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A comparative approach expands the protein-protein interaction node of the immune receptor XA21 in wheat and rice

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Abstract

The rice (*Oryza sativa*) OsXA21 receptor kinase is a well-studied immune receptor that initiates a signal transduction pathway leading to resistance to *Xanthomonas oryzae* pv. *oryzae*. Two homologs of *OsXA21* were identified in wheat (*Triticum aestivum*): *TaXA21-like1* located in a syntenic region with *OsXA21,* and *TaXA21-like2* located in a non-syntenic region. Proteins encoded by these two wheat genes interact with four wheat orthologs of known OsXA21 interactors. In this study, we screened a wheat yeast-two-hybrid (Y2H) library using the cytosolic portion of TaXA21-like1 as bait to identify additional interactors. Using full-length *T. aestivum* and *T. monococcum* proteins and Y2H assays we identified three novel TaXA21-like1 interactors (TaARG, TaPR2, TmSKL1) plus one previously known in rice (TaSGT1). An additional full-length wheat protein (TaCIPK14) interacted with TaXA21-like2 and OsXA21 but not with TaXA21-like1. The interactions of TaXA21-like1 with TmSKL1 and TaSGT1 were also observed in rice protoplasts using bimolecular fluorescence complementation (BiFC) assays. We then cloned the rice homologs of the novel wheat interactors and confirmed that they all interact with OsXA21. This last result suggests that inter-specific comparative interactome analyses can be used not only to transfer known interactions from rice to wheat, but also to identify novel interactions in rice.

Key words: protein-protein interactions, disease resistance, comparative genetics, signaling networks, yeast-two-hybrid, OsXA21.

Introduction

Plants have evolved a variety of defense mechanisms against pathogens. A key component of this defense response is the presence of pattern recognition receptors (PRRs), which recognize conserved microbial signatures. In plants, such PRRs typically consist of receptor-like kinases (RLKs) that carry a non-arginine aspartate (non-RD) motif in the kinase domain.

The OsXA21 protein is a well-studied PRR that confers broad-spectrum resistance to *Xanthomonas oryzae pv. oryzae* (henceforth, *Xoo*). OsXA21 contains extracellular leucine-rich repeats (LRRs), a transmembrane and juxtamembrane (JM) domains and a non-RD Ser/Thr kinase domain (Song et al.1995) (Figure 1). The cytosolic portion of OsXA21 (amino acids 668 to stop codon, henceforth OsXA21K668), which includes part of the JM domain and the complete kinase domain, has previously been shown to serve as a high affinity binding site for several downstream signaling proteins, including OsXB3, OsXB10, OsXB15 and OsXB24 (Wang et al. 2006; Park et al. 2008; Chen et al. 2010*b*; Park et al.2010). OsXB24, an ATPase, interacts with the OsXA21 JM domain and uses ATP to promote phosphorylation of specific Ser/Thr sites on OsXA21, which is associated with an inactive state (Chen et al. 2010*b*). Upon pathogen recognition, the OsXA21 kinase disassociates from OsXB24 and is activated, triggering downstream defense responses (Chen et al. 2010*b*). OsXB3, a RING finger ubiquitin ligase, is transphosphorylated by the OsXA21 kinase *in vitro* (Wang et al. 2006). OsXB15, a PP2C phosphatase, binds to OsXA21 and dephosphorylates OsXA21 negatively regulating the OsXA21-mediated innate immune responses (Park et al. 2008). Recent studies suggest that upon infection, OsXA21 is cleaved and that the intracellular kinase domain is translocated to the nucleus where it interacts directly with the transcription factor OsWRKY62 (XB10) (Park and Ronald 2012). So far, 12 OsXA21 binding (OsXBs) proteins have been shown to interact with

OsXA21 by yeast-two-hybrid (Y2H) or co-immunoprecipitation analyses (Lee at al. 2011). Although some of the OsXBs have been well characterized, the direct downstream target proteins phosphorylated by OsXA21 have not yet been identified.

Previously, we identified two OsXA21 homologs from wheat, designated *TaXA21-like1* (JX424300) and *TaXA21-like2* (JX4243001) and tested their interactions with known interactors of OsXA21 in rice (Cantu et al. 2013*b*). We showed that both TaXA21-like genes interact with rice OsXB3, OsXB15 and OsXB24, and OsWRKY76 suggesting that TaXA21-like genes may have some overlapping functions with rice OsXA21. We report here the identification of additional wheat proteins that interact with TaXA21-like1 and TaXA21-like2 from a screening of a wheat Y2H library. We also validated in rice the new interactions discovered in wheat, demonstrating that comparative protein interaction studies can be useful not only for accelerating the transfer of knowledge from rice to wheat, but also to enrich the interactome map of rice.

Material and Methods

The sources of the different clones, genes and proteins used in this study are indicated by the following two-letters prefixes: *Os* (Oryza sativa L.), *Tm* (diploid wheat *Triticum monococcum* L.), and *Ta* (hexaploid wheat, *T. aestivum* L.). The A^m genome of *T. monococcum* is more than 99% identical to the A genome of *T. aestivum* and more than 97% identical to the B and D genomes (Dubcovsky and Dvorak 2007) and therefore, limited differences are expected in the structure of orthologous wheat proteins and their corresponding protein-protein interactions.

Wheat yeast- two-hybrid cDNA library

RNA for the wheat cDNA library construction was extracted from leaves of *Triticum turgidum* ssp. *durum* cv. Langdon (line RSL65, Cenci et al. 2003). RNAs extracted from healthy wheat leaves were pooled with RNAs extracted from leaves infected by *Puccinia striiformis* f. sp. *tritici* (PST), a fungal pathogen of wheat responsible for recent stripe rust epidemics (Wellings 2011). The rationale for pooling RNAs from both tissues in a single cDNA library was to include wheat transcripts that were induced under biotic stress as well as constitutively expressed wheat transcripts. We used this strategy to increase the probability of detecting TaXA21 interactors that are relevant in the disease response.

The Langdon line RSL65 used in this study carries the *Yr36* resistant gene that confers partial resistance to PST at temperature higher than 25°C (Fu et al. 2009). RSL65 plants were grown in controlled conditions with 10°C nocturnal temperature, 15°C diurnal temperature, and 16 hours of light. At this growth conditions *Yr36* resistance is not functional and plants are partially susceptible to PST. RSL65 plants at the 3-5 leaf stage were inoculated with urediniospores of race PST-113 as described in Fu et al. (2009). Infected leaves from RSL65 were collected at 0, 3, 9, and 15 days post-inoculation. Total RNA was prepared using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) and its integrity was evaluated by gel electrophoresis.

RNAs from healthy leaves and leaves at different stages of PST-infection were pooled and reverse transcribed into cDNA using the "Make Your Own Mate & Plate Library System" following the company's protocol (Clontech, Mountain View, CA, USA). The cDNA was then recombined into the library prey vector (*pGADT7-Rec*) using Clontech's SMART technology included in the library system. The final library was transformed into the yeast strain Y187 (*MATα*) following the Clontech protocol.

Yeast-two-hybrid screening

The wheat *TaXA21-like1* (from amino acid 681to stop codon, ortholog of rice OsXA21k668) was cloned into pENTR™ Directional TOPO® vector (Invitrogen). The Gateway® LR ClonaseII™ reaction (Invitrogen) was used to clone the *TaXA21-like1* into yeast cloning vector pLAW10 to generate the bait construct (Cantu et al. 2013*b*). The Gateway (Invitrogen) compatible Y2H vectors pLAW10 (BD) and pLAW11 (AD) were kindly provided by Dr. Richard Michelmore (Perroud and Michelmore, unpublished). The bait constructs were introduced into the yeast strain "Y2H Gold" (Clontech, Mountain View, CA, USA) using the lithium acetate method (Gietz et al. 2002). Colonies from the Y2HGold baits were grown to approximately $1x10^8$ cfu/ml in 50 mL SD media lacking tryptophan (SD-Trp). Cells of the Y2HGold baits were pelleted, washed once with sterile H_2O and resuspended in 50 mL rich yeast media 2×YPAD. One aliquot of the Y187 target yeast (>2 $x10^7$cells) was combined with the baits. Yeast strains were allowed to mate for 20-24 hrs at 30^{o}C with slight shaking. Yeast cells were then isolated and washed twice with sterile water and plated on SD media without leucine, tryptophan, histidine, and adenine (SD/-Leu/-Trp/-His/-Ade).

Yeast putative positive diploids from the primary screens were isolated and plasmids extracted using Zymoprep I™ Yeast Plasmid Minipreparation Kit (Zymo Research,CA,USA). The Matchmaker AD LD primers (AD5:5'CTATTCGATGATGAAGATACCCCACCAAACC-C-3' and AD3: 5' GTGAACTTGCGGGGTTTTTCAGTATCTACGAT-3') were used to amplify the inserted wheat gene fragments. To confirm the observed interactions and eliminate false positives, yeast plasmids were transformed into *E. coli* DH5α, and the rescued plasmids together with the bait were co-transformed into yeast strain Y2H Gold. Transformants were selected on SD media lacking leucine and tryptophan (SD/-Leu/-Trp). Co-transformed cells were pooled in

300 µl sterile water from which three dilutions of 1:50, 1:250, and 1:500 were made (the original undiluted sample was considered 1:1). Dilutions were spotted onto SD agar media without leucine, tryptophan, and histidine (SD/-Leu/-Trp/-His) for detection of HIS3 reporter activation, or SD media without leucine, tryptophan, histidine, and adenine (SD/-Leu/-Trp/-His/-Ade) for detection of both HIS3 and ADE2 reporter activation. Cells were also spotted onto SD/-Leu/-Trp as a loading standard. After the secondary screens, the prey plasmids were isolated and sequenced. Sequence annotation and phosphorylation site prediction were carried out with BLASTN / BLASTP homology searches against the GenBank nr database and with NetPhos 2.0 (Blom et al. 1999), respectively.

Identification of full-length wheat genes

The screen of the wheat cDNA library was performed using the TaXA21-like1 (from amino acid 681 to stop codon) as bait on media that lacked both His and Ade (SD/-Leu/-Trp/-His/-Ade). Positive clones were picked after a 10-day growth period. All clones were sequenced. Duplicated sequences, clones that were not in frame, that include frame shift mutations, or that showed auto-activation (see below) were discarded.

Most of the selected cDNA clones contained partial transcripts. Therefore, we first identified and cloned full-length wheat genes for each of the identified interactors and then tested them for their interactions with both TaXA21-like1 and TaXA21-like2. The primers used for the PCR-based cloning of the full length wheat genes (Table 1) were designed based on available wheat nucleotide sequences in the NCBI EST database. Only sequences with more than 97% identity to the Y2H cDNAs were selected to avoid paralogs. The full length transcripts for the *Arginase*

(*TaARG*), *β-1,3-glucanase (TaPR2)*, *CBL-interacting protein kinase 14* (*TaCIPK14*) and the *DnaJ protein homolog2* (*TaDNAJ*) genes were cloned from *T. aestivum* cv. Fielder. The full length transcripts for the *Shikimate kinase like 1* (*TmSKL1*) and *Late embryogenesis abundant protein* (*TmLEA*) genes were isolated from *T. monococcum* accession DV92, which is the same accession used for cloning other TaXA21-like interactors in Cantu et al. (2013*b*).

Full length target genes were PCR amplified using the primers described in Table 1 using the Phusion® High-Fidelity PCR Kit. Amplification products were then cloned using the Gateway-compatible pENTR™ Directional TOPO® vector (Invitrogen). The coding sequences of the full-length wheat genes cloned in this study have been deposited in GenBank (accession numbers JX879752 to JX879756, Table 1).

TaXA21-like1 (JX424300) was previously synthesized based on available sequence from *T. aestivum* cv. Chinese Spring, whereas *TaXA21-like2* (JX424301) was cloned from *T. aestivum* hard red spring line UC1041 from the UC Davis breeding program (Cantu et al. 2013*b*). The four full-length wheat homologs of previously known rice interactors of OsXA21L668 (*TmWRKY76*, JX424311; *TmXB3*, JX424304; *TmXB15*, JX424307 and *TmXB24*, JX424309) were cloned from *T. monococcum* accession DV92 (Cantu et al. 2003*b*). The three members of the protein chaperon complex RAR1/SGT1/HSP90 were cloned from *T. aestivum* and are described in Tai 2008 (*TaRAR1*, EF202841 and *TaSGT1*, EF546432) and Wang et al. 2011 (TaHSP90s, ADF31758 and ADF31760). Finally, the OsXA21K668 rice construct used in this study was described before by Seo et al. (2011).

Isolation of full length rice OsARG, OsSKL1, OsPR2, and OsCIPK14 genes

The full length cDNAs of *OsARG* (CAH67831.1) and *OsSKL1* (NP_001041750.1) genes were isolated from synthesized templates (GenScript USA Inc., Piscataway, NJ, USA) using primers described in Table 1. The *OsPR2* gene (NP_001045372.1) was isolated form rice cultivar Kitaake and the *OsCIPK14* gene (Q2QYM3.1) from rice cultivar Nipponbare using primer pairs described in Table 1. The four full-length genes were also cloned into Gateway recombination Entry vector pENTR/d-TOPO (Invitrogen).

Validation of Y2H interactions with full-length proteins

Yeast vectors pLAW10 (DNA-binding domain, BD) and pLAW11 (activation domain, AD) and yeast strain Y2HGold were used in Y2H assays to test the interactions between the XA21 partial proteins (OsXA21K668, TaXA21-like1, TaXA21-like2) and the six wheat full-length proteins identified from the screening of the Y2H cDNA library (Table 1). The first three partial proteins were used as bait in the pLAW10 vector, while the six wheat full-length proteins (Table 1), as well as TaRAR1, TaSGT1, and TaHSP90s were recombined as preys in the pLAW11 vector. The vectors used and transformation methods were as described in Cantu et al. (2013*b*). Autoactivation tests of prey (pLAW11-AD) and bait (pLAW10-BD) constructs were performed using empty pLAW10 vector as bait and empty pLAW11 as prey, respectively. We also included as positive control the interaction test between the murine p53 (pGBKT7-53) and SV-40 large T-antigen (pGAD-T) provided in the Matchmaker kit (Clontech).

Interactions between OsXA21K668 and full-length OsARG, OsSKL1, OsPR2, and OsCIPK14

The interactions between OsXA21K668 and the rice homologous proteins of the four full-length

TaXA21-interactors identified in this study were tested using Y2H LexA system with vectors and transformation methods described before (Park et al. 2008). OsXA21K668 was used as Y2H bait in the pNLex vector, while OsARG, OsSKL1, OsPR2, and OsCIPK14 were used as preys in the pB42AD vector. Bait and prey were co-transformed into yeast EGY48/p8op-lacZ (Clontech) by using the Frozen-EZ yeast transformation II kit (Zymo Research). Autoactivation tests of bait (pNLex-BD) constructs were performed using empty pB42AD vector as prey. Transformed yeast cells were initially placed on SD media (SD/-His/-Ura/-Trp) and then patched to SD induction media to assay LacZ activity.

Bimolecular fluorescence complementation (BiFC) assay

Bimolecular fluorescence complementation (BiFC) assays, also known as split yellow fluorescent protein (YFP) assays, were performed using rice protoplasts as described before (Ding et al. 2009). The full length *TaARG, TaPR2, TmSKL1, TaCIPK14* and *TaSGT1* cDNAs were recombined with the N-terminal part of the Yellow Fluorescence Protein (YFP) in the vector pSY736; and the *TaXA21-like1* and *TaXA21-like2* cDNA was recombined with the C-terminal part of YFP in the vector pSY735. The fusion proteins were co-transformed into rice protoplasts using the polyethylene glycol (PEG) method. Fluorescence was monitored between 24 and 48 h after transformation using a Zeiss Axiovert 25 fluorescence microscope using the Zeiss YFP filter cube 46HE (excitation, BP500/25; beam splitter, FT515; emission, BP535/30).

Real-Time Quantitative Reverse Transcription PCR (qRT-PCR)

T. aestivum (cv. Fielder) plants (fully susceptible to stripe rust) were inoculated at seedling stage

using urediniospores of *Puccinia striiformis* f.sp. *tritici* race PST130 as described in Cantu et al (2013*a*). Leaves were harvested at 0, 5, 8, 15 and 22 days post inoculation (dpi). Primer design (Table 2) and qRT-PCR were carried out as described in Cantu et al. (2011). Primer efficiencies were calculated using five 4-fold cDNA dilutions (1:1, 1:4, 1:16, 1:64 and 1:256) in duplicate as well as checking for amplification in a negative control without DNA. Specificity was checked by analyzing dissociation curves ranging from 60°C to 94°C. *ACTIN* (gene AB19881.1) was used as endogenous control for gene expression analysis. Transcript levels for all genes are expressed as linearized fold-*ACTIN* levels calculated by the formula $2^{(ACTIN\text{-}\ C_T - TARGET\text{-}\ C_T)}$ as described before (Chen and Dubcovsky 2012).

Results

Similarity of TaXA21-like and OsXA21 proteins

We previously reported the identification and isolation of *TaXA21-like1* (JX424300) and *TaXA21-like2* (JX424301) as the closest wheat homologs of the *OsXA21* gene (Cantu et al. 2013*b*). The full length *OsXA21* is predicted to encode a 1,025 amino acid protein, whereas the full length *TaXA21-like1* and *TaXA21-like2* encode proteins that are 1,033 and 1,013 amino acid long, respectively (Figure 1). Several critical amino acids identified in previous studies of OsXA21 (Ser686, Thr688, Ser689, Thr705 and Lys 736) were perfectly conserved in TaXA21-like1 and TaXA21-like2 (Figure 2). The residues that determine serine-threonine specificity (underlined in Figure 2) and the 15 amino acids that are highly conserved among kinase proteins (in bold and marked with arrows in Figure 2) were also conserved among OsXA21, TaXA21-like1 and TaXA21-like2. In contrast, only one of the three N-glycosylation sites (gray letters in

rectangles in Figure 2) of OsXA21 was conserved in TaXA21-like1 and TaXA21-like2.

All three genes have a similar gene structure with two exons (Figure 1). The first exon comprises the LRR, the transmembrane, the JM and the start of the kinase domains, whereas the smaller second exon encodes the end of the kinase domain. The TaXA21-like1 is slightly more similar than TaXA21-like2 to OsXA21. Identity values between OsXA21 and wheat TaXA21-like1 and TaXA21-like2 amino acid sequences were lowest for the regions including the small transmembrane and JM domains (TaXA21-like1: 47%, TaXA21-like2: 47%); intermediate for the extracellular LRR domain (TaXA21-like1: 59%, TaXA21-like2: 58%); and highest in the kinase domain (TaXA21-like1: 70%, TaXA21-like2: 67%). The overall identity between TaXA21-like1 and TaXA21-like2 kinase domains is 71%.

BLASTN searches of the two wheat genes against the sequence assemblies of flow sorted telocentric chromosomes of wheat (http://urgi.versailles.inra.fr/Species/Wheat/Sequence-Repository, IWGSC) indicated that *TaXA21-like1* is located in the long arm of homoeologous group 5 chromosomes and *TaXA21-like2* in the long arm of homoeologous group 2. The *OsXA21* gene was mapped on rice chromosome 11 which is colinear with wheat chromosome 5, suggesting that *TaXA21-like1* is more likely to be the true orthologous copy of *OsXA21* than *TaXA21-like2*. Based on this syntenic chromosome location and its higher similarity with OsXA21, we prioritized TaXA21-like1 for the screening of the wheat Y2H library.

Interactors of TaXA21-like1 identified in the Y2H library screening

The multiple-alignment of the amino acid sequences of TaXA21-like1 region used to screen the Y2H cDNA library (from amino acid 681 to stop codon) with OsXA21K668 (from amino acid

668 to stop codon) and TaXA21-like2 (from amino acid 661 to stop codon) is shown in Figure 2. This region correspond to the cytosolic portion of OsXA21, which has been shown before to serve as high affinity binding region for different downstream signaling proteins (Park et al. 2010).

The Y2H library screening yielded 138 positive clones. After sequencing, we identified six different wheat partial cDNA clones that were on frame and that encoded protein fragments that interact with TaXA21-like1 (Table 1). Based on the annotation of the closest homologs identified by BLASTX searches in the GenBank NCBI nr database, the six genes were tentatively annotated as: *Arginase* (*ARG*), *β-1,3-glucanase* (*PR2*), *CBL-interacting protein kinase 14* (*CIPK14*), *Late embryogenesis abundant protein* (*LEA*,), *Shikimate kinase like 1* (*SKL1*), and *DnaJ protein homolog 2-like* (*DNAJ*).

Validation of the interaction between TaXA21-like1 and the full-length proteins encoded by the novel interactors identified in the Y2H cDNA library screen

To validate the interactions identified in the Y2H screen, we cloned the full-length cDNAs for each of these six genes from either *T. aestivum* cv. Fielder (TaARG, TaCIPK14, TaDNAJ, and TaPR2) or *T. monococcum* DV92 (TmLEA and TmSKL1) leaf-RNA samples. All sequences were deposited in GenBank and the accession numbers are indicated in Table 1.

No autoactivation was detected for any of the six wheat full-length cDNAs cloned into vector *pLAW11* as prey when tested against empty bait using vector *pLAW10* (Figure 3). Out of the six original interactions detected with the partial cDNA clones, only the TaARG, TaPR2, and TmSKL1 full-length proteins showed strong interaction with TaXA21-like1 in SD/-Leu/-Trp/-

His media (Figure 3; Table 3). Among these three, only TmSKL1 interacted with TaXA21-like1 in the more-stringent SD/-Leu/-Trp/-His/-Ade selection media (Figure 3).

Since XA21 has an active serine/threonine protein kinase domain, we characterized the potential serine and threonine phosphorylation targets in the full-length wheat TaXA21 interactors using the NetPhos2.0 software (Blom et al. 1999). Although putative phosphorylation sites were predicted by NetPhos2.0 for all identified wheat protein interactors (Table 2), the final confirmation of these sites will require experimental validation.

TaXA21-like1 and TaXA21-like2 interactions with members of the TaRAR1/TaSGT1/TaHSP90 complex

The protein chaperon complex RAR1/SGT1/HSP90 is required for the proper folding of many proteins, including resistant genes (Shirasu and Schulze 2003). It has been previously shown that both OsSGT1 and OsRAR1 can interact with the OsXA21 cytosolic domain (Seo et al. 2011). In wheat, the TaRAR1/TaSGT1/TaHSP90 proteins are known to interact with each other (Cantu et al. 2013*b*; Tai 2008; Wang et al. 2011). In this study we tested the interactions between TaXA21-like1 and TaRAR1 (EF202841.1), TaSGT1 (EF546432.1), TaHSP90.2 (ADF31758.1) and TaHSP90.3 (ADF31760.1). TaSGT1 interacted with TaXA21-like1, but no member of this complex interacted with TaXA21-like2 (Figure 4; Table 3). This result suggests some functional differentiation between the two wheat paralogs.

Bimolecular fluorescence complementation (BiFC) assay (split YFP) in rice protoplasts

We used (BiFC) assays in rice protoplasts to test *in planta* the Y2H interactions detected

between TaXA21-like1 and the four full-length wheat proteins TaARG, TaPR2, TmSKL1 and TaSGT1 (Table 3). We also tested the BiFC interactions between TaCIPK14 and TaXA21-like2 (TaXA21-like1 and full-length TaCIPK14 showed no interaction in Y2H). Clear positive fluorescence signals were observed only for the interactions between TaXA21-like1 and both TmSKL1 or TaSGT1 (Figure 5, Table 3).

Interactions of full-length TaXA21-like1 interactors with TaXA21-like2 and OsXA21

A previous study of four wheat homologs of known OsXA21 interactors (TmXB3, TmXB15, TmXB24 and TmWRKY76) showed conserved interactions between these four wheat proteins and both TaXA21-like1 and TaXA21-like2 (Cantu et al. 2013*b*). In this study, we confirmed that these four full-length wheat proteins also interact with OsXA21 (Table 3).

We then tested if the full-length wheat proteins encoded by the six clones identified in the wheat cDNA library were able to interact with TaXA21-like2 (from amino acid 661 to stop codon) and OsXA21 (OsXA21K668). Both TaXA21-like2 and OsXA21 showed no interactions with full length proteins TaDNAJ and TmLEA, and positive interactions with full-length proteins TaPR2, TmSKL1, and TaCIPK14 (Figure 6; Table 3). This last full-length protein did not interact with TaXA21-like1 (Figure 3a), but the peptide encoded by the partial *TaCIPK14* gene identified in the Y2H cDNA library showed a weak interaction between these two proteins. For the sixth protein, TaARG, we detected a positive interaction with OsXA21 (Figure 6b) and no interaction with TaXA21-like2 (Figure 6a). The additional TaSGT1 protein showed a similar interaction profile as TaARG: no interaction with TaXA21-like 2 (Figure 4b) and a weak interaction with OsXA21 in the LexA Y2H system (figure not shown).

Y2H interactions between OsXA21 and rice homologs of the novel TaXA21 interactors

The ARG, SKL1, PR2 and CIPK14 proteins identified in this study are not among previously known OsXA21 interactors. To test if these interactors were specific to wheat, we isolated their rice homologs and tested their interaction with OsXA21K668 using Y2H. The rice proteins OsARG (92% amino acid identity with TaARG), OsSKL1 (79% identity with TmSKL1) and OsCIPK14 (84% identity to TaCIPK14) all interacted with the cytosolic portion of OsXA21 (OsXA21K668, Figure 6c). The cloned rice OsPR2 protein interacted weakly with OsXA21K668 (Figure 6c). The additional interactions between OsXA21K668 and OsXB3, OsXB15, OsXB24, OsWRKY76 and OsSGT1 shown in Figure 8d have been previously summarized by Seo et al. (2011).

Transcriptional profiles of TaXA21-likes interactors after stripe rust inoculation

To determine if the identified wheat interactors are co-expressed during wheat interactions with pathogens, we determine the transcriptional profiles of the TaXA21-like interactors across multiple time points during the compatible interaction between hexaploid wheat (*T. aestivum*) and PST using quantitative RT-PCR. We were able to determine the transcriptional profiles of eight of the nine TaXA21-like1/2 interactors (Fig. 7), but could not reliably determine the expression of *TaWRKY76* (probably because of the presence of close paralogs). Note that all the genes described in this section have a Ta prefix since they refer to expressed *T. aestivum* genes.

Among the eight transcription profiles analyzed, only TaCIPK14 was consistently down-regulated during the first 15 days post inoculation (dpi). Among the other seven genes, the

transcript levels of *TaPR2*, *TaXB3*, *TaXB15* and *TaSGT1* showed a significant up-regulation relative to time zero at five and/or eight dpi; whereas the transcript levels of *TaSKL1* and *TASGT1* showed a significant down-regulation relative to time zero at fifteen and twenty-two dpi (Figure 7). These results indicate that the transcriptional profiles of six out of the eight genes encoding *TaXA21* interactors are affected by stripe rust.

Discussion

In this study we identified four full-length wheat proteins that interact with the cytosolic portion of TaXA21-like1. Together with the four previously reported interactions between TaXA21-like1 and TmXB3, TmXB15, TmXB24, and TmWRKY76 (Cantu et al. 2013*b*), our new results indicate that the TaXA21-like1 and TaXA21-like2 nodes include at least eight and seven protein interactors, respectively (Figure 8, Table 3).

XA21 homologs have been identified in several grass species, including rice, *Brachypodium*, sorghum, and maize (Tan et al. 2012). Changes in copy number and frequent structural changes in the grass XA21 homologs suggest that this gene family is undergoing rapid evolutionary changes (Tan et al. 2012). Therefore, it is not surprising that even the closest homologs between wheat and rice XA21 proteins (TaXA21-like1, TaXA21-like2 and OsXA21) showed some differentiation in their interaction profiles. Of the eight interactions described in Figure 8 for TaXA21-like1, only six were observed in TaXA21-like2 (TaSGT1 and TaARG1 did not interact with TaXA21-like2). In contrast, TaXA21-like2 and OsXA21 proteins showed interactions with the full-length TaCIPK14 protein that were not observed with TaXA21-like1. The OsXA21 protein combined all the interactions observed in TaXA21-like1 and TaXA21-like2, suggesting

the possibility of some sub-functionalization of wheat TaXA21-like1 and TaXA21-like2 paralogs.

The conservation of most of the interactions involving the cytosolic portions of these three OsXA21 homologs parallels the conservation of critical amino acid positions in the three proteins (Figure 2). The OsXA21 JM residues Ser686, Thr688, and Ser689, which are important to stabilize the OsXA21 protein (Xu et al. 2006) are all conserved in the TaXA21-like1 and TaXA21-like2 proteins (Figure 2). The residue Thr705 in the OsXA21 JM region, which is required for binding OsXB3, OsXB10, OsXB15 and OsXB24 to OsXA21 (Park et al. 2008; Chen et al. 2010*b*) is also conserved in the two TaXA21 paralogs. Interestingly, three of these four known interactions in rice were validated in the wheat homologs. We were not able to test if the TmXB10 interaction was also conserved since we were unable to obtain a complete sequence of this gene (Cantu et al. 2013*b*).

Description of the TaXA21-like1 interactors

Although mutants and transgenic studies will be required to validate the function of these new TaXA21 wheat interactors, the knowledge their function in other species may provide initial clues about their potential roles in the disease resistance response. Among the previously known rice interactors of OsXA21, OsXB3 is a positive regulator of OsXA21-mediated resistance (Wang et al. 2006), whereas OsXB15, OsXB24, and OsWRKY76 are negative regulators of OsXA21-mediated resistance (Park et al. 2008; Chen et al. 2010*b*; Seo et al. 2011).

Among the new wheat interactors identified in this study, the *ARG* gene is known to be involved in response to infection. In mammalian cells, induced expression of *ARG* in response to wound

trauma and pathogen infection plays an important role in regulating the metabolism of L-arginine to either polyamines or nitric oxide (NO). In animal systems, increased ARG expression stimulates the production of polyamines that promote cell proliferation and wound healing (Satriano 2004). Because ARG and NO synthase (NOS) compete for a common substrate, L-Arginine, increased ARG expression can effectively inhibit the NO-pathway. Many animal pathogens, for example, induce ARG expression to evade NO-mediated host defenses (Vincendeau et al. 2003). Less is known about the role of the *ARG* gene in plants. However, it has been reported that the tomato *ARG2* gene is massively induced in foliar tissue in response to mechanical wounding and methyl jasmonate treatment (Chen et al. 2004). The increase in ARG activity is abolished in the *jasmonic acid insensitive1* (*jai1*) mutant that is defective in the jasmonate signaling pathway (Li et al. 2004). It would be interesting to test if knock-out mutation in the rice *ARG* gene will impede or reduce the *OsXA21* mediated resistance to *Xoo.*

The second interactor identified in this study, *β*-1,3-glucanase (PR2), belongs to the pathogenesis-related (PR) protein 2 family (Higa-Nishiyama et al. 2006). The *β*-1,3-glucanase has been shown to be part of the early stages of the defense response of wheat to stem rust (*Puccinia graminis f. sp. tritici, Pgt*) (Münch-Garthoff et al. 1997). The plant response included a rapid increase in *β* -1,3-glucanase activity between 24 and 48 h after *Pgt* inoculation (Münch-Garthoff et al. 1997), approximately 16 h before the typical hypersensitive response was observed. In this study, the transcriptional levels of *TaPR2* suggested that it responded to stripe rust infection and maintained relative higher expression levels before the 15 dpi. It would be interesting to generate mutants for the *β*-1,3-glucanase to test its role in the wheat and rice *OsXA21*-mediated signaling pathway.

The wheat *Shikimate kinase like 1* (SKL1) identified in this study as an interactor of TaXA21-like1 and TmSKL1 has very low amino acid similarity to OsXB12 (=OsSKL2) a different Shikimate kinase-like protein known to interact with OsXA21. OsSKL1 and OsSKL2 proteins share limited similarity (~50%) over two short regions including only 71 amino acids. The TmSKL1 gene is likely to be orthologous to the rice OsSKL1 (79% similarity; Fucile et al. 2008). Mutants of OsXB12 are lethal in rice and therefore its function is not well characterized (Chern and Ronald unpublished). Mutations of *AtSKL1* in Arabidopsis result in albino phenotypes, suggesting a role in chloroplast physiology (Fucile et al. 2008). The possibility to generate mutants in only one of the genomes of wheat can be used to alter the dosage of these *SKL* genes and clarify their roles in plant defense, even if the complete null-mutant is lethal. The qRT-PCR results for *TmSKL1* indicate that the transcript levels of this gene are up-regulated by the PST infection during the first eight dpi but are then significantly down-regulated below the initial levels after 15 dpi (Figure 7).

A partial clone coding for the TaCIPK14 protein was identified as an interactor of TaXA21-like1 but that interaction was not confirmed with the complete protein. However, the full-length TaCIPK14 protein was able to interact with both the TaXA21-like2 paralog and OsXA21. The expression profile of *TaCIPK14* was consistently down-regulated during the first 15 days of the stripe rust infection. It would be interesting to investigate if the opposite transcription profile during the first eight dpi of *TaCIPK14* compared to *TaPR2*, *TaXB3*, *TaXB15* and *TaSGT1* reflects contrasting functions during pathogen infection. Since the CIPK14 interactions are conserved in wheat and rice, the role of this gene can be now studied in rice, where more tools are available. It was reported that *OsCIPK14* was rapidly induced by MAMPs and involved in various MAMP-induced immune responses (Kurusu et al. 2010). Based on these results it is

tempting to speculate that *OsCIPK14* may provide a link between the OsXA21-mediated pathway and the CBL-CIPK-mediated Ca^{2+} pathway in the rice disease response.

In plants, the RAR1/SGT1/HSP90 molecular chaperone complex is required for the correct folding of many NBS-LRR resistance proteins such as barley Mla6 and Mla12, which are effective against powdery mildew (Azevedo et al. 2002; Halterman and Wise 2004 ; Shirasu 2009) and wheat Lr21, which is effective against leaf rust (Scofield et al. 2005). RAR1/SGT1/HSP90 interactions are conserved in wheat (Tai 2008; Wang et al. 2011; Cantu et al. 2013*b*), which suggests a conserved function of this complex. The interaction between TaXA21-like1 and TaSGT1 may suggest that the RAR1/SGT1/HSP90 complex is required for the proper folding of TaXA21-like1.

In summary, the conservation of most of the OsXA21 and TaXA21 protein interactions in rice and wheat after more than 50 million years of divergence suggests that these genes may have some overlapping functions in the disease response. This hypothesis is also supported by the observed up- and down-regulation of the transcript levels of the genes coding for these novel XA21 interactors during PST infection. A final validation of this hypothesis will require the development and characterization of RNAi transgenic wheat plants or TILLING mutants for the *TaXA21-like1* and *TaXA21-like2* genes. Finally, our wheat-rice comparative interactome analyses were useful not only to transfer known interactions from the well-studied rice model to wheat, but also to identify novel rice interacting proteins.

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Tables

Table 1. Genes recovered from the Y2H screening of the wheat cDNA library using the cytosolic domain of TaXA21-like1 as bait and primers used to recover full length genes in wheat and rice.

Gene	**Primers for cloning of full length protein** (5' to 3')	**Species used for cloning & GenBank No.**
Arginase		
TaARG	F-CACCATGGGCGGCGCGGCGG R-TCATTTTGAGATCTTGGC	*T. aestivum* cv. Fielder (JX879752)
OsARG	F-CACCATGGGCGGCGTGGCGGC R-TCACTTGGAGATCTTGGCTGTGAGC	*O. sativa cv. Nipponbare* (synthesized)
Endo-beta-1,3-glucanase		
TaPR2	F-CACCATGGCGCGGCAGCGTACGTGC R-CTAATTGGTAAACCTTATAGGG	*T. aestivum* cv. Fielder (BAE96094)
OsPR2	F-CACCATGGCCAGGAGACAGGGAGTT R-TTAGAACCGGATGGGATACACG	*O. sativa* cv. Kitaake (NP_001045372.1)
Shikimate kinase		
TmSKL1	F-CACCATGGCGATGGCGATGCGG R-CTAGAAGGGCTTTGGAGCAG	*T. monococcum* accession DV92 (JX879753)
OsSKL1	F-CACCATGGAGAGTAGAGGGAAGATACTAATGG R-CTATTCATCTCCATGCCAGGCCAA	*O. sativa cv. Nipponbare* (synthesized)
CBL-interacting protein kinase 14		
TaCIPK14	F-CACCATGGCAAACAGAGGGAAGATTC R-CTACTCTAGCTGCTGCTGGT	*T. aestivum* cv. Fielder (JX879754)
OsCIPK14	F-CACCATGGAGAGTAGAGGGAAGATACTAATGG R-CTATTCATCTCCATGCCAGGCCAA	*O. sativa* cv. Kitaake (Q2QYM3.1)
Late embryogenesis abundant protein		
TmLEA [1]	F-CACCATGGCGAGCCTGATGGACAAG R-CTAGGCGGTGGCCTCCTCGG	*T. monococcum* accession DV92 (JX879755)
DnaJ protein homolog 2-like		
TaDNAJ [1]	F-CACCATGCCAAGGAAGACCAGCAAC R-TTACTGCTGGGCGCACTGCAC	*T. aestivum* cv. Fielder (JX879756)

[1] Interactions he for the last two genes were not detected for the full length clones and therefore they were not tested in rice.

Table 2. qRT-PCR primers for TaXA21-likes interactors

Names	Primer sequence for qRT-PCR (5' to 3')
TmXB3 (JX424304)	F-CGCCATCGTGTCGGCATTG R-GCCGAAGAAGGGTTCAGCAGTG
TmXB15 (JX424307)	F-TCCATGCCGGAGGAGGTTC R-TTCCATTTCGGCTGTTTCAAGA
TmXB24 (JX424309)	F-CCTCAAGTGCGAGAAGACCG R-CTGCGAAGTCCTGGAAATGC
TaSGT1 (EF546432)	F-TCGGAAGGGCTCTGCTTGC R-CTGGCTAGCCTCCTCAGCAATAC
TaARG (JX879752)	F-CGTGCTCAACATCCTCCA R-GACCATAGCCGTCATCCC
TmSKL1 (JX879753)	F-AAGTCGAGACCGAGGGGC R-CTCAGTAGCCCTAAGTTGGTGGA
TaPR2 (BAE96094)	F-AGTCTTGGCGTCCATCCCTATCG R-CGCTGATGCCCTTGGTCCTGTA
TaCIPK14 (JX879754)	F-TATGTGAAAGGCGGTGAG R-TTGGCTGTGGCAGTAATC
ACTIN (AB181991.1)	F-ACCTTCAGTTGCCCAGCAAT R-CAGAGTCGAGCACAATACCAGTTG

Table 3. Summary of predicted phosphorylation sites and interactions for TaXA21 proteins identified in this and previous studies.

Names	Description	Number of predicted phosphorylation sites [1]	TaXA21-like1 Y2H interaction strength	TaXA21-like1 interaction in *planta*	TaXA21-like2 Y2H interaction strength	OsXA21 Y2H interaction strength
TmXB3	E3 ubiquitin ligase	18 S, 2T [1]	++ [2]	N	++	+++
TmXB15	Phosphatase 2c	25S, 7T	+++	Y [3]	+++	++
TmXB24	ATPase	6S, 4T	++	Y	++	++
TmWRKY76	WRKY transcription factor 76	18S, 7T	++	N	++	++
TaSGT1	Plant ortholog of yeast cell cycle regulator SGT1	18S, 2T	++	Y	-	++
TmSKL1	Shikimate kinase	12S, 2T	+++	Y	++	++
TaARG	Arginase	7S, 3T	++	N	-	++
TaPR2	Endo-beta-1,3-glucanase	12S, 4T	++	N	++	++
TaCIPK14K	CBL-interacting protein kinase 14	12S, 7T	-	N[4]	+++	++
TaDNAJ	DnaJ protein homolog2	16S, 8T	-	N/A	-	-
TmLEA	Late embryogenesis abundant protein	2S, 3T	-	N/A	-	-

[1] S=serine; T=threonine.

[2] Interactions in the Y2H assays were classified in: no interaction (-); weak interactions (++) and strong interactions (+++). Weak interactions were detected only on SD-L-T-H media whereas strong interaction (+++) were observed also on SD-L-T-H-A media.

[3] Interactions observed in rice protoplast are indicated with "Y" (yes), whereas those interactions where the signal was not clearly different form the background fluorescence were marked with "N". Interaction between TaCIPK14K, TaDNAJ, and Tm LEA with TaXA21-like2 were not tested ("N/A") by BiFC because the initial interactions with the proteins encoded by partial cDNA clones were not validated with the full-length proteins in the Y2H system.

[4] The CIPK14 interaction by BiFC was tested against TaXA21-like 2, since TaXA21-like1 did not interact with the full length CIPK14 in Y2H.

Figure Legends

Figure 1 Schematic diagram comparing the structure of OsXA21, TaXA21-like1 and TaXA21-like2 genes. The two horizontal gray rectangles represent exons and black lines represent introns. The length of the genomic DNA sequence is indicated at the end of each gene. The LRR, transmembrane and juxtamembrane, and the kinase domains were delimited with different gray shades following Song et al. (1995, 1997).

Figure 2 Alignment of intracellular regions of proteins OsXA21 (OsXA21K668), TaXA21-like1, and TaXA21-like2. The aligned regions included amino acids 668-1025 from OsXA21K668, 681-1033 from TaXA21-like1 (TaXA21-1), 661-1013 from TaXA21-like2 (TaXA21-2). Alignment was determined using ClustalW2. The 15 amino acids that are invariant among most protein kinases are in bold and marked with arrows. The amino acids indicating serine-threonine specificity are underlined, and the N-glycosylation sites are indicated in gray and marked with rectangles as described by Song et al. (1995). The location of the intron is indicated by a "/". The OsXA21 JM critical residues Ser686, Thr688 and Ser689 are marked with black triangles. The OsXA21 amino acids Thr705 is marked with a black star and the Lys736 is marked with a black rhombus. Identical amino acids in at least two out of the three homologs are highlighted with gray shading.

Figure 3 Yeast-two-hybrid tests of interactions between the cytosolic domain of wheat XA21-like proteins, and the full length proteins of candidate interactors identified in the screen of the wheat cDNA library. (a) Interaction tested between the cytosolic domain of TaXA21-like1 with full length proteins of candidate interactors. (b): Autoactivation tests using empty bait (-/) or prey vectors (/-). (a & b) Positive co-transformation in the Gal4 based system

was tested in SD-L-T media, whereas positive interactions were tested in SD-L-T-H (absence of histidine) and SD-L-T-H-A (absence of both histidine and adenine) media.

Figure 4 Yeast-two-hybrid tests of interactions between wheat XA21-like proteins and the members of the RAR1/HSP90/SGT1 complex. (a) Interactions between TaXA21-like1 and members of wheat RAR1/SGT1/HSP90 complex**.** (b) Interactions between TaXA21-like2 and components of wheat RAR1/SGT1/HSP90 complex**.** Lack of autoactivation for these three constructs (TaRAR1, TaSGT1, TaHSP90.2 and TaHSP90.3) had been reported before (Cantu et al. 2013*b*)

Figure 5 BiFC assay showing positive interaction between TaXA21-like1 and both TmSKL1 and TaSGT1. Bimolecular fluorescence complementation assay (BiFC) was used to visualize protein-protein interactions in rice protoplasts. Non-fused YFP-N and YFP-C empty vectors were used as negative controls.

Figure 6 Yeast-two-hybrid tests of interactions between the cytosolic domain of wheat XA21-like2 and rice XA21 and the full length proteins of candidate interactors identified in the screen of the wheat cDNA library. (a) Interaction tested between the cytosolic domain of TaXA21-like2 with full length proteins of candidate interactors. (b) Interaction tested between the cytosolic domain of OsXA21 with full length proteins of candidate interactors. Autoactivation tests for all clones (a & b) are shown in Figure 3b. (c) Interactions between OsXA21 and rice homologs of the wheat interactors of TaXA21-like1 identified in the wheat Y2H screen**.** Bait OsXA21K668 is fused to the LexA protein *pNLex*-BD vector and all rice homologs of wheat interactors of TaXA21-like1 are fused to B42AD protein in the *pB42AD*

vector as prey. Empty pNLex-BD as bait is used to test autoactivation with all preys. Blue colors indicate positive interactions.

Figure 7 Transcript levels of TaXA21-like1 and TaXA21-like2 interactors across a *P. striiformis* f.sp. *tritici* infection time course in hexaploid wheat variety Fielder determined by qRT-PCR. (A) *TaARG*; (B) *TaPR2*; (C) *TaSKL1*; (D) *TaCIPK14*; (E) *TaXB3*; (F) *TaXB15*; (G) *TaXB24*; and (H) *TaSGT1*. X axis scale indicates days post inoculation Y axis scale indicates transcript levels expressed as linearized fold-*ACTIN* levels calculated by the formula $2(^{ACTIN\text{-}C_T - TARGET\ C_T})$ as described before (Chen and Dubcovsky 2012). Each data point is an average of four plants (± SEM). * $P \leq 0.05$, ** $P \leq 0.01$ in t-tests comparing different time points versus initial time of inoculation (0 dpi).

Figure 8 The XA21 interaction node in wheat and rice. (a) Interaction node of TaXA21-like1 (wheat-wheat interactions). (b) Interactions between TaXA21-like2 and TaXA21-like1 interactors (wheat-wheat interactions). (c) Interactions between OsXA21 and TaXA21-like1 interactors (wheat-rice interactions) (d) Interactions between OsXA21 and rice homologs of the TaXA21-like1 interactors (rice-rice interactions).