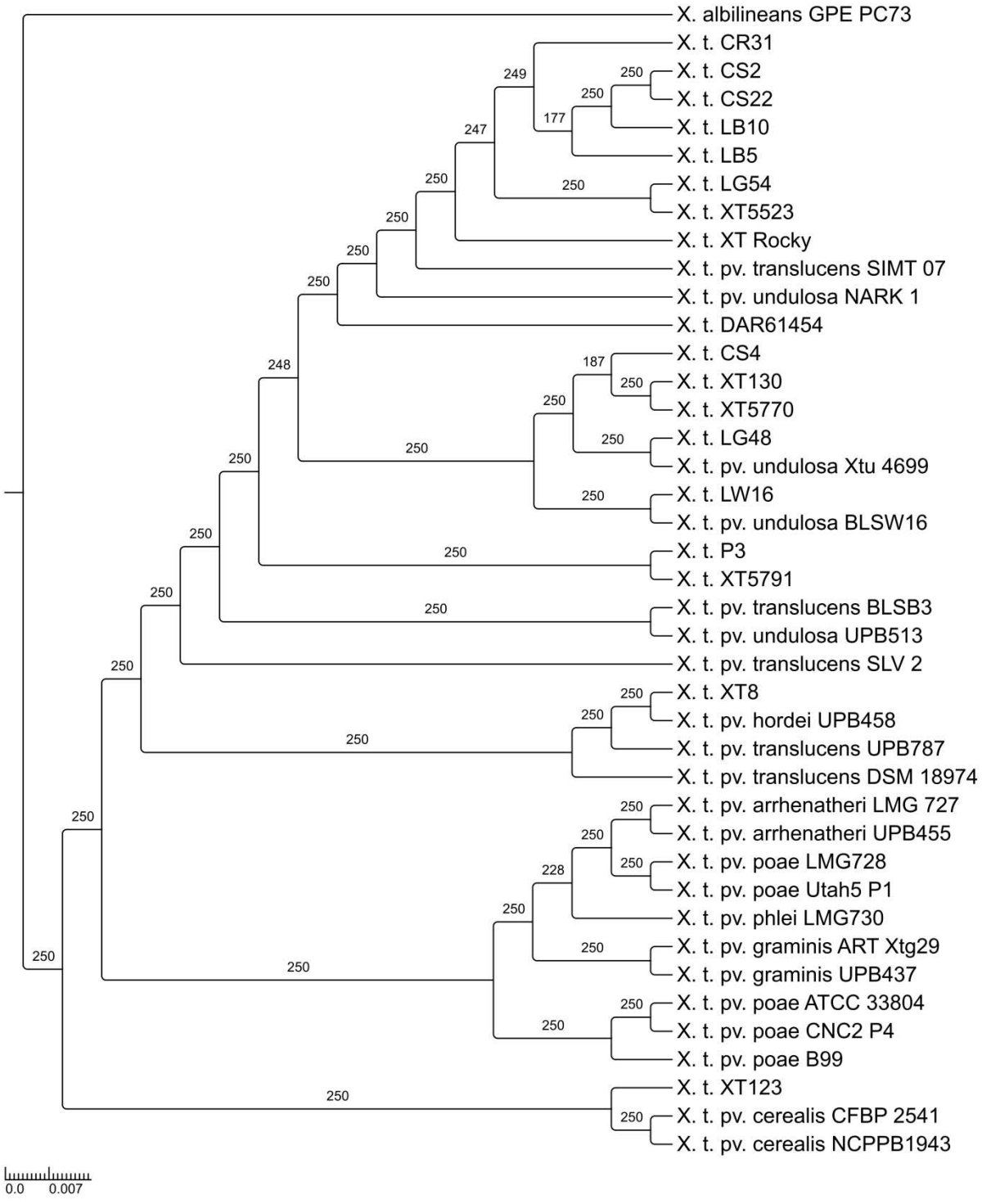


Figure 4. Prediction of detection of Real-Time thermal cycler- LAMP primers using serial dilutions starting from 10^8 CFU mL^{-1} of *Xanthomonas translucens* heat-killed cells (A) *ina*-Xt, showing amplification using barley seed isolated *X. t.* pv. *translucens*, (B) *gyrB*-Xt, showing amplification using barley seed isolated *X. t.* pv. *translucens*, (C) Xt-Cerealis, showing amplification of *X. t.* pv. *cerealis* strain B50; (D) Xt-Poae, showing amplification of *X. translucens* pv. *poae* strain ATCC-33804; (E) Xt-CLS, showing amplification using barley seed isolated *X. t.* pv. *translucens*. Each dilution was tested three times. Bars represent the standard error of the mean and associated R^2 values obtained after linear regression analysis are provided.



Supplemental Figure S.1. Phylogenetic tree done in EDGAR based on 1092 CDS from 41 strains of *Xanthomonas translucens*. The 1,092 core gene sets were concateneated and aligned using the MUSCLE software. The resulting multiple alignment was used to generate a phylogenetic tree.

Supplemental Table S.2. Bacterial strains used in this study and LAMP assay results using the different *X. translucens* group primers. Amplification (+, positive and -, none) with each set of LAMP primers are the results of a minimum of two independent assays for each strain.

					LAMP amplification				
					Xt species -pv. *graminis*	All *Xt* species	*Xt* pv. *cerealis*	*Xt* pv. *poae*	Xt CLS pvs.
***X. translucens* pv.**	Strain	Origin	Host	Source	*ina*-Xt	*gyrB*-Xt	Xt-Cerealis	Xt-Poae	Xt-CLS
arrhenatheri	UPB455[a]	Switzerland	*Arrhenatherum elatius*	C. Bragard	+	+	-	-	-
cerealis	B50		*Bromus inermis*	N. Tisserat	+	+	+	-	-
cerealis	UPB721	Japan	*Bromus* sp.	K. Miyagima	+	+	+	-	-
cerealis	UPB945/ NCPPB 1944[T]	United States	*Bromus inermis*	J.R. Wallin	+	+	+	-	-
cerealis	LMG7393/ NCPPB 1943[a]		*Hordeum vulgare*	B. Cunfer	+	+	+	-	-
cerealis	CFBP2541[T]	United States	*Bromus inermis*	J.R. Wallin	+	+	+	-	-
graminis	UPB437[a b]	Switzerland	*Lolium multiflorum*	T. Egli	+	+	-	-	+
graminis	Utah5 P2	United States		N. Tisserat	-	+	-	-	-
graminis	UPB1018	Belgium	*Lolium perenne*	H. Maraite	-	+	-	-	-
graminis	UPB1019	Belgium	*Lolium perenne*	H. Maraite	-	+	-	-	-
graminis	UPB1020	Belgium	*Lolium perenne*	H. Maraite	-	+	-	-	-
graminis	UPB1174	Belgium	*Lolium perenne*	Vanbellinghem C.	-	+	-	-	-
graminis	UPB1175	Belgium	*Lolium multiflorum*	Vanbellinghem C.	-	+	-	-	-
graminis	UPB1176	Belgium	*Lolium multiflorum*	Vanbellinghem C.	-	+	-	-	-
graminis	UPB1177	Belgium	*Lolium perenne*	Vanbellinghem C.	-	+	-	-	-
graminis	UPB1192	Belgium	*Lolium perenne*	Vanbellinghem C.	-	+	-	-	-

graminis	UPB1194	Belgium	*Lolium perenne*	Vanbellinghem C.	-	+	-	-	-
graminis	UPB1195	Belgium	*Lolium perenne*	Vanbellinghem C.	-	+	-	-	-
graminis	UPB1156/ CFBP2053[T a]	Switzerland	*Dactylis glomerata*	T. Egli	-	+	-	-	-
phlei	UPB1157	Norway	*Phleum pratense*	D. Smidt	-	+	-	-	-
phleipratensis	PDDCC 5744		*Phleum pratense*	L.E. Claflin	+	+	-	-	-
phleipratensis	UPB441[b]	United States	*Phleum pratense*	J.R. Wallin	+	+	-	-	+
phleipratensis	UPB950	United States	*Phleum pratense*	J.R. Wallin	+	+	-	-	-
poae	CNC2-P4[a]	United States	Turfgrass	N. Tisserat	+	+	-	+	-
poae	UTAH5-P1[a]	United States	Turfgrass	N. Tisserat	+	+	-	+	-
poae	BF05	United States	Turfgrass	N. Tisserat	+	+	-	+	-
poae	BF01	United States	Turfgrass	N. Tisserat	+	+	-	+	-
poae	LMG728/ CFBP2057/ ATCC-33804[T a]	Switzerland	*Poa trivialis*	ATCC	+	+	-	+	-
poae	B94	United States	Turfgrass	N. Tisserat	+	+	-	+	-
poae	B99[a]	United States	Turfgrass	N. Tisserat	+	+	-	+	-
poae	B100	United States	Turfgrass	N. Tisserat	+	+	-	+	-
poae	UPB454	Switzerland	Turfgrass	C. Bragard	+	+	-	+	-
poae	UPB952	Switzerland	*Poa trivialis*	J. Herzog	+	+	-	+	-
secalis	B43		*Secale cereale*	N. Tisserat	+	+	-	-	+
secalis	UPB469		*Secale cereale*	V. Verdier	+	+	-	-	+
translucens	B69/ SLV-2[a]	United States	*Hordeum vulgare*	N. Tisserat	+	+	-	-	+
translucens	ATCC9000-4402	Canada	*Hordeum vulgare*	N. Tisserat	+	+	-	-	+
translucens	B96	United States	*Hordeum vulgare*	N. Tisserat	+	+	-	-	+

translucens	UPB458/ NCPPB2389[a]	India	*Hordeum vulgare*	G.S. Shekhawat	+	+	-	-	+
translucens	UPB545	Mexico	*Hordeum vulgare*	E. Duveiller	+	+	-	-	+
translucens	UPB684	Iran	*Hordeum vulgare*	V. Verdier	+	+	-	-	+
translucens	UPB763	United States	*Hordeum vulgare*	D. Sands	+	+	-	-	+
translucens	UPB787[a]	Paraguay	*Hordeum vulgare*	C. Bragard	+	+	-	-	+
translucens	UPB820	Iran	*Hordeum vulgare*	Ali	+	+	-	-	+
translucens	UPB886	Iran	*Hordeum vulgare*	Ali	+	+	-	-	+
translucens	UPB906	Iran	*Hordeum vulgare*	Ali	+	+	-	-	+
translucens	CFBP2054[T]	United States	*Hordeum vulgare*	C.S. Reddy	+	+	-	-	+
translucens	MC6/ BLSB3[a]	United States	*Hordeum vulgare*	T. Adhikari	+	+	-	-	+
translucens	SIMT-07[a]	United States	*Hordeum vulgare*	N. Tisserat	+	+	-	-	+
undulosa	UPB600	Mexico	*Secale cereale*	E. Duveiller	+	+	-	-	+
undulosa	UPB681	South Africa	*Triticum aestivum*	V. Verdier	+	+	-	-	+
undulosa	B71	United States	*Triticum spp.*	N. Tisserat	+	+	-	-	+
undulosa	NARK-1[a]	United States	*Triticum spp.*	N. Tisserat	+	+	-	-	+
undulosa	MC5/ BLSW16[a]	United States	*Triticum spp.*	T. Adhikari	+	+	-	-	+
undulosa	UPB 426	Argentina	*Triticum spp.*	E. Duveiller	+	**nt**	-	-	+
undulosa	UPB513[a]	Mexico	*Triticum spp.*	V. Verdier	+	+	**nt**	**nt**	**nt**
undulosa	UPB 727	Ethiopia	*Triticale*	C. Bragard	+	+	-	-	+
undulosa	UPB 882	Yemen	*Triticum durum*	Mamluk	+	+	-	-	+
undulosa	BZ319 (56)	Brazil	*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	BZ343 (75)	Brazil	*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	BZ344 (76)	Brazil	*Triticum spp.*	E.A. Milus	+	+	-	-	+

undulosa	BZ345 (79)	Brazil	*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	BZ346 (80)	Brazil	*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	BZ347 (1)	Brazil	*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	BZ348 (3)	Brazil	*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	BZ349 (5)	Brazil	*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	BZ350 (8)	Brazil	*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	BZ351 (10)	Brazil	*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	BZ352 (11)	Brazil	*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	BZ353 (13)	Brazil	*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	BZ354 (16)	Brazil	*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	E-20 (26)		*Triticum spp.*	E.A. Milus	+	+	-	-	-
undulosa	FFA-5 (23)		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	11.15 (28)		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	ID215A (36)		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	ID224 (41)		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	ID246 (43)		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	ID248A		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	LA030A (22)		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	LA034 (24)		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	LA037A (26)		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	LA042A (28)		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	LA044A (30)		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	LA-90-01A (32)		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	LA-90-06A (34)		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	MX317 (53)	Mexico	*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	MX318 (54)	Mexico	*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	MX321 (59)	Mexico	*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	MX322 (60)	Mexico	*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	MX323 (62)	Mexico	*Triticum spp.*	E.A. Milus	+	+	-	-	+

undulosa	MX324 (64)	Mexico	*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	MX325 (66)	Mexico	*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	MX326 (68)	Mexico	*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	MX327 (71)	Mexico	*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	MX328 (72)	Mexico	*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt003		*Triticum spp.*	Jones	+	+	-	-	+
undulosa	Xt004		*Triticum spp.*	Jones	+	+	-	-	+
undulosa	Xt005		*Triticum spp.*	Jones	+	+	-	-	+
undulosa	Xt-1 ga		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt-2 ga		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt-7 ga		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt 9		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt 12		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt 13		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	X-26 (30)		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	X-31 (31)		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	X-56 (18)		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	X-58 (20)		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt102		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt103		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt104		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt105 ga		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	X-106 (21)		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt108		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt109		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt111		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt112		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt113		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt114		*Triticum spp.*	E.A. Milus	+	+	-	-	+

undulosa	Xt115		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt116		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt118		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt121		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt125		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt126		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt127		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt129		*Triticum spp.*	E.A. Milus	+	+	-	-	+
undulosa	Xt132		*Triticum spp.*	E.A. Milus	+	+	-	-	+

Other bacteria tested	Strain	Origin	Host	Source	*ina*-Xt	*gyrB*-Xt	Xt-Cerealis	Xt-Poae	Xt-CLS
Acidovorax avenae* pv. *citri	94-21	United States	*Citrullus lanatus*	R. Walcott	-	-	-	-	-
B. andropogonis	3549	United States	*Saccharum officinarum*	L.E. Claflin	-	-	-	-	-
E. coli	DH5α	United States	n/a	Life Technologies	-	-	-	-	-
Erwinia herbicola			*Malus domestica*		-	-	-	-	-
Lonsdalea quercina	B83	United States	*Quercus rubra*	N. Tisserat	-	-	-	-	-
Pseudomonas syringae* pv. *syringae	1517			L.E. Claflin	-	-	-	-	-
Pseudomonas fuscovaginae	SE-1	Philippines	*O. sativa*	Gavin Ash	-	-	-	-	-
Ralstonia solanacearum	K60	United States	*S. lycopersicum*	J.E. Leach	-	-	-	-	-
X. arboricola* pv. *corylina	B105				-	-	-	-	-
X. axonopodis* pv. *vesicatoria	1123			A. Bogdanove	-	-	-	-	-
X. axonopodis* pv. *alli	0177	United States		H. Schwartz	-	-	-	-	-
X. oryzae	X4-2c	United States	*O. sativa*	J.E. Leach	-	-	-	-	-
X. oryzae	X11-5a	United States	*O. sativa*	J.E. Leach	-	-	-	-	-

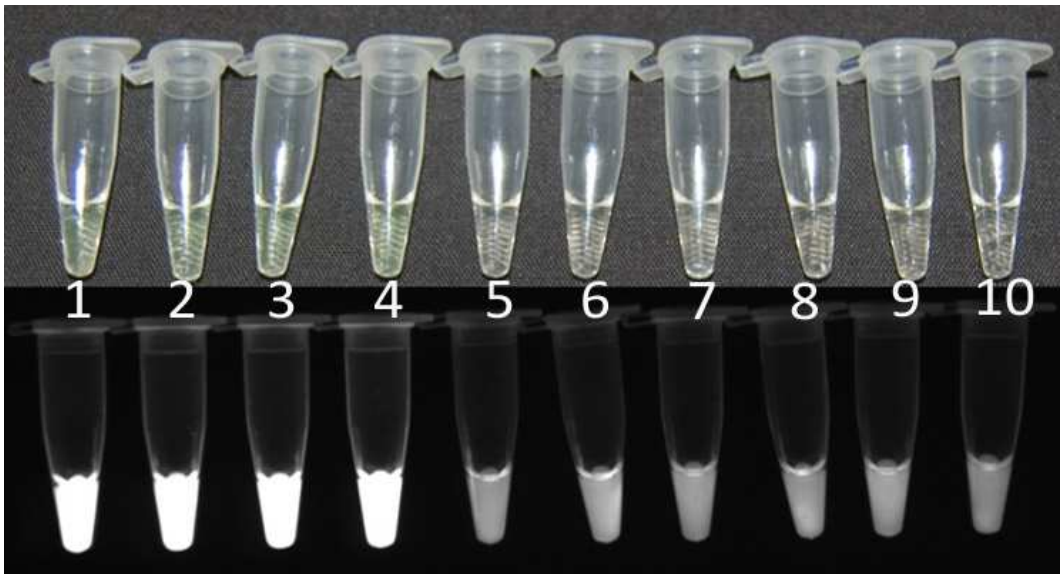
X.oryzae* pv. *oryzae	PXO99A	Philippines	*O. sativa*	J.E. Leach	-	-	-	-	-
X. oryzae* pv. *oryzicola	BLS256	Philippines	*O. sativa*	C. Vera Cruz	-	-	-	-	-
X. campestris* pv. *carotae	4464/ NZ5723	New Zealand	*Daucus carota*	L.E. Claflin	-	-	-	-	-
X. campestris* pv. *carotae	9925		*Daucus carota*	L.E. Claflin	-	-	-	-	-
X. campestris*. pv. *campestris	X1910	United States	*Brassica oleracea*	N. Dunlop	-	-	-	-	-
X. fragaria	462		Strawberry	L.E. Claflin	-	-	-	-	-
X. campestris* pv. *geranium	426		*Geranium* spp.	L.E. Claflin	-	-	-	-	-
X. campestris* pv. *holcicola	116	Machache, Lesotho	*Sorghum*	L.E. Claflin	-	-	-	-	-
X. campestris* pv. *holcicola	118	Machache, Lesotho	*Sorghum*	L.E. Claflin	**nt**	-	**nt**	**nt**	**nt**
X. campestris* pv. *holcicola	128		*Sorghum*	L.E. Claflin	-	**nt**	-	-	-
X. campestris* pv. *pelargonii	X5	United States	*Geranium* spp.	L.E. Claflin	-	-	-	-	-
X. campestris* pv. *phaseoli	454		*Phaseolus vulgaris*	L.E. Claflin	-	-	-	-	-
X. campestris* pv. *sojense			*Glycine max*	L.E. Claflin	-	-	-	-	-
X. campestris* pv. *vasculorum	NCPPB206		*Saccharum officinarum*	L.E. Claflin	-	-	-	**nt**	-

nt = not tested, [T] = type strain, [a]= draft genome sequence generated in this study, [b] = strain with uncertain historical identification

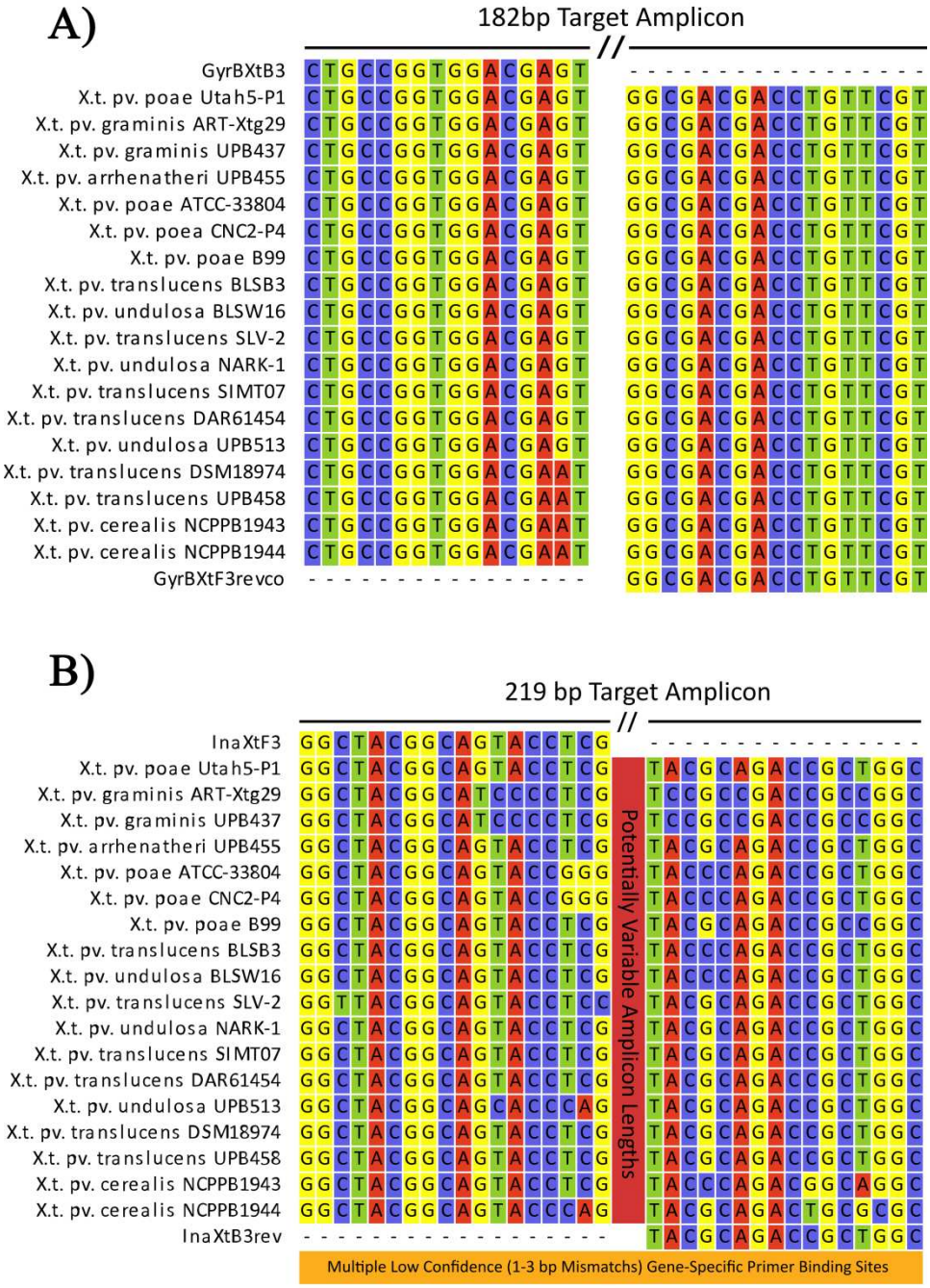
Supplemental Table S.3. Validation of LAMP assays and isolation of *Xanthomonas translucens* with field-derived tissues. Each sample was tested at least two times per primer set. Sample sources and details in Supplemental Table S.3.

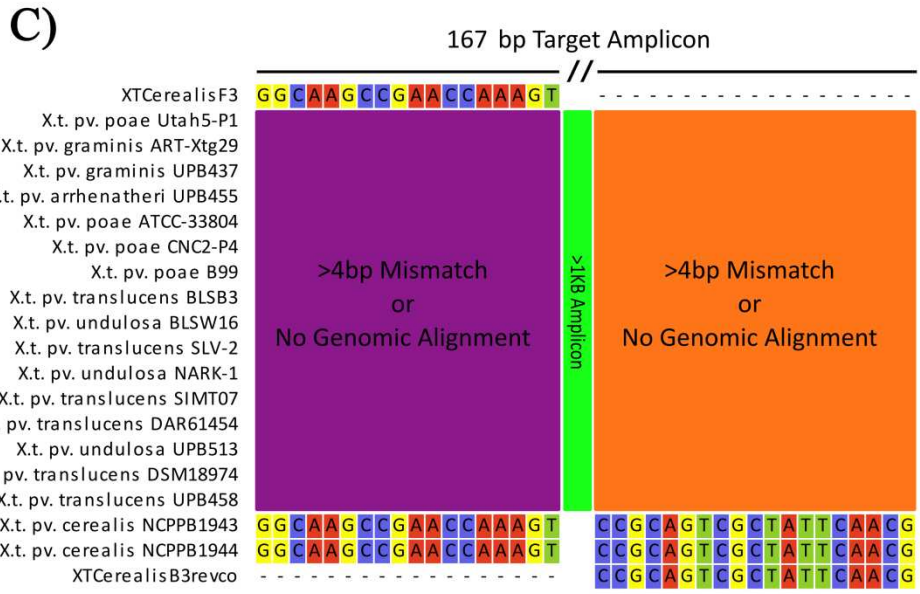
					LAMP amplification from both tissue and isolated bacterial cultures[a]				
					Xt species -pv. *graminis*	All *Xt* species	*Xt* pv. *cerealis*	*Xt* pv. *poae*	Xt CLS pvs
Sample ID	Origin	Source	Sample Type	Symptoms	*ina*-Xt	*gyrB*-Xt	Xt-Cerealis	Xt-Poae	Xt-CLS
Lf. C[a]	Montana	Anheuser Busch	Barley Leaf	+	+	+	-	-	+
Lf. D[a]	Montana	Anheuser Busch	Barley Leaf	+	+	+	-	-	+
Lf. E[a]	Idaho	Anheuser Busch	Barley Leaf	+	+	+	-	-	+
Lf. F[a]	Idaho	Anheuser Busch	Barley Leaf	+	+	+	-	-	+
Lf.W[a]	North Dakota	Anheuser Busch	Wheat Leaf	+	**nt**	**nt**	**nt**	**nt**	+
ID1[a]	Montana	Anheuser Busch	Barley seed	+	+	+	-	-	+
ID3[a]	Montana	Anheuser Busch	Barley seed	+	+	+	-	-	+
ID4[a]	North Dakota	Anheuser Busch	Barley seed	+	+	+	-	-	+
ID9[a]	North Dakota	Anheuser Busch	Barley seed	+	+	+	-	-	+
ID11[a]	North Dakota	Anheuser Busch	Barley seed	+	+	+	-	-	+
ID12[a]	North Dakota	Anheuser Busch	Barley seed	+	+	+	-	-	+
Annex 1	Montana	MSU	Barley seed	-	-	**nt**	**nt**	**nt**	**nt**
Annex 2	Montana	MSU	Barley seed	-	-	**nt**	**nt**	**nt**	**nt**
Brown[a]	Colorado	Miller Coors	Barley seed	+	+	+	-	-	+
JDSA[a]	Colorado	Miller Coors	Barley seed	+	+	+	-	-	+
McNitt[a]	Colorado	Miller Coors	Barley seed	+	+	+	-	-	+
Ponderosa[a]	Colorado	Miller Coors	Barley seed	+	+	+	-	-	+
Scidmore[a]	Colorado	Miller Coors	Barley seed	+	+	+	-	-	+

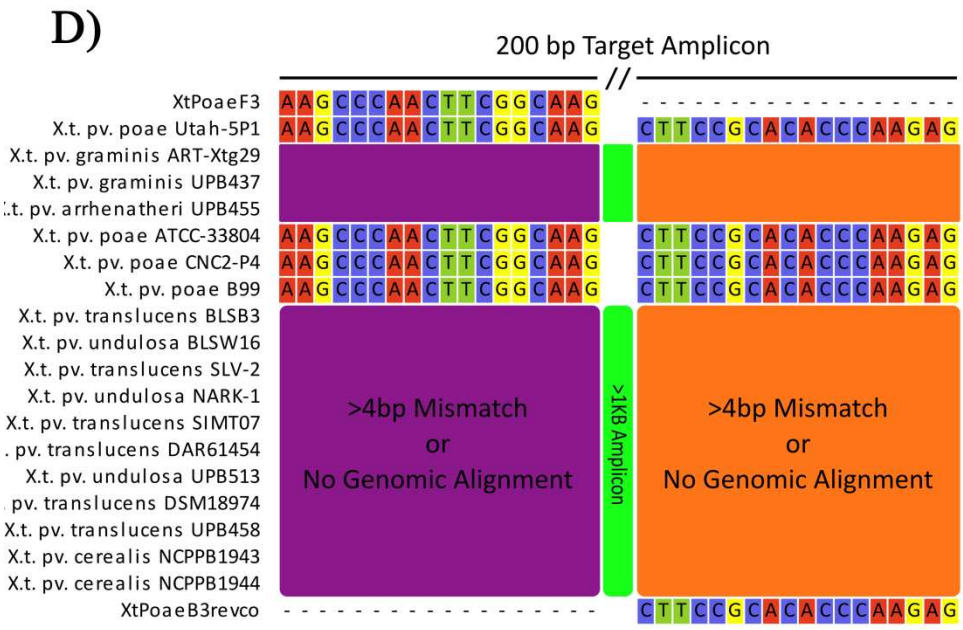
nt = not tested, [a]=Bacteria were isolated from field tissue samples, and pure cultures were tested by LAMP.

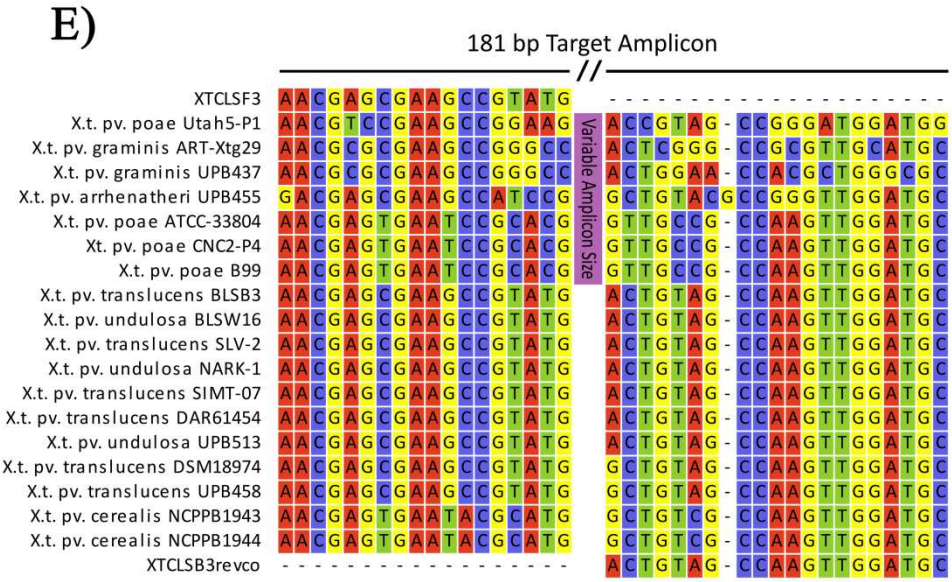


Supplemental Figure S.4. Visual detection *X. translucens* pv. *poae* ATCC-33804 heat killed cells amplified using the *Xt* pv. *poae* LAMP assay. A dilution series was tested consisting of 10^8 (1), 10^7 (2), 10^6 (3), 10^5 (4), 10^4 (5), 10^3 (6), 10^2 (7), 10^1 CFU ml^{-1} (8), negative control (9), and a no template control (10). Products were visualized after the addition of 1 µl Quant-IT™ Pico Green Reagent (Invitrogen, Carlsbad, CA, USA) under visual light (A), where a positive result changes from orange to green; ultra-violet light (B) where a positive result fluoresces. Reactions were ran for 60 min in a 65° C water bath. Detection can be achieved at lower dilutions with an extended reaction time.


A)
182bp Target Amplicon
GyrBXtB3
X.t. pv. poae Utah5-P1
X.t. pv. graminis ART-Xtg29
X.t. pv. graminis UPB437
X.t. pv. arrhenatheri UPB455
X.t. pv. poae ATCC-33804
X.t. pv. poea CNC2-P4
X.t. pv. poae B99
X.t. pv. translucens BLSB3
X.t. pv. undulosa BLSW16
X.t. pv. translucens SLV-2
X.t. pv. undulosa NARK-1
X.t. pv. translucens SIMT07
X.t. pv. translucens DAR61454
X.t. pv. undulosa UPB513
X.t. pv. translucens DSM18974
X.t. pv. translucens UPB458
X.t. pv. cerealis NCPPB1943
X.t. pv. cerealis NCPPB1944
GyrBXtF3revco
B)
219 bp Target Amplicon
InaXtF3
X.t. pv. poae Utah5-P1
X.t. pv. graminis ART-Xtg29
X.t. pv. graminis UPB437
X.t. pv. arrhenatheri UPB455
X.t. pv. poae ATCC-33804
X.t. pv. poae CNC2-P4
X.t. pv. poae B99
X.t. pv. translucens BLSB3
X.t. pv. undulosa BLSW16
X.t. pv. translucens SLV-2
X.t. pv. undulosa NARK-1
X.t. pv. translucens SIMT07
X.t. pv. translucens DAR61454
X.t. pv. undulosa UPB513
X.t. pv. translucens DSM18974
X.t. pv. translucens UPB458
X.t. pv. cerealis NCPPB1943
X.t. pv. cerealis NCPPB1944
InaXtB3rev
Potentially Variable Amplicon Lengths
Multiple Low Confidence (1-3 bp Mismatchs) Gene-Specific Primer Binding Sites


C)
167 bp Target Amplicon
XTCerealisF3
X.t. pv. poae Utah5-P1
X.t. pv. graminis ART-Xtg29
X.t. pv. graminis UPB437
X.t. pv. arrhenatheri UPB455
X.t. pv. poae ATCC-33804
X.t. pv. poae CNC2-P4
X.t. pv. poae B99
X.t. pv. translucens BLSB3
X.t. pv. undulosa BLSW16
X.t. pv. translucens SLV-2
X.t. pv. undulosa NARK-1
X.t. pv. translucens SIMT07
X.t. pv. translucens DAR61454
X.t. pv. undulosa UPB513
X.t. pv. translucens DSM18974
X.t. pv. translucens UPB458
X.t. pv. cerealis NCPPB1943
X.t. pv. cerealis NCPPB1944
XTCerealisB3revco
>4bp Mismatch or No Genomic Alignment
>1KB Amplicon
>4bp Mismatch or No Genomic Alignment


D)
200 bp Target Amplicon
XtPoaeF3
X.t. pv. poae Utah-5P1
X.t. pv. graminis ART-Xtg29
X.t. pv. graminis UPB437
X.t. pv. arrhenatheri UPB455
X.t. pv. poae ATCC-33804
X.t. pv. poae CNC2-P4
X.t. pv. poae B99
X.t. pv. translucens BLSB3
X.t. pv. undulosa BLSW16
X.t. pv. translucens SLV-2
X.t. pv. undulosa NARK-1
X.t. pv. translucens SIMT07
. pv. translucens DAR61454
X.t. pv. undulosa UPB513
pv. translucens DSM18974
X.t. pv. translucens UPB458
X.t. pv. cerealis NCPPB1943
X.t. pv. cerealis NCPPB1944
XtPoaeB3revco
>4bp Mismatch or No Genomic Alignment
>1KB Amplicon
>4bp Mismatch or No Genomic Alignment



Supplemental Figure S.5. Alignments of each LAMP F3 and B3 primer set with target region of *X. translucens* genomes.
a) *gyrB*-Xt b) *ina*-Xt c) Xt-Cerealis d) Xt-Poae e) Xt-CLS

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CHAPTER THREE

CONCLUSIONS AND FUTURE PERSPECTIVES

With the release of the genomes sequenced in this study and the phylogenetic analyses we present, we have demonstrated that the cereal and non-cereal isolates of *X. translucens* are genetically distinct from each other, and that the pathovar *cerealis*, which can be found on cereal and non-cereal hosts, is more closely related to, but, genetically distinct from the cereal pathovars. The pathovar *cerealis* falls within the clade that contains cereal hosts, which includes the pvs. *translucens* and *undulosa*, but groups in its own separate subclade. This distinction of the *cerealis* pathovar from others was confirmed in our five pathovar comparison using EDGAR analysis.

Utilizing unique regions found in our sequenced strains and the available genomes at the time, we developed sensitive diagnostic assays that are specific to *X. translucens* subgroups. These diagnostics greatly improve on the current methods of detection and differentiation available for *X. translucens*, and they are readily adapted for use in the laboratory or field. The availability of these assays now provide a reliable and rapid means of detection that can be used in high throughput and give results within 30 min or less. Detection can be done using ground-up leaf tissue or supernatant from seed washes due to the specificity of the primers, and the robustness and sensitivity of the assays, therefore reducing sample preparation time.

In addition to better understanding *X. translucens* phylogeny and the development of diagnostic assays, the release of the 15 genomes will provide information to greatly improve on the

understanding of the true genealogical relationships that the currently named pathovars of *X. translucens* share. At this time, including our own submissions, there are 42 *X. translucens* genomes. With the use of this information, just like in the case of *X. oryzae*, pathogenicity factors and phytospecializations can now be better uncovered and understood. This can help decipher virulence mechanisms to enable better understanding of host range specificity and lend knowledge to improve crop protection and resistance.

Future directions to better understand how *X. translucens* interacts with plants include next-generation sequencing (NGS) technologies, specifically long read/ Pac-Bio sequencing. Single Molecule Real-Time (SMRT) sequencing developed by Pac-Bio is being used very successfully for sequencing transcription activator-like (TAL) effectors. Based on published genome data, the *X. translucens* genome has been shown to contain TAL effectors. Strain CFBP2541 contains two type III transcription activator-like (TAL) effector genes, while strain XT4669 contains eight (Peng et al. 2016; Pesce et al. 2015). TAL effector genes encode proteins that are injected via a type III secretion system (T3SS) into the host cell, and that move to the nucleus where they bind to the promoters of host plant genes to regulate transcription of resistant (R) and/ or susceptible (S) genes (Wilkins et al. 2015; Sebra et al. 2015; Peng et al. 2016; Scholze and Boch 2011). These genes were first discovered in *Xanthomonas*, and are a very important research focus because they are often directly involved with plant resistance and susceptibility and are thought to contribute to host specialization. TAL effector genes contain a central repeat region (CRR) that determines pathogen specificity, but is very difficult to sequence and assemble with short read technologies. Recent reports show resolution in specific TAL effector sequences because of Pac-Bio sequencing, and, in some cases, having accurate sequence of the repeat region of these

genes can reveal their host plant gene targets (Sebra et al. 2015; Wilkins et al. 2015; Hutin et al. 2015; Peng et al. 2016). Particularly, a report done by Peng et al this year, released the complete genome of strain XT4699, belonging to *X. translucens* pv. *undulosa* using PacBio and Illumina sequencing technologies. PacBio data enabled accurate assembly of multiple TAL effector genes. From the assembly of TAL effector genes, they were able to identify two candidate plant target genes in which transcriptional activity was altered due to one of the identified TALs, Tal6 (Peng et al. 2016).

In the case of *X. translucens*, nearly all of the named pathovars cause varying degrees of symptoms on non-cereal hosts and cereal hosts in artificial inoculations (Bragard et al. 1997). This along with the broad host ranges observed with many of these pathovars, goes against the traditional pathovar concept. Although there are many historically named pathovars, not enough screening has been done using differential host range inoculations to validate their taxonomy. Thus, poor classification driven by this "new host-new pathovar" concept, led to much confusion in the nomenclature and true relatedness of these pathovars that were formerly classified under the *campestris* species (Bragard, Verdier, and Maraite 1995). Genetic information has provided a vital foundation for reassessment and reclassification of many pathogenic bacteria including *Xanthomonas* (Vauterin et al. 1995).

By the end of this year, 42 genomes of *X. translucens*, including seven pathovars, will become available. This vast amount of genomic data will allow scientists to question if more reclassification of strains within pathovars is needed, or reassessment of whether pathovar designation is even appropriate for many strains in *X. translucens.* The data generated to address

taxonomy and classification will provide a great foundation for more discovery of the genomic and pathogenic tools of *X. translucens*. With this foundational genomic platform now being available, the "effectorome" of *X. translucens* may begin to be deciphered, and the TAL targets discovered. This will lead to the discovery of host R and S genes that can greatly improve crop resilience and resistance. This is important because although the genetic similarity may be very high among and within pathovars, large variation in the host range for disease exists. Therefore, if the *X. translucens* complex were to undergo heavy genomic mining, including the utilization of these new sequencing and assembling methods to uncover TAL effectors and other pathogenicity factors, as well and large-scale, standardized, differential host pathogenicity tests with all pathovars in *X. translucens*, a greater understanding of the species' host specialization and true phylogeny could finally be uncovered.

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APPENDICES

AGROBACTERIUM-MEDIATED TRANSFORMATION OF *ORYZA SATIVA* SPP. *INDICA* AND SPP. *JAPONICA*

I. Background and differences in protocols

Throughout my undergraduate and graduate research I was interested in genetic transformations of various plant species to discover gene function. In the time I spent researching in Dr. Jan Leach's lab I performed two successful rice transformation studies, and attended the Advanced *indica* Rice Transformation Course at the International Rice Research institute (IRRI) in the Philippines. Transformation success and efficiency rates in rice (*Oryza sativa* L.), vary widely between *indica* and *japonica* subspecies. In the case of *indica* varieties, they tend to be much more difficult to transform, involve more intensive techniques and media, and have lower transformation efficiency rates than *japonica* varieties. In both transformation studies I performed at CSU, we used *Oryza sativa* ssp. *japonica* cultivar Kitaake, that is commonly used in research. The Kitaake variety has a very fast seed maturity time and is small in stature which makes it an ideal cultivar for various biological studies as well as rice transformations. However, *indica* rice is more agronomically important because it is grown in more quantity than *japonica* varieties (Calpe, 2006). In the case of rice transformation studies, using *indica* varieties can be more ideal due to the fact that the results obtained are more relevant to varieties that are more agronomically important.

We followed a protocol for *japonica* varieties modified by Amanda Broz from the Bush Lab at Colorado State University, originally from Pamela Ronald. In *Agrobacterium tumefaciens*

mediated rice transformation, undifferentiated cells called calli must first develop which are vulnerable to infection by *Agrobacterium*. These undifferentiated calli are more easily transformed and have a high rate of successful gene transfer and incorporation into the nucleus, compared to differentiated cells. In the case of *japonica* transformations, the calli are generated from either immature green seed or mature seed. Immature green seed is preferred as the tissue contains more cells that have not undergone cell differentiation, and therefore produce more calli, more easily, and also often proliferate more, rather than if the calli used were derived from mature seed. IRRI has developed two protocols for *indica* rice transformations, that have shown to be very successful and have better transformation efficiency than previously used methods. In taking this Advanced *indica* transformation course we performed all the tasks and techniques of the protocol, however the course was only one week and therefore we did not do some steps like preparing the complex media, or observe the growth process of the calli cells. In this work I will show the protocols, discuss the fundamentals, processes, and observations I have experienced with both the *indica* and *japonica* transformations I have been a part of and studied over my time at Colorado State University and at IRRI.

The Plant Breeding, Genetics and Biotechnology Division (PBGB) at IRRI uses two protocols for rice transformations. One is specifically the *indica* protocol that utilized immature embryos, and will be the only protocol of theirs I cover in this work. In my training we performed the *Agrobacterium*-mediated transformation of indica rice using immature embryos (Protocol A02) from the course training manual. This protocol utilizes immature embryos from fresh rice panicles taken that morning from the greenhouse or field. In this course we were working with one of the most grown and consumed *indica* rice varieties, IR64. The panicle stems are put in

water and brought into the laboratory for isolation of the embryo. The seed germ is high in moisture which allows the embryo to easily be isolated from the endosperm. Panicles are selected and freshly cut, that are transitioning between the milk stage and the dough stage. These seed stage is great for embryo isolation and remains mature enough and hydrated enough to extract from the embryo without damage. Using fresh embryos from this milk-dough rice stage ensures good embryogenic calli formation which is ideal for transformation. The seed are separated from the panicles and are surface sterilized in ethanol and bleach. The embryos are then isolated carefully under a disecting microscope and plated on co-cultivation medium (media A201 in the protocol) with the scutellum side facing upward. In this protocol the immature embryos are infected with *Agrobacterium* early on, unlike in the *japonica* protocol. Each individual embryo is co-cultivated with 5 µl of 3 x 10^9 cfu mL^{-1} of *Agrobacterium* for 7 days. After this co-cultivation step the elongated shoots are excised to prevent future contaminations, and calli are dried by blotting on moist sterile filter paper. The calli are blotted throughout a few layers in succession, and then are allowed to grow and rest on resting medium (media A202 in the protocol). Throughout the resting stage process, the calli grow and are split into 4 equal parts about 2 different times. Doing this helps reduce the number of shoots that generate in regeneration thus reducing or even eliminating the possiblility of one plantlet having multiple transformation events in a clump of shoots. Each time the calli clumps are split into four, a label stays with them so they can be tracked back to which calli it originated from. For example, calli chunk 1, was split into four, so each new piece is labeled as 1-1, 1-2, 1-3, and 1-4. Later on each of those will be split again. So if calli chunk 1-3 is split later on, the peices from that calli would be labeled as 1-3-1, 1-3-2, 1-3-3, and 1-3-4, etc. Also when observing the calli over time something important is happening that may not be very apparent. This is that embryogenic calli

proliferate from the embyro, as well as other cells that proliferated from cells surrounding it. Embryogenic calli are most efficient at T-DNA encorporation and should be watched more carefully and/or noted or separated from other calli. It is difficult to distinguish embryogenic calli from non-embryogenic calli without having observed both before or having a well trained eye. Embryogenic calli are often more white and translucent or cloudy, rather than creamy or slightly yellow tinted. Either type of calli is able to encorporate the T-DNA, but the instructors from the course at IRRI insist that embryogenic calli have higher rates of efficiency, faster growth, and have better regeneration. As the calli grow more and are later plated on the appropriate medias, no matter the amount of splitting of calli done previously, many shoots can develop in regeneration. It is still very important, and better for screening purposes, to separate out these shoots into individual plantlets as much as possible, when plating in rooting medium. IRRI does separations in a way that appeared to be somewhat rough and haphazard. However, it is due to routine, experience, and confidence that allows them to remain gentle and undamaging to the plant shoots. Another major part to this protocol is since IRRI does transformations in high-throughput they use a Yoshida culture solution in which the rooted and developed plants are put into in greenhouse conditions for screening purposes. This allows them to grow many plants at a time in a small area, enabling them to remove any rogue plants that may have somehow survived the transformation process even on the selection media. There are other underlying differences in the protocols, but these mostly include the complexity of ingredients and preparation that goes into making the media, among other techniques that are used in this *indica* rice transformation protocol.

In the case of the *japonica* transformations I performed in the Leach Lab we used *Oryza sativa* ssp. *japonica* cultivar Kitaake. In these processes we used both immature green rice seed and mature rice seed. Both can be used for transformation and produce of callus tissue. However green immature seed has a faster rate of calli cell proliferation, higher efficiency of transformation, and presumably produces more embryogenic calli due to lack of cell differentiation. This method starts out by using immature green seed of the variety you would like to transform. I would suggest using the freshest green seed you have, and avoid using it if it is older than 4 months. Seeds are first dehulled, then surface sterilized with bleach and a surfactant, and are then washed again with successive washes of sterile distilled water. Seeds are placed on calli induction media and are allowed to grow for a few weeks. After calli have been vigorously growing they are cultured with the *Agrobacterium* culture for about 1-2 hours on a shaker. This is a major difference in the *indica* and *japonica* protocols due to the fact that calli are first grown and then inoculated, where as in the *indica* protocol, the embryos are first isolated, sterilized, inoculated, and then are grown to produce calli. The calli are then removed and blot dried on sterile filter paper and placed on co-cultivation media for 2-3 days. After co-cultivation the calli are either placed on selection media as is, or depending on the *Agrobacterium* strain used and how aggressively it grows, a carbenicillin wash may be needed to kill the rest of the bacteria to prevent it from overgrowing on the callus tissue. The calli stay on selection media for about 2-8 weeks and then are transferred onto rooting and then later onto regeneration media.

II. *Indica* rice transformation protocol using immature embryos

Advanced *indica* Rice Transformation Course: Training manual. *Agrobacterium*-mediated transformation of *indica* rice using immature embryos: Protocol A02. Sept 3-8, 2012. (pg. 4-7, 26, 28-29). Plant Breeding, Genetics and Biotechnology Division (PBGB), International Rice Research institute (IRRI). Los Baños, Laguna Philippines.

i. Materials

Equipment	
Laminar flow hood	Oven
Sterilizer	Refrigerator
Autoclave	Incubator
pH meter	Rotator
Balances (toploading and analytical)	Cell density meter/ Spectrophotometer
Stirrer/ hot plate	
Laboratory ware	
Beakers	Test tube cap
Graduated cylinder	Centrifuge tube, 50ml
Media bottles	Forceps
Petri dish	Scalpel/blade
Volumetric flask	Spatula
Test tube	Wire loop

Consumables	
Petri dish (100x15, 100x200)	Filter paper
Syringe	Syringe filter
Micropore tape	Pipette tips
Filter with receiver	2ml Eppendorf tubes
Cuvette	Parafilm

ii. Preparation of stock solutions and media

Stock solutions: Components are dissolved in MQ water and stored at 4°C

Stock solution (vol)	**Component**	**Weight**
N6 major 1 (1 liter)	KNO_3	141.5 g
N6 major 2 (1 liter)	$MgSO_4 \cdot 7H_2O$ $(NH_4)_2SO_4$	18.5 g 46.3 g
N6 major 3 (1 liter)	KH2PO4	40.0 g
N6 major 4 (1 liter)	$CaCl_2 \cdot 2H_2O$	16.6 g
B5 minor 1 (1 liter)	$FeSO_4 \cdot 7H_2O$ $Na_2EDTA \cdot 2H_2O$	2.785 g 3.725 g
B5 minor 2 (1 liter)	$MnSO_4 \cdot 4H_2O$ $ZnSO_4 \cdot 7H_2O$ H_3BO_3	1.0 g 0.2 g 0.3 g
B5 minor 3 (1 liter)	KI	0.075 g
B5 minor 4 (1 liter)	$CuSO_4 \cdot 5H_2O$ $Na_2MoO_4 \cdot 2H_2O$ CoCl2·6H2O	0.0025 g 0.025 g 0.0025 g
B5 vitamins (100 mL)	Thiamine HCl Pyridoxine HCl Nicotinic acid Myo-inositol	200 mg 20 mg 20 mg 2000 mg
AA macro salts (1 liter)	$CaCl_2 \cdot 2H_2O$ $MgSO_4 \cdot 7H_2O$ $NaH_2PO_4 \cdot 2H_2O$ KCl	1.5 g 2.49 g 1.7 g 29.5 g
AA micro salts (1 liter)	$CoCl_2 \cdot 2H_2O$ $CuSO_4 \cdot 5H_2O$ H_3BO_3 KI $MnSO_4$	25.0 mg 25.0 mg 3000.0 mg 750.0 mg 8.9 mg

	$Na_2MoO_2 \cdot 2H_2O$ $ZnSO_4 \cdot 7H_2O$	250.0 mg 2000.0 mg
AA iron stock (1 liter) *Same as B5 minor 1*	$FeSO_4 \cdot 7H_2O$ $Na_2EDTA \cdot 2H_2O$	2.78 g 3.73 g
Glycine (1 liter) (filter-sterilized and store at -20°C)		7.5 g
MS1 (1 liter)	KNO_3 NH_4NO_3	95.0 g 82.5 g
MS2 (1 liter)	$MgSO_4 \cdot H_2O$ $MnSO_4 \cdot 4H_2O$ $ZnSO_4 \cdot 7H_2O$ $CuSO_4 \cdot 5H_2O$	37.0 g 2.23 g 0.86 g 0.0025 g
MS3 (1 liter)	$CaCl_2 \cdot 2H_2O$ KI $CoCl_2 \cdot 6H_2O$	44.0 g 0.083 g 0.0025 g
MS4 (1 liter)	KH_2PO_4 H_3BO_3 $Na_2MoO_2 \cdot 2H_2O$	17.0 g 0.62 g 0.025 g
MS vitamins (100 mL)	Nicotinic acid Pyridoxine HCl Thiamine HCl Glycine Myo-inositol	10.0 mg 10.0 mg 2.0 mg 40.0 mg 2000.0 mg

Media preparation

Stock Solution	**Amount of stock to take per liter of medium (mL)**						
	A200 (infection)	**A201** (co-cultivation)	**A202** (resting medium)	**A203** (selection)	**A204** (pre-reneration)	**A205** (regeneration)	**MS0** (rooting)
N6 major 1		20	20	20			
N6 major 2		10	10	10			
N6 major 3		10	10	10			
N6 major 4		10	10	10			
B5 minor 1		10	10	10	10	10	10
B5 minor 2		10	10	10			
B5 minor 3		10	10	10			
B5 minor 4		10	10	10			
B5 vitamins	1	5	5	5			
AA macro salts stock	100						
AA micro salts stock	1						
AA iron stock *Same as B5 minor 1*	10						

Glycine	1						
MS1					20	20	20
MS2					10	10	10
MS3					10	10	10
MS4					10	10	10
MS vitamins					5	5	5

Chemical	**Amount of chemical to add per liter of medium (mg)**						
	A200 (infection)	**A201** (co-cultivation)	**A202** (resting medium)	**A203** (selection)	**A204** (pre-reneration)	**A205** (regeneration)	**MS0** (rooting)
L-glutamine	876		300	300			
Aspartic acid	260						
Arginine	174						
Casamino acid	500	500	500	500			
L-proline		500	500	500			
Sucrose	20000	20000				30000	3000
D-glucose monohydrate	10000	10000					
Mannitol			36000	36000			
Maltose			20000	20000	30000		
Sorbitol					20000		
	Adjust volume to 1 liter						
pH	5.2	5.2	5.8	5.8	5.8	5.8	5.8
Agarose type 1		5500			10000		
Gelrite			5000	5000		3000	2000 Microwave medium to melt gelrite

Stock solution	**Amount of stock to add per liter of medium (mL)**						
Stock solution	**A200** (infection)	**A201** (co-cultivation)	**A202** (resting medium)	**A203** (selection)	**A204** (pre-reneration)	**A205** (regeneration)	**MS0** (rooting)
							Dispense in big test tubes at 20 mL/tube
	Filter sterilize	Autoclave at 115°C for 15 min, then cool to 55°C					Autoclave at 115°C for 15 min, then cool at room temperature
acetosyringone	100 µM (add before use)	19.62 mg dissolved in 1 mL DMSO					
Growth regulator	**Amount of to add per liter of medium (mL)**						
2,4-D		2	1	1			
NAA		1	1	1	5	1	
BAP		1	0.2	0.2			
Kinetin					2	2	
Antibiotic	**Amount of to add per liter of medium (mL)**						
Claforan			1	1	1	1	
Carbenicillin			1	1			
Hygromycin				0.6	1	1	
		Dispense 25 ml in 100x15 petri dish	Dispense 25 ml in 100x15 petri dish	Dispense 25 ml in 100x15 petri dish	Dispense 25 ml in 100x15 petri dish	Dispense 25 ml in 100x15 petri dish	

iii. Procedure

Panicle harvesting and immature embryo sterilization and isolation

1. Harvest panicles 8-12 days after anthesis. (about 1.3-1.8mm embryo size).
2. Dehull immature embryos (IEs) and put in 50 mL Falcon tubes.
3. Sterilize in 70% ethanol for 1 min, rinse with sterile water and then add 1% sodium hypochlorite (containing 1-2 drops Tween 20) solutions and put 50-ml tubes on a rotator for 5 min.
4. Rinse at least five times with plenty of sterile distilled water.
5. Isolate IEs (under a stereomicroscope, if needed).

Immature embryo transformation - co-cultivation

1. Three days before IE transformation, streak the *Agrobacterium* from glycerol stock on to the AB plate with suitable antibiotics (e.g., Spectinomycin 50mg L^{-1} or Kanamycin and also hygromycin 50mg L^{-1}, depending on the plasmid). Incubate *Agrobacterium* culture in the dark at 28 °C for 2-3 days.
2. An hour before infection fo IEs, take about one full loop (3mm loop size) of *Agrobacterium* culture from the AB plate and suspend it in A200 medium contained in a Falcon tube; pipette or invert the tube gently several times for even mixing. Adjust bacterial density to 3 x 10^9 cfu mL^{-1}. (OD_{600} 0.3). Incubate the suspension in the dark at 25 °C for 1h (incubator) prior to infecting the IEs.
3. Place the IEs with the scutellulm side up onto forced air dried A201 medium. Drop 5 µl of *Agrobacterium* suspension to each IE. Incubate cultures in the dark at 25°C for 7 days. Place 50 IEs per plate.

Resting culture and selection

1. After the co-cultivation period, place IEs on a sterile filter paper and remove the elongated shoots with a scalpel.
2. Quick-dry the IEs several times by gently pressing them between two layers of sterile filter papers.
3. Transfer IEs with the scutellum side up onto the A202 resting medium. Place 16 IEs per petri dish. Incubate under continuous light at 30°C for 10 days (2^{nd} selection).
4. Separate the embryogenic call from the black callus tissue and transfer the embryogenic calli to fresh A203 selection medium. Incubate continuous light at 30°C for 10 days (3^{nd} selection).

Plant regeneration and transfer to plants to pots

1. Transfer resistant calli to A204 pregeneration medium at 6-8 callus lines per petri dish. Incubate under continuous light at 30°C for 10 days.
2. Select proliferating calli with green spots and transfer to A205 regeneration medium. Place four callus lines per petri dish. Label each regenerable callus line.
3. Carefully select one plantlet from each callus line and inoculate into a tube containing MS0 rooting medium. Barcode-label the plant ID on each test tube. Keep the plantlets under continuous light at 25°C for 14 days. Keep original petri dishes with plantlets as backup.
4. If a callus line has not regenerated plants but has small shoots, transfer these to a fresh A205 medium to allow the shoots to grown into plantlets. Incubate under

continuous light at 30°C for 14 days and transfer to MS0 once plantlets have developed.

5. Transfer the plants in plastic trays containing Yoshida culture solution and grow plants therein your growing area for 14 days. Culture solutoin should be changed weekly or as often as needed.
6. Collect leaf samples from each plant for PCR analysis. Confirmation by PCR should be done within the 2 weeks that the plants are in culture solution.
7. Transfer confirmed transgenic plants to pots and grow until maturity.

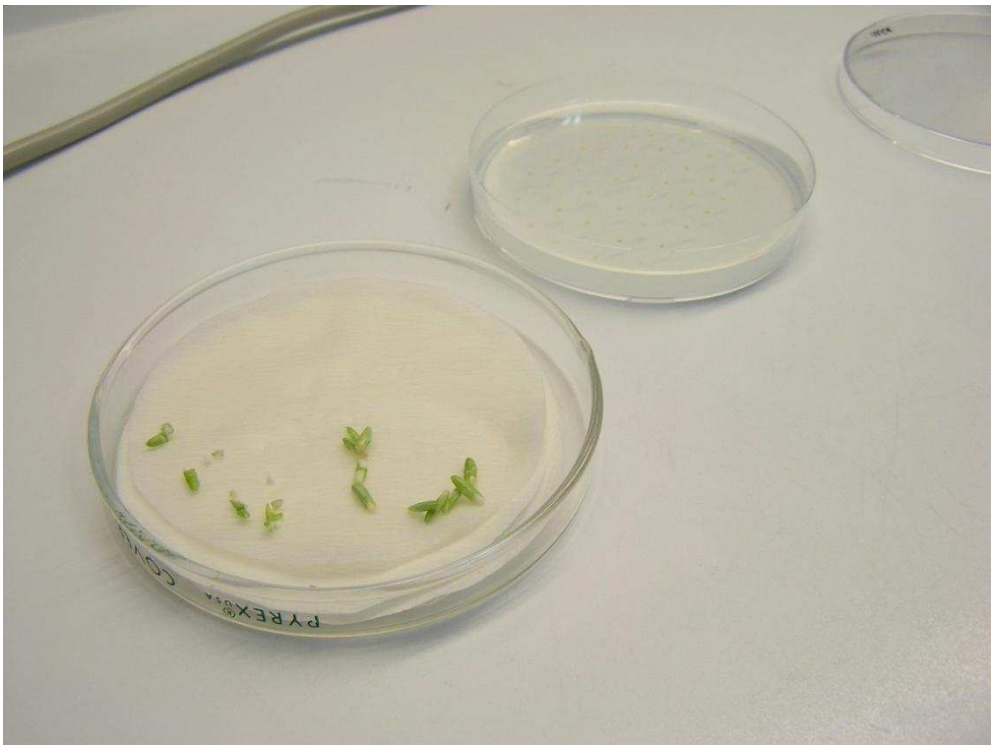

Figure A1. Embryo isolation from fresh rice seed in the milk-dough stage.

Figure A2. Inoculation of immature embryos with *Agrobacterium*.

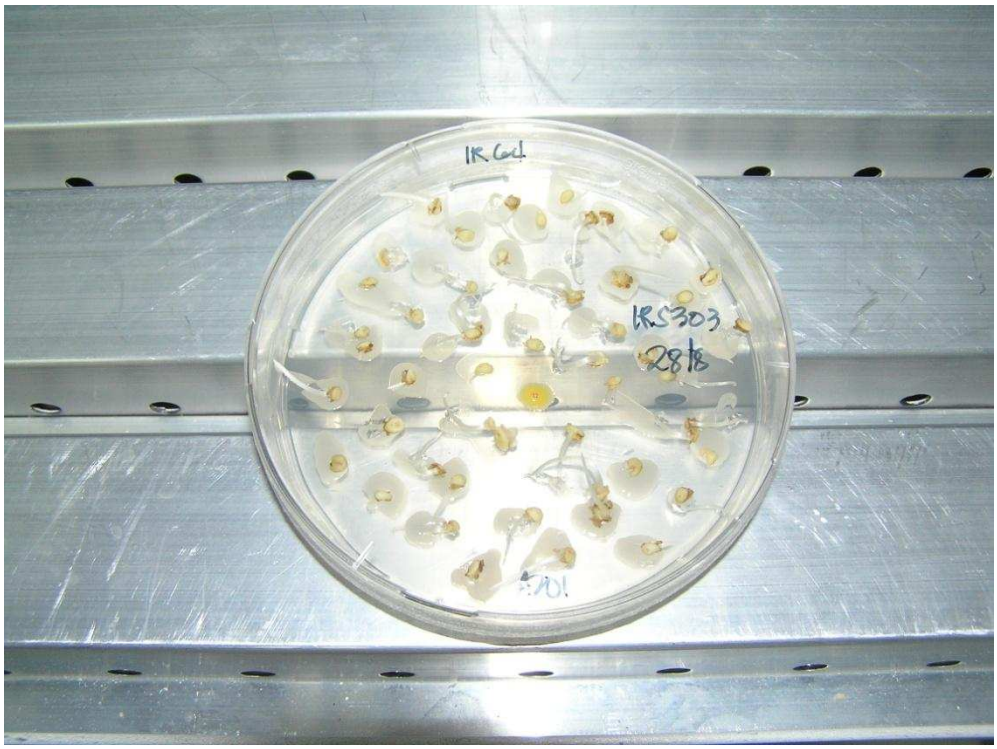

Figure A3. *Agrobacterium* growth during co-cultivation, the middle yellow colony is a contamination that was removed and thrown away.

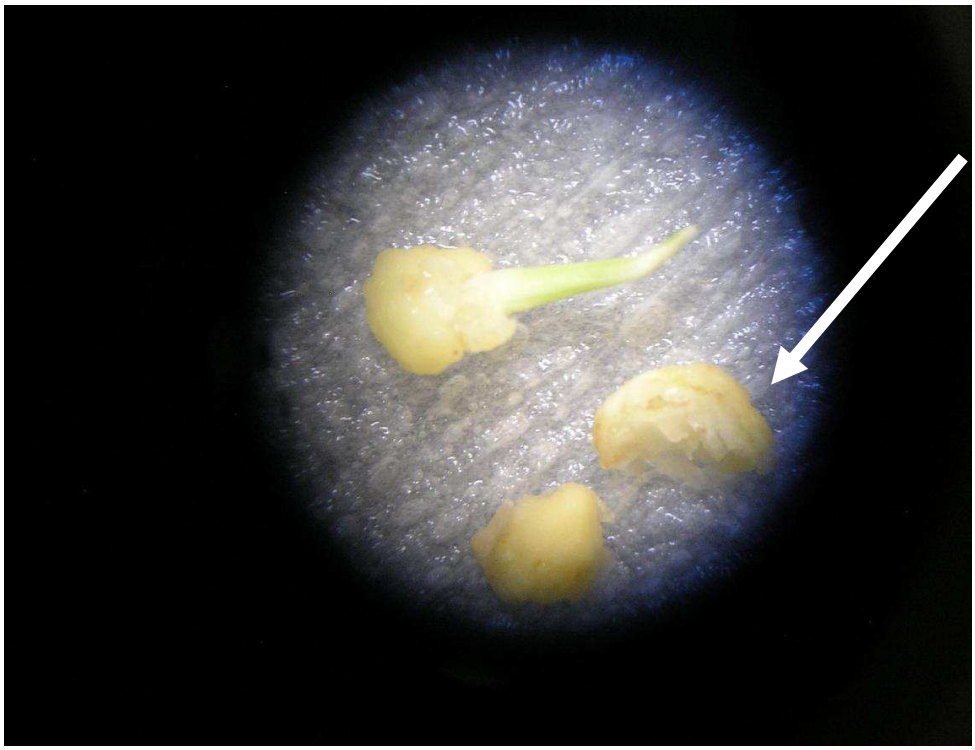

Figure A4. Calli growth observed under a dissecting microscope. The arrow is pointing to the scutellum side of the embyro in which cells grow from.

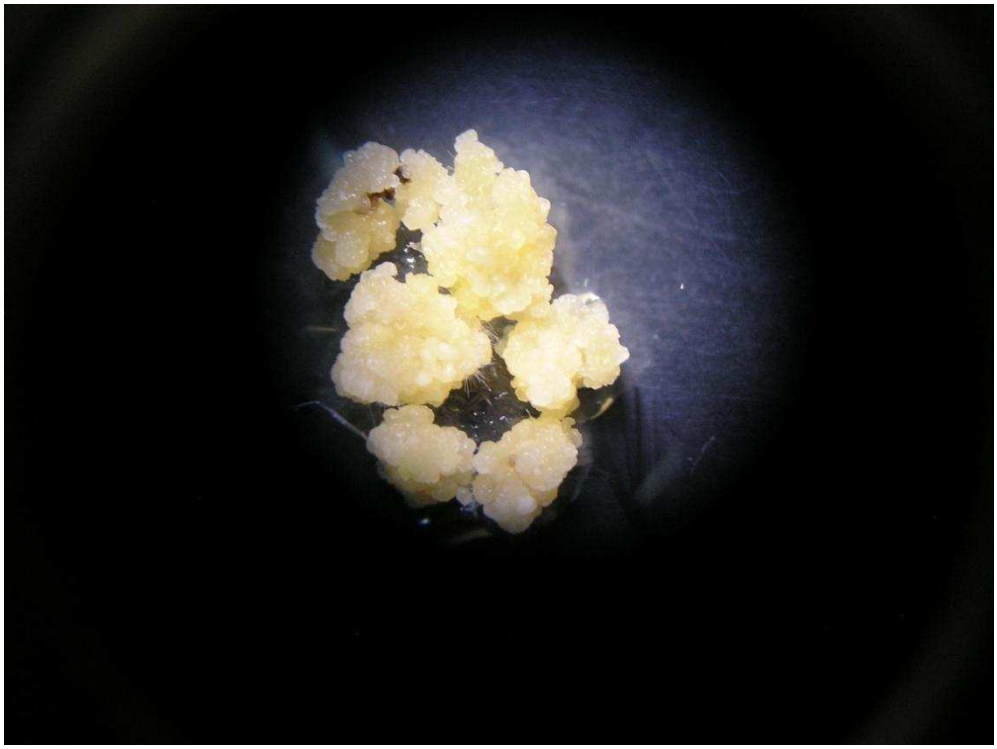

Figure A5. Calli growth taking off of A202 resting medium. Ready to transfer to A203 selection media. The whiter cells are embyrogenic cells.

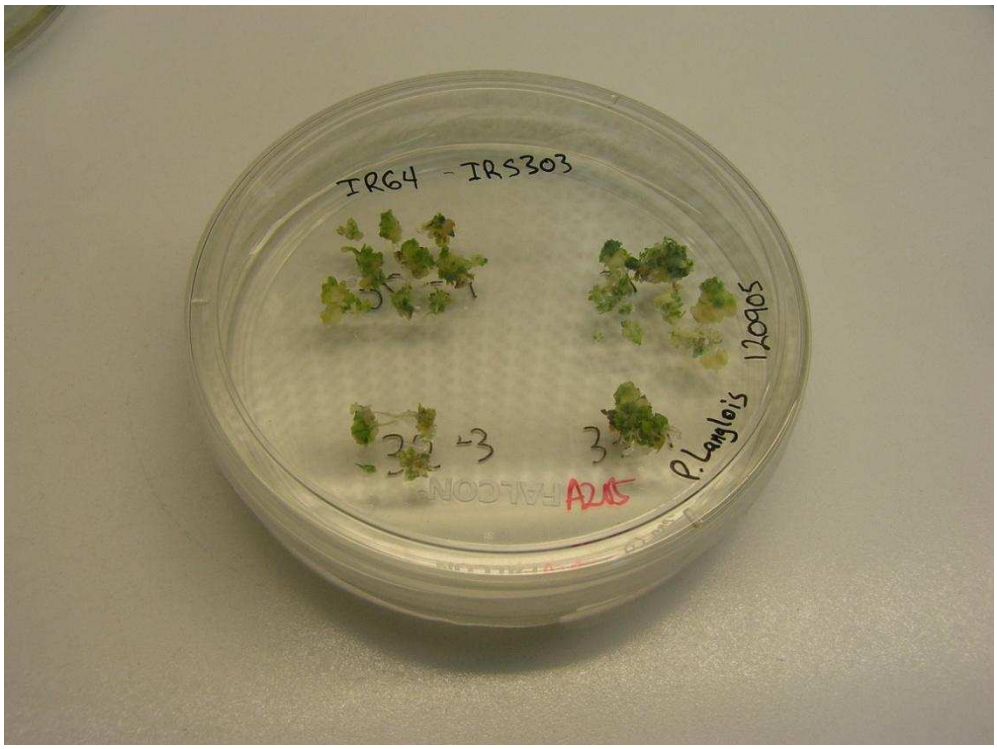

Figure A6. Leaf primordia growth on A205 regeneration media. Note that the calli are grouped and numbered by which emybro they originated from.

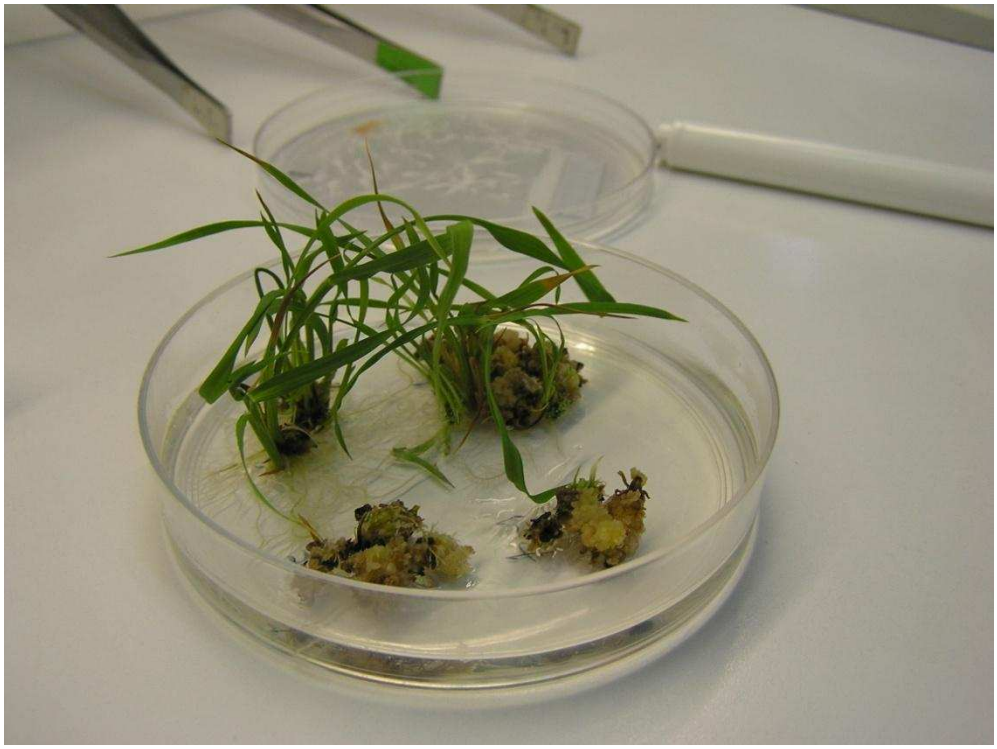

Figure A7. Regenerated rice plantlets ready for separation and transfer to MS0 rooting medium.

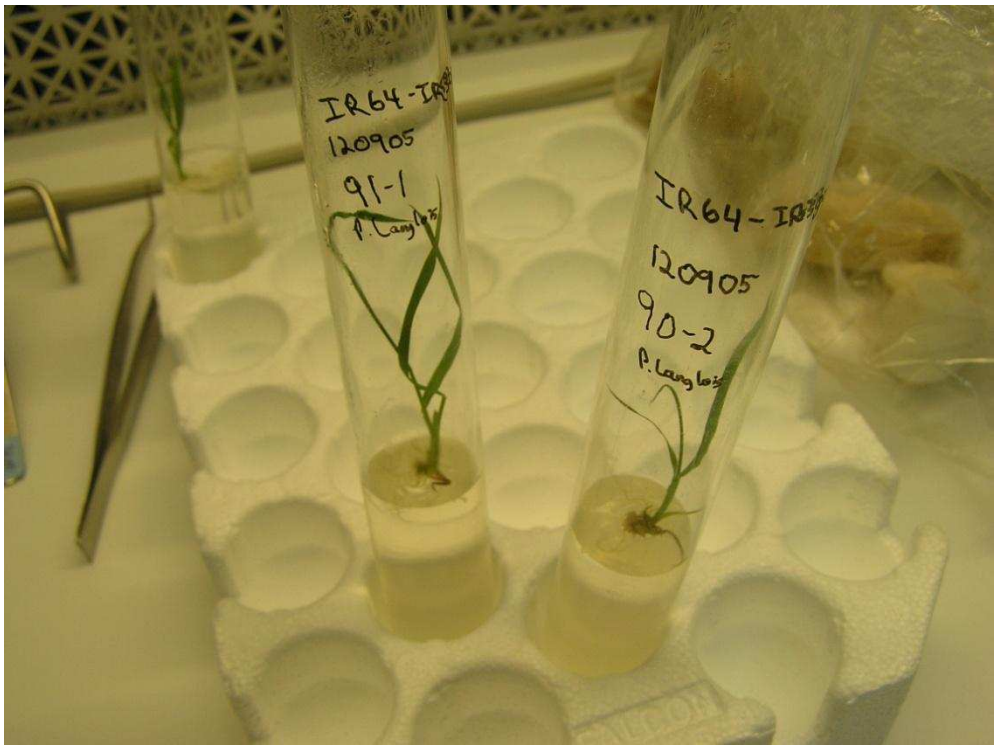

Figure A8. Single-transformant plantlets placed in MS0 rooting medium, ready for root proliferation. Note that the plantlets are numbered and still can be tracked back to the emybro they originated from.

Figure A9. Vigourous root growth, plants are now ready for transfer into Yoshida solution to be grown in greenhouse conditions.

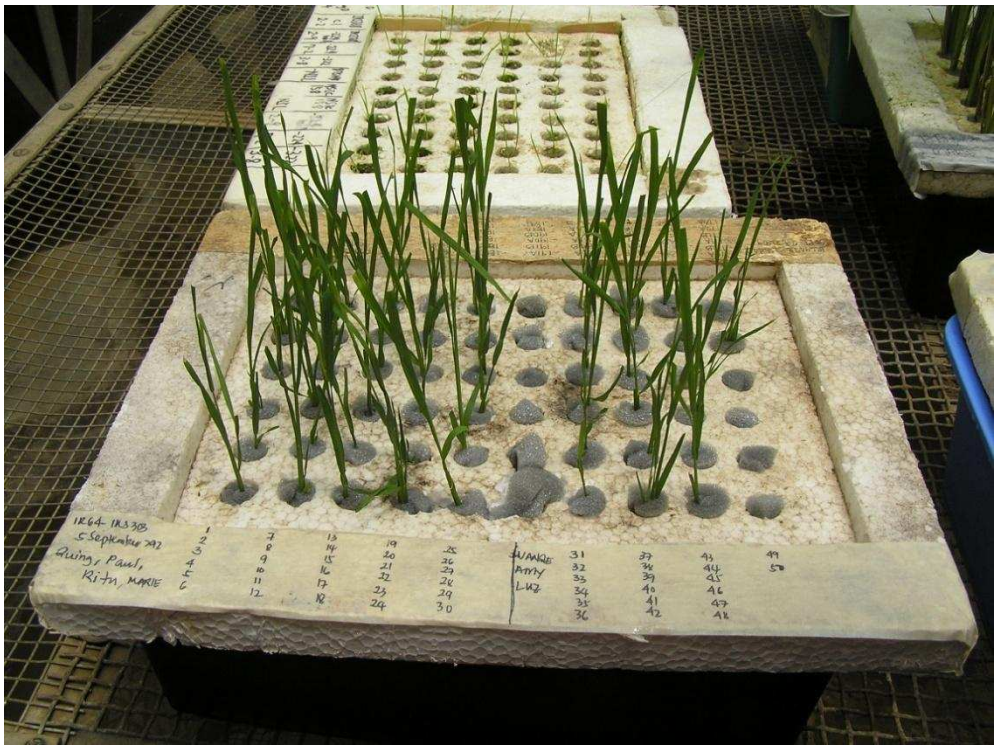

Figure A10. Plants growing in greenhouse conditions in Yoshida solution for later genotyping.

III. *Japonica* rice transformation protocol

Originally from Patrick Canlas (PC), Pam Ronald Laboratory, UC Davis
Adapted by Amadou Seck (2007), Jan Leach Laboratory, CSU
Revised by Amanda Broz (2011), Dan Bush Laboratory, CSU
Additions and revisions by Paul Langlois (2016), Jan Leach Laboratory, CSU

i. Materials

Equipment	
Biosafety cabinet	Laminar Flow hood
Sterilizer	Refrigerator
Autoclave	Incubator
pH meter	Shaker
Laboratory balances	Spectrophotometer
Stirrer/ hot plate	
Laboratory ware	
Beakers	Centrifuge tube, 50ml
Graduated cylinder	Spatula
Media bottles	Forceps
Scalpel/blade	
Consumables	
Petri dish (100x15, 100x200)	Filter paper
Syringe	Syringe filter
Micropore tape	Pipette tips
Parafilm	2ml Eppendorf tubes
Cuvette	Paper towels

ii. Preparation of stock solutions and media

Stock solutions: Components are dissolved in sterile water and stored at 4°C

Stock solution	Component	Amount
YM (for LBA4404 growth) pH to 7.0; Autoclave for making solid YM, add 8g/L agar before autoclaving	Yeast Extract Mannitol NaCl $MgSO_4 \cdot 7H_20$ K_2HPO_4	0.40 g/L 10.0 g/L 0.10 g/L 0.20 g/L 0.38 g/L
LB (for EHA105 growth) pH to 7.0; Autoclave for making solid LB, add 8g/L agar before autoclaving	LB premade media	25.0 g/L
TY Medium, for co-cultivation with calli pH to 5.5 with HCl; Autoclave and cool Add 1 mL of 200mM Acetosyringone stock solution per liter of TY for a final concentration of 200 uM immediately before use	Tryptone Yeast Extract Acetosyringone, add after cooling (final conc. 200 µM)	5.0 g/L 3.0 g/L 1 mL
Carbenicillin Calli-*Agro* overgrowth wash (optional) *only if additional control of *Agrobacterium* is needed* pH to 5.8; Autoclave, cool to ~50 °C Add 2 mL (per L) of 200 mg/mL carbenicillin	MS (salts+vitamins) Sucrose	4.4 g/L 30 g/L
Callus Induction Medium (1, MSD) pH to 5.8 with KOH; Autoclave and cool Add 1 mL of 200mM Acetosyringone (final conc. 200uM)	MS (salts+vitamins) Sucrose Gelrite/ Phytagel 2,4-D (1mg/mL stock)	4.4 g/L 30 g/L 4.0g/L 2 mL
Co-cultivation Medium (2, MSD + Acetosyringone) pH to 5.8 with KOH; Autoclave,cool to ~50 °C Add 1 mL of 200mM Acetosyringone (final conc. 200uM)	MS (salts+vitamins) Sucrose Sorbitol Gelrite/ Phytagel 2,4-D (1mg/mL stock)	4.4 g/L 30 g/L 50 g/L 6.0g/L 2 mL
Selection Medium (3. MSD + Carbenicillin + Drug)	MS (salts+vitamins) Sucrose	4.4 g/L 30 g/L

pH to 5.8 with KOH; Autoclave,cool to ~50 °C Add 1.25 mL (per L) of 200 mg/mL Carbenicillin (final conc. 250mg) Add 1.0 mL (per L) PPM (optional) Add 1.0 mL (per L) of 50 mg/mL Hygromycin (or 1mL of 20mg/mL Glufosinate)	Gelrite/ Phytagel 2,4-D (1mg/mL stock)	50 g/L 2 mL
Regeneration Medium (4, BN + S) pH to 5.8 with KOH; Autoclave,cool to ~50 °C Add 625 µL (per L) of 200 mg/mL Carbenicillin (final conc. 125mg/L- less is OK) Add 1.0 mL (per L) of 50 mg/mL Hygromycin (or 1mL of 20mg/mL Glufosinate)	MS (salts+vitamins) Sucrose Sorbitol BAP (1mg/mL stock) NAA (1mg/mL stock) Gelrite/ Phytagel	4.4 g/L 30 g/L 50 g/L 3mL 0.5mL 6.0 g/L
Rooting Medium (5, MS + CG) pH to 5.8 with KOH; Autoclave, cool to ~50 °C Add 625 µL (per L) of 200 mg/mL Carbenicillin (final conc. 125mg/L- less is OK) Add 1.0 mL (per L) of 50 mg/mL Hygromycin (or 1mL of 20mg/mL Glufosinate) Swirl and pour into sterile containers for plant growth. We use SOLO® cups with dome lids.	MS (salts+vitamins) Sucrose Gelrite/ Phytagel	4.4 g/L 30 g/L 2.0 g/L

iii. Drugs and other reagents

2-4-D
Stock solution 1mg/mL (Phytotechnology Labs #D295)
Also available as a powder from other chemical companies
Working concentration 2mg/L
Store solution at 4°C
Purpose: plant hormone causing induction of calli

Acetosyringone
(aka: 3', 5'-Dimethoxy-4'-hydroxyacetophenone; Sigma D134406)
Stock solution 200mM: dissolve 39.2mg in 1mL DMSO
Working concentration 200µM
Store stock at 4°C or -20°C (DMSO crystallizes at 4°C)
Store chemical at room temperature
Purpose: induces expression of virulence genes in *Agrobacterium*
Note: may require hand warming to dissolve after freezing
Also soluble in 70% ethanol

BAP
(aka; benzylaminopurine; cytokinin B)
Leach lab uses pre-made solution of 1mg/mL (Phyotechnology Labs #B130)
I make up my own 1mg/mL stock from powder;
Sigma (852430-1G-A) – dissolve in 0.1M NaOH
Working concentration 3mg/L
Store chemical at 4°C
Store stock solution at 4°C
Purpose: induces shoot formation during calli regeneration

Carbenicillin
Stock solution 200mg/mL: dissolve 200mg in 1 mL water (Phytotechnology labs #C346)
Working concentration varies (generally 100-500mg/L)
Store stock at -20°C
Store chemical at 4°C
Purpose: kills agrobacterium after co-cultivation with calli
Note: literature says that 100mg/L is enough to kill bacteria; cannot make higher concentration stock solution (than 200mg/ml) as drug will not dissolve.

Glufosinate
(aka; glufosinate-ammonium, 'BASTA', L-phosphinothricin, ppt)
Stock solution 20mg/mL: dissolve 20mg in 1 mL water
Working concentration 20µg/mL
Store stock at -20°C
Purpose: PLANT selection marker in many plant transformation vectors

Hygromycin
Stock solution 50mg/mL: dissolve 50mg in 1mL water
Working concentration 50ug/mL
Store stock at -20C in dark/foil (light sensitive!)
Store chemical at 4°C in dark
Purpose: PLANT selection marker in many plant transformation vectors
(we get this chemical from GoldBio; #H-270-1)

Kanamycin
Stock solution 50mg/mL: dissolve 50mg in 1mL water
Working concentration 50ug/mL
Store stock at -20°C
Purpose: BACTERIA selection marker in many plant transformation vectors

MS media (salts plus vitamins)
(aka; Murashige and Skoog media for plant growth)
Phytotechnology labs #M519
Store at 4C
Ideally powder should be white and fluffy
Warm to RT before opening to prevent water from getting in MS (it gets yellow)
I do not know if the salts and vitamins are critical for good calli growth, but can't hurt…

NAA
(aka; Naphthaleneacetic acid)
Pre-made solution of 1mg/mL (Phytotechnology Labs #N605)
Working concentration 0.5mg/L
Store solution at 4°C
Purpose: induces regeneration of calli

Phytagel/ Gelrite
Sigma product number P8169-250g
Purpose; Gelling agent for media
Gel-rite should be able to be used interchangeably
Agar can also be used but amounts will differ and media is cloudier

PPM (Preservative for plant tissue culture)
(aka; 5-chloro-2-methyl-3(2H)-isothiazolone and 2-methyl-3(2H)-isothiazolone)
Plant Cell Technology Inc.
Working concentration 1 mL/L
Store solution at room temperature
Purpose: kills agrobacterium and other contaminants in tissue culture
can also be used in selection media

Rifampcin
Stock solution 30mg/mL: dissolve 30mg in 1mL DMSO
Working concentration 30 µg /mL
Store chemical at 4°C in dark
Make stock solution fresh as needed (has short half life, store only 1 day at 4°C wrapped in foil; possible use up to 1 month according to others; can also use 50mg/mL stock)
Purpose: keep appropriate *Agrobacterium* strain from contamination (this resistance gene is generally on the chromosome and is present in EHA105 and LBA4404)

Streptomycin
Stock solution 100mg/mL: dissolve 100mg in 1 mL water
Working concentration 100µg/mL
Store stock at -20°C
Purpose: keeps virulence plasmid in certain strains of *Agrobacterium* (LBA4404)

iv. Procedure

I. Seed sterilization and Calli Induction

1. Remove rice hulls (by hand or using a grinder) and place in a falcon tube or similar [a]
2. Cover seeds with 40% bleach plus one drop of TWEEN-20 per 50 mL solution [b]
3. Shake horizontally at room temperature (~125 rpm) for 30 min [c]
4. Rinse three (or more) times in sterile water, decant in between rinses
5. Tap sterilized seed onto a sterile piece of filter paper or sterile paper towel to dry
6. Transfer seed to calli induction media (MSD) plates [c]
7. Seal plates with ¾ inch vent tape [d]
8. Place under continuous fluorescent light around 25 µE at 28-30º C [e]
9. Routinely check MSD plates for contamination [f]
10. After two weeks or so, separate calli from the rest of the germinating seed (ie; coleoptile and endosperm) and transfer to new calli induction media (MSD) 4-7 days before co-cultivation [g]

Notes:

[a] Immature green seeds are preferred as they have better rates of calli induction, cell proliferation, growth, and transformation efficiency. Ideally, immature seeds are fresh or less than 6 months old.

[b] More dilute bleach, such as 20%, works well and can be substituted.

[c] Plate 50 seeds per large petri dish (100x15mm). Use a smaller seed density if you are worried about contamination (as that can save you some transferring time later if contaminants arise). When plating on media, lay the seed flat on the media and gently press it down. It is important to not push the embryo all the way under the media because this is where calli formation originates. If you are unsure of where the embryo is on the seed, hold the seed up to the light and find the dark area at one of the ends of the seed. This is the embryo.

[d] Vent tape is highly recommended, however parafilm can be used only if some small holes are poked into it after wrapping, to allow air transfer. Calli formation is better with gas exchange.

[e] The growth chambers can range from 10-50 µE. 'Fried calli' can be a result form too much light or drying out from excessive airflow.

[f] If a seed/calli on a plate gets contaminated, it is better to just transfer all the non-contaminated calli to a new plate. Removing the contaminated calli from the plate doesn't stop contamination or work well.

[g] Use calli that are between 2-4 weeks old for transformation. This varies as there is literature as there are reports of using calli that are 4-5 days old. Other studies have used calli slightly over one month old. Plates may be dry after 2 weeks; if so, transfer calli.

***Agrobacterium* culture and calli transformation**

1. Inoculate 1mL LB + selection antibiotic with *Agrobacterium* and shake at 250 rpm at 28-30º C for 24 hrs (may take longer if done at room temp)

   Alternative step 1. Streak out *Agrobacterium* on LB + selection antibiotic plate and grow at 20-30º C for 2-3 days

   Alternative step 2. Scrape *Agrobacterium* off of plate with a sterile loop, resuspend in 5-10mL TY+AS[a] and shake for 2-4 hours or until OD_{600} is close to 0.1

2. Add 150uL *Agrobacterium* culture to 5 mL of TY+AS[a] and shake at 250 rpm at ~25º C for 2-4 hrs (OD_{600} should be 0.1-0.2; if not, dilute with TY+AS or grow longer).

3. Add your 2-4 week old calli to the tube of *Agrobacterium* TY+AS and shake gently (either by using a very low shaker rpm, or simply swirling the tube by hand periodically) for 5-30 min. [b]
4. Remove agro solution (by decanting or pipette) and blot dry calli on sterile filter paper to eliminate excess bacteria.
5. Transfer the calli to co-cultivation (MSD+AS) plates and seal with vent tape.
    a. **Positive Control:** Co-cultivate some calli with your strain of *Agrobacterium* with no plasmid on plain selection media with only carbenicillin and no drug/antibiotic.
6. Keep plates in the dark [c] at room temperature (21-25º C) for 2-3 days. [d]

Notes:

[a] To make TY+AS, add 10uL of 200mM acetosyringone (AS) to 10 mL TY media (final concentration of AS=200uM) – There is no need to add antibiotic to this media.

[b] There are various incubation times in the literature for this step, but the timing doesn't seem to be critical. I generally incubate for 30 min. and invert or swirl the tube by hand every 5 min. or so.

[c] Wrapping plates in foil in an unused drawer that is room temperature works well.

[d] For more vigorous agro strains (EHA105) you probably only want to incubate for 2 days maximum. LBA4404 tends to overgrow to a lesser extent so you may be able to get away with 3 days of co-cultivation. Two days is often optimal over the weekend for instance, with LBA4404.

Additional notes:

- *Agrobacterium* take longer to grow at cooler temperatures, so plan ahead. If there are no available 30°C shakers use the ‘alternative method'. It is also easier to see how much growth are on a plate rather than in culture. If you use the alternative method, do NOT store the plates in the fridge and then scrape off the bacteria; they should be growing when they go into the TY+AS. LBA4404 tends to be clumpy so vortex it once it is in liquid culture (**Alternative step 2**).
- It is best to make multiple glycerol stocks of your construct +*Agrobacterium* strain. In the case of not having success in your first few attempts, you will then have multiple glycerol stock back-ups to streak out on plates. This is a good idea because you don’t want to defrost your glycerol stock multiple times.

Selection

1. Very gently wash calli with MS+carbenicillin solution until the liquid is somewhat clear. Don't wash too hard as infected micro-calli may come off and are valuable for transformation due to low surface area.
    a. If no, or very little *Agrobacterium* overgrowth is visible on plate [a], this step can be skipped.
2. Transfer calli to selection media (MSD+carb+drug [b]), seal plates with surgical tape or parafilm [c].
    a. **Positive control:** plate half of the calli treated with *Agrobacterium* lacking the plasmid (from ***Agrobacterium* culture and calli transformation**, Step 5.a.) on your plain selection media with only carbenicillin and no drug/antibiotic. This is important to carry out throughout the experiment to demonstrate completion of the tissue culture process.
    b. **Negative control:** plate the other half of the calli treated with *Agrobacterium* lacking the plasmid (from ***Agrobacterium* culture and calli transformation**, Step 5.a.) on your normal selection media (carb + drug/antibiotic). These should all die and turn black eventually.
3. Incubate at 30º C under continuous fluorescent light around 25 µE. Transfer calli to new selection media if there is any sign of *Agrobacterium* overgrowth. Otherwise transfer every 10-21 days.
4. Keep calli on selection media for 2-8 weeks total [d]

Notes:

[a] *Agrobacterium* may be visible on the plates after co-cultivation depending on the strain being used for transformation. If there is some growth, proceed with the Step 1. In most cases if there

is *Agro.* overgrowth, you just need to keep washing the calli until the media becomes clear. However sometimes growth is difficult to manage and it may be worth starting over. As always keep checking the plates for overgrowth. In the case of overgrowth, transfer everything that is not covered in *Agrobacterium* to a new plate. Also, you can increase the carbenicillin in your wash or selection media if you continue to have problems. (EHA105 has tendency to overgrow versus LBA4404)

[b] Some reports use PPM (1mL L^{-1}) in this media, but it is optional and not necessary. It may be helpful if you are having problems with *Agro.* overgrowth or contamination. If carbenicillin isn't working, there are multiple drugs out there that kill *Agrobacterium* or prokaryotes, that may be worth a try as well.

[c] Depending on your growth chamber and its humidity, vent tape may be ok for this step. Parafilm is preferred because the media often dries out somewhat quickly. However, if you have condensation is present on plates or a large amount of *Agrobacterium* overgrowth, surgical tape may be better.

[d] After 2-6 weeks resistant microcalli will begin to appear. There seems to be a fine line between life and death for calli and it is difficult judging which calli/microcalli are going to make it. Additionally, some calli that are not transformed are able to grow on selection plates, this step sometimes is not extremely selective. However it does allow the calli to grow a bit and recover from their bacterial attack before proceeding to regeneration.

Regeneration

1. When calli have been rapidly growing and proliferating and/or when resistant microcalli are observed (approximately 2-8 weeks on selection media, 4 weeks on selection media is typical), transfer to regeneration media and seal plates with parafilm [a]
2. Incubate at 30º C under continuous light (same conditions it's been at) for 2-3 weeks.
3. Transfer calli to new regeneration media [b]. At this point (2-3 weeks) hopefully non-resistant calli will be dead/black in color [c], and resistant ones will be white and creamy as well as golden/ bright yellow in color and may even be exhibiting small green leaf primordia and becoming greenish in color.
4. Keep calli on regeneration media until small 'leaves' emerge (this may take up to 2 months)[d].

Notes:

[a] At this point resistant microcalli should be 2-5mm wide. Pick them off the original callus and place on regeneration media.

[b] Calli themselves do well dry and in this case it is ok to seal with parafilm because the media contains sorbitol and a larger amount of agar. However media shouldn't ever get dry, and by using parafilm calli don't need to be transferred as much.

[c] Sometimes the non-resistant calli do actually turn black and die, however sometimes they no longer grow and turn a beige-cream color and may seem 'normal' looking. However these can often not be recovered in the long run. Resistant calli should start turning a bright white/ yellow. This may take a while for some of the calli, often resistant calli become more white/ yellow after 3 weeks, or even 5-6 weeks. The yellow color always comes before the tiny green cells. If you don't see yellow cells after 2 months, it may be good to start again.

[d] Leaves should be at least 5-20mm before transferring to rooting media. Plantlets can stay in regeneration longer, as they will likely sprout many 'leaves' over time. Plantlets will grow a root system and more foliage and do well once they get growing.

Additional notes:

- Regeneration often seems to have more selection than the Selection stage
- If no leaf primordia/leaves are seen within 2 months, or microcalli stop producing, it is likely that the calli will not generate at all.
- Selecting the bright yellow calli clumps away from any non-growing/non-yellow tissue and put them on a new plate of media, is good for the calli and to segregate them from the old non-growing calli. Do not throw out the non-growing calli at this point, just seal the plate back up and wait. Some calli grow/regenerate faster than others, so keep them around until you are sure they are not going to regenerate.

Rooting and growing

1. When calli are greenish and are developing leaves, transfer to rooting medium.[a]

2. Incubate at 30º C under continuous light, some reports switch the light schedule to 16hr light- 8h dark cycle at this time.

3. After a few weeks plantlets should develop a nice root system and bigger leaves.

4. Once plants have a good root system, transfer to soil and place in growth chamber(s) or a greenhouse. [b]

Notes:

[a] Depending on how big the calli are, how many 'leaves' they have, and if you feel comfortable, carefully break them up into one leaflet per calli chunk before putting them in rooting media. This helps ensure the separation of individual transformation events which are important for leaf collections for genotyping later on.

[b] Plants are still very delicate at this time. Keeping them under a humidity dome for 3-7 days to ensure low transplanting stress and keeping high humidity, and prevention of damage from air movement is highly suggested.

Additional notes:

- Breaking off individual plants when placing in a growth chamber or greenhouse helps separating plantlets that may have originated from a calli that received a different transformation insertion event, but it must be done gently and carefully. If you have many stems coming out of one former calli clump it is a good idea to separate as good as possible, or break it into smaller pieces
- Very high management of separating microcalli and individual shoots from plantlets for planting, can higly improve selection for single transformation events. However, it can be difficult to manage.
- Most of the plants coming out of tissue culture are often atypical looking due to the process it underwent and the stresses involved. So for a trait like biomass or anything phenotypic, it is almost always inappropriate to do any phenotyping at the T_0 stage.

Experiment Controls

Co-cultivate some calli with your strain of *Agrobacterium* (no plasmid)

1. For your positive control, plate these on Selection media with carbenicillin only (without selection drug/antibiotic)

2. For your negative control, plate on your normal selection media (carb + drug/antibiotic)

3. For another positive control, co-cultivate calli with *Agrobacterium* containing an empty vector or a tagged construct (I use pUbiNC1300RFCA-GUS) and proceed as normal. This is a good control, particularly if you are using a silencing or over-expression construct that could be harmful to the plant.

It is important to make sure to include all appropriate control experiments when doing all transformation studies. This is very important to ensure a properly executed and successful

experiment. In the case of the positive control, it undergoes the transformation process but does not receive a gene product. Therefore in undergoing all of the other transformation steps, it demonstrates completion of proper tissue culture, from seed, to calli, to plant. In the case of negative controls, they ensure that the resistance marker that you are using is actually being selected for on media and is therefore selecting for your transformants. If you have a failed negative control, and all calli live and regenerate, you cannot have confidence if the calli received the gene product and makes genotyping much more strenuous and large.

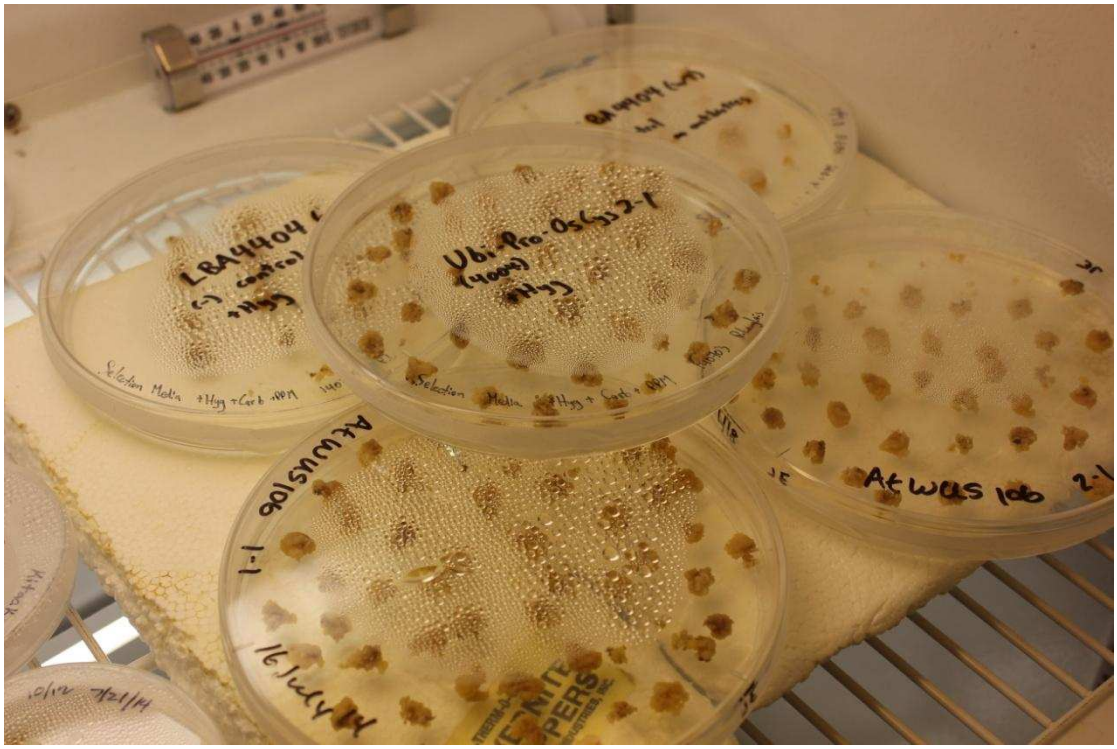

Figure B1. *Agrobacterium* treated calli undergoing selection.

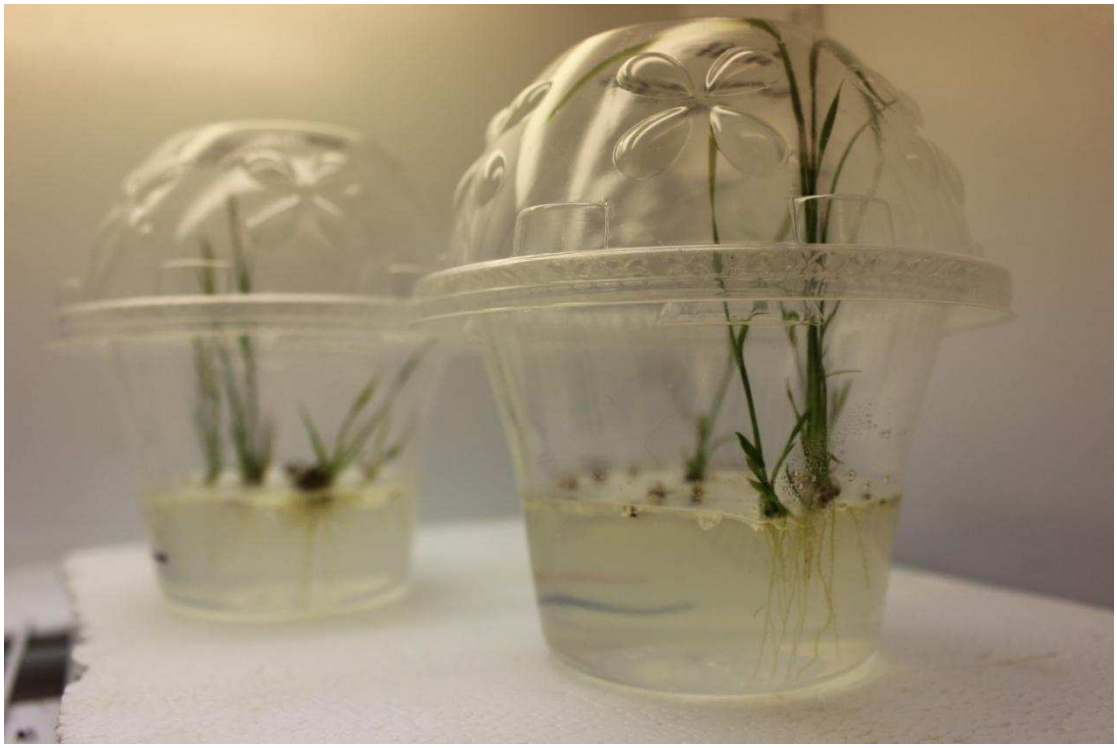

Figure B2. Successful transformation plantlets growing on rooting media, nearly ready for planting.

Figure B3. Separated plantlets transplanted into the greenhouse with humidity domes to insure lots of moisture and avoid excessive air movement. Be sure to reduce stresses in the transition that these vulnerable plantlets experience.

Figure B4. From left to right, a wild type Kitaake plant, successful transformant containing *Os*Cys, and a T2 plant that segregated without the gene it recieved in the initial T0 generation.

IMPACT OF VOZ TRANSCRIPTION FACTORS ON LIGNIN BIOSYNTHESIS IN RICE

We wanted to determine if reduced expression of *Os*VOZ transcription factors altered lignin composition. Lignin is a component found in the secondary cell walls of plants and is an important consideration in the biofuel industry. It is a complex polymer that is classified based on the three types of monomers it consists of: sinapyl (S), coumaryl (H), and coniferyl (G) (Tanger, 2014). In the case of plants that contain high amounts of lignin, ethanol producing microbes cannot efficiently reach cellulose fibers very well, and inhibit fermentation. However, lignin in high amounts can be beneficial when burned, used in gasification, or in pyrolysis because of the high energy bonds that lignin monomers make with each other when forming polymers (Tanger, 2014). Therefore, using rice as a model for lignin deposition in grasses, may help with gene modifications or marker assisted selection to improve a plant's phenotype for either more or less lignin in related monocot bioenergy crops such as sorghum, switchgrass, and *Miscanthus*.

VOZ (Vascular plant One-Zinc finger) transcription factors are part of the NAC family of transcription factors that are major regulators of flowering regulation, abiotic and biotic stress responses, as well as drought and cold tolerance (Nakai et al. 2013; Mitsuda et al. 2004). The VOZs are also thought to be involved with regulation of high or low lignin content. We observed lignin content using histological staining approaches, as well as gene expression data based on genes involved in the phenylpropanoid pathway, responsible for lignin production. We wanted to determine if VOZ transcription factors are regulators of lignin deposition, and in knocking out the VOZs, is the amount of lignin in rice affected. If lignin content is altered, how does the distribution of lignin change?

My work in this project was performing histological tests on different ages of both leaf and stem tissues of rice lines with VOZ1, VOZ2, and VOZ1 & VOZ 2 knock-down or knock-out lines, as well as Kitaake lines with normal VOZ expression (wild type, WT). I observed the relative amounts of G lignin in the knock-down plants compared to the WT Kitaake (the genetic background of the knock-outs). We also collected tissue for RNA extractions to compare the relative transcript levels through real-time PCR of various genes involved in the phenylpropanoid pathway to see if these transcription factors had an impact on the biosynthesis pathway of lignin at any given step.

The rice lines with altered expression we included in this study were plants with VOZ1 or VOZ2 silenced by RNAi, for two different silenced lines in the T1 and T2 generation, and a complete double knock-out for VOZ1 & VOZ 2. We also had a heterozygous mutant for *Os*PAL4 in IR64, an *indica* variety. The concept behind the *Os*PAL4 mutant is that lignin expression should be reduced due to a loss of 1, of the 2 copies of an important gene in the beginning of the phenylpropanoid pathway. The two control plants for RNA expression and lignin analysis were therefore Kitaake and IR64 (WT). All plants were grown under greenhouse conditions in the CSU Plant Growth Facilities at about 24°C, 85% RH, and 16 hr daylight cycles. Approaches I took to visualize lignin in rice stems and leaves included mäule staining, phloroglucinol staining, and autofluorescence of lignin under ultraviolet (UV) light. Phloroglucinol staining reacts with the cinnamaldehyde end groups of lignin to give rise to a red-violet color (Gahan, 1974). In the case of Mäule staining, it is often more red in the presence of a higher S:G lignin ratio, and is more brown with a lower S:G lignin ratio (Guo, 2001). Lignin can also be visualized under UV light, however differentiating amounts of lignin between lines was difficult to assess. Therefore,

of these methods, phloroglucinol staining showed the most differentiation of lignin amounts between lines and was our chosen method for observing lignin content. However, getting this to work was not trivial. We began the experiment planning to use a microtome for sectioning rice leaf and stem samples at different developmental stages, then proceed with staining sections that were cut and deparaffinized. We couldn't stain tissue prior to using the microtome for a number of reasons. One being that the tissue degrades and loses integrity due to the use of hydrochloric acid that is a part of the phloroglucinol stain, also the stain loses intensity over a short period of time. We were also unsure if the stain would be compromised throughout various xylene and ethanol baths used to deparaffinize the slides because it is not a permanent stain. For these reasons we stained tissue after sectioning, mounting, and deparaffinizing. Unfortunately, the rice samples would not take up the phloroglucinol stain after the microtome process. This may have been because the rice leaves and stems were stored in formalin, or it may have been due to the chemicals used in the deparaffinizing process.

We also had the surprising opportunity to use a cryostat to make our sections. This method did work and the stain worked well, as we had read in previous reports. However, sections were often highly fragmented in leaf samples and did not look optimal in most cases. As for the stem samples, the stem section would routinely fall out of the O.C.T. compound due to the large diameter of the stems. Often, the section was not able to even be laid down on the slide in the proper manner. We then began doing fresh hand-cut sections that were taken within 2-3 days and stored in sterile water with a surfactant. These samples remained fresh and hydrated and contained all chlorophyll. At first we thought it would be important to remove the chlorophyll for stain visibility and imaging purposes, however after using these fresh handcut sections, we could

see the stain better than all the other methods. We also wanted to microtome and/or use the cryostat initially but we wanted to have consistent section thickness sizes to interpret staining intensity better, and thus compare lignin between lines better. However, we noticed that samples that had sat in ethanol to remove the chlorophyll, would not take up the phloroglucinol stain. Therefore, it was determined that for our case, fresh hand cut sections showed the clearest staining, and differences in the lines could be best observed best.

In our observations the knock-out lines for VOZ seemed to show a higher lignin content than in wild type. This was mostly evident in the outer sclerenchyma cells at the outside of the vascular bundles and in some cells close to the vascular bundles in both leaf and stem samples, but the bundles themselves didn't appear to show much change (Figure C1). There was of course some variation between samples, and it was not evident in silenced VOZ lines. This did however support our original hypothesis that the VOZs are involved in negative regulation of lignin (Figure C2), and that with knocking out both VOZ1 and VOZ2, we observed higher lignin content. As for the *Os*PAL4 heterozygous mutant line, we observed what we expected to see. In this case the heterozygous mutant, showed considerably less lignin than in the wild type IR64 background (Figure C3). This was intuitive because PAL4, being a key enzyme in the beginning of the phenylpropanoid pathway, is overall responsible for lignin production, and in the loss of one of the gene copies, total lignin content was affected.

Gene expression results using RT-PCR were somewhat inconclusive, however. Lines that were supposed to be silenced for the VOZs did not show a drastic difference in VOZ expression compared to WT, therefore not much information about VOZ influenced lignin production, could

be derived from the VOZ expression data (Figure C4). We did demonstrate again however, that in silenced VOZ lines, PAL4 expression is increased. This was noticeable in both the leaf samples at 21 days and 6 weeks after planting, but no difference was obvious in the stem samples. (Figure C4).

Overall this experiment was very valuable in gaining experience and expertise in histological and sectioning methods with rice and lignin. Work involved in using the cyrostat was still very useful, however lots of practice and meticulousness is required to achieve high quality sections for leaves and stem. If we had more time to practice and optimize our sections with the cyrostat, I'm sure using this method could have turned out very well. I hope that others doing research in this area can take something away from our trials of finding the most appropriate and clearest methods for sectioning and staining rice.

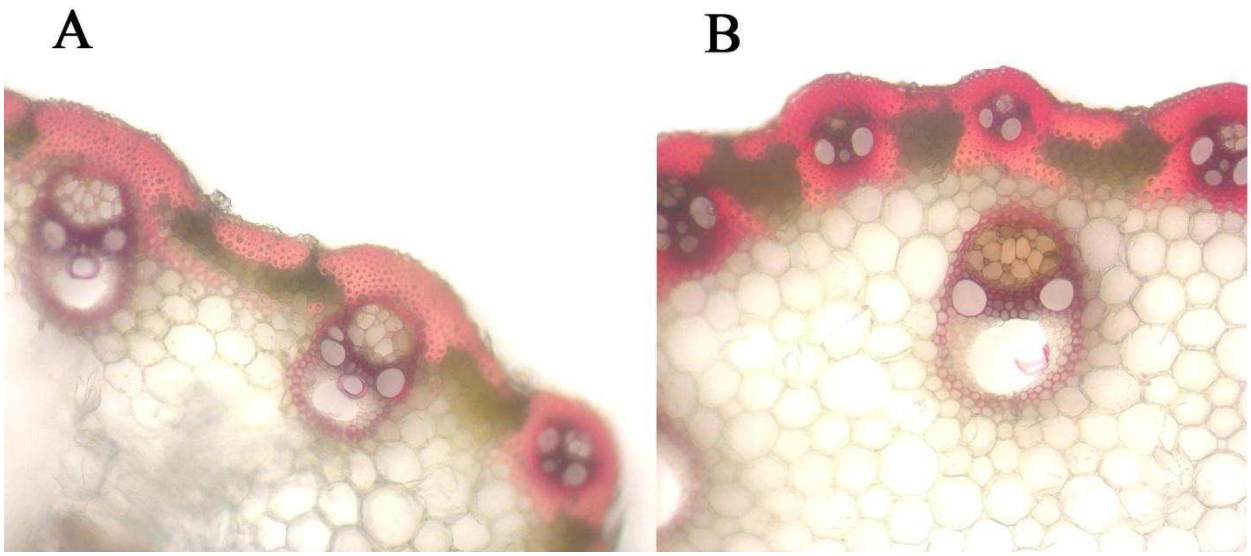

Figure C1. Phloroglucinol staining showing relative amounts of G-subunits in lignin, in both wild type (WT) (A) and a double knockout line for VOZ 1 & VOZ 2 (B) in Kitaake rice background. Pictures were taken on a 20x objective on a compound microscope.

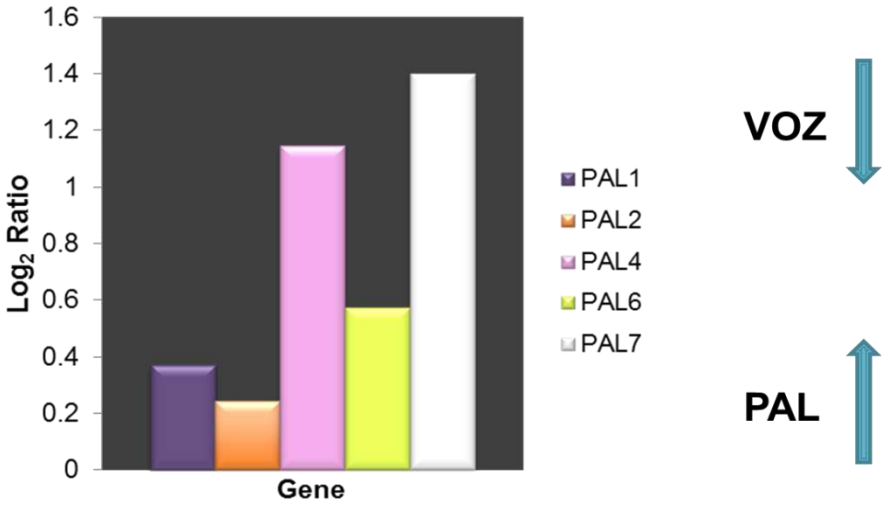

Figure C2. Transcriptome analysis suggests VOZs may regulate PAL expression.

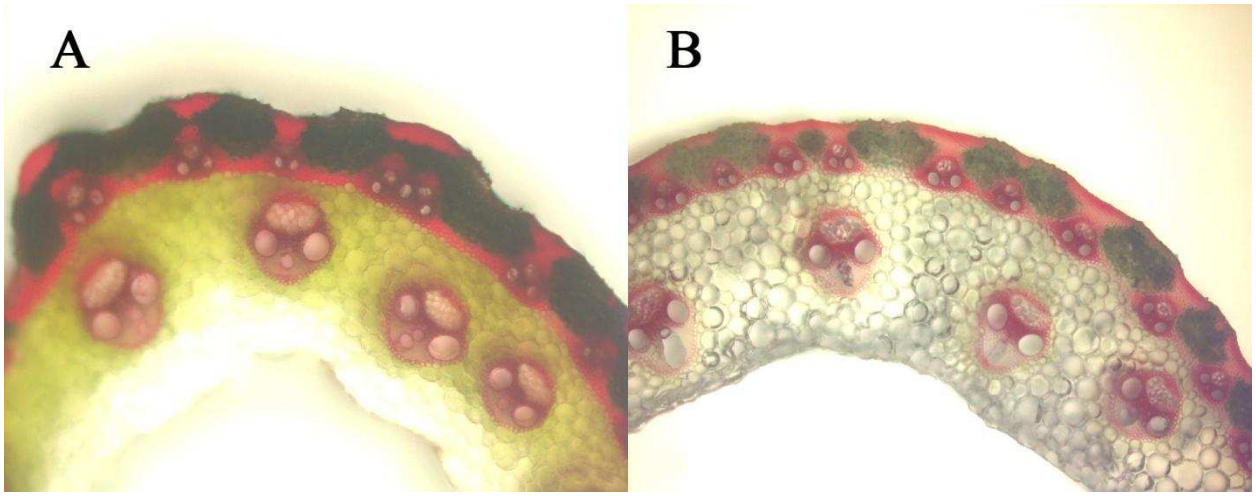

Figure C3. Phloroglucinol staining showing relative amounts of G-subunits in lignin, in both wild type (WT) (A) and heterozygous mutant for *Os*PAL4 (B) in IR64 rice background. Pictures were taken on a 10x objective on a compound microscope.

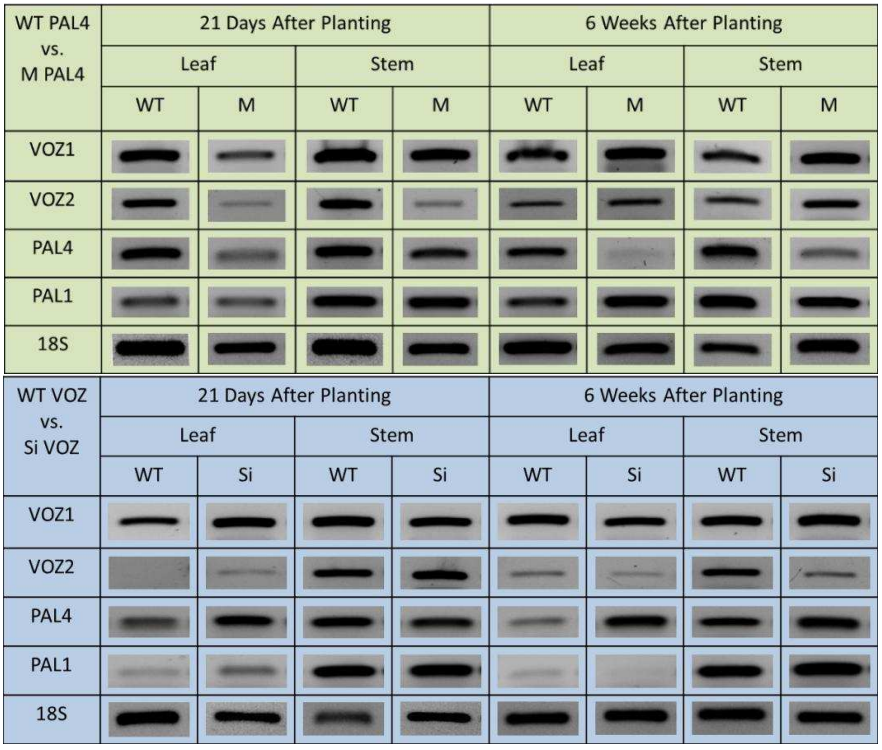

Figure C4. RT-PCR depicts VOZ and PAL gene expression in rice leaf and stem tissue at 21 days and 6 weeks after planting, in VOZ knock down lines (RNAi) and PAL4 mutant (M) lines relative to wild type (WT) plants.

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