Functional characterization of an inducible bidirectional promoter from *Fusarium oxysporum* f. sp. *cubense*

Ashutosh Dash, Vartika Gurdaswani, Jacinta S. D’Souza & Siddhesh B. Ghag*

Bidirectional promoters (BDPs) are regulatory DNA sequences (~1000 bp long) intervening two genes arranged on opposite strands with their 5’ ends in close proximity. These genes are mostly co-expressed; but, instances of anti-correlation and independent transcription have been observed. In fungal systems, BDPs have shown to provide an improved genetic circuit by assembling and regulating transcription of different genes of a common metabolic pathway. We have identified an intergenic region (1063 bp) from the genome of *Fusarium oxysporum* f. sp. *cubense* (Foc), a banana root pathogen. This intergenic region regulates the expression of a gene pair required for the breakdown of hemicellulose. For characterization, it was cloned into pCSN44 vector backbone between two reporter genes, namely β-glucuronidase (GUS) and enhanced green fluorescent protein (EGFP). The newly formed vector was transformed into Foc and tested for its bidirectional expression activity. Using histochemical staining and fluorescence microscopy, the kinetics for both, GUS and EGFP expression were tested under different growth conditions respectively. The activity was differentially regulated by inducers such as xylan, arabinogalactan and pectin. This is the first report on the isolation of the intergenic region with inducible bidirectional promoter activity from *Fusarium*. Characterization of such BDPs will find applications in genetic engineering, metabolic engineering and synthetic biology using fungal systems.
between At4g35985 and At4g35987 genes of Arabidopsis contains a number of environmental stress-responsive and tissue-specific cis-regulatory elements which showed differential regulation of two genes under study15. A 1429 bp intergenic region between the genes Ghrack1 and Ghuhrf1 in Gossypium hirsutum was cloned and used for expressing reporter genes in Arabidopsis19. A BDP was built by arranging in tandem three copies of a modified enhancer from the promoter region of the Arabidopsis PLDZ2 gene, which strongly responds to phosphate starvation. The induction of transcription is controlled by the availability of phosphate in the medium14. Such regulatory sequences work as a circuit switch to drive the expression or stop transcription.

Filamentous fungi are one of the best eukaryotic systems known to express recombinant proteins, especially enzymes of industrial scale in the cheapest possible means17. Moreover, filamentous fungi are genetically modified to restructure the metabolic fluxes which eventually results in expression and secretion of the protein of interest into the medium, making downstream processing easier. Bidirectional promoters would be of great advantage in this aspect. Few BDPs have been identified and isolated from fungi. Most of these promoters are seen to co-regulate functionally related genes such as those involved in a common metabolic pathway. Most of the secondary metabolite genes are clustered in the fungal genome and few of them are regulated by divergent promoters. The Aspergillus nidulans penicillin biosynthesis genes acvA and ipmA are divergently oriented and separated by an intergenic region of 872 bp16. The pcbAB and pcbC genes involved in penicillin biosynthesis in Penicillium chrysogenum and Penicillium nalgiovense are expressed from a 1.16 kb BDP region which is controlled by both nutritional and developmental factors19. The nitrite reductase (niiA) and nitrate reductase (nirA) genes in Aspergillus nidulans are transcriptionally regulated by a 1.2 kb intergenic region20. This intergenic region behaves as a switch; ‘on’ state in presence of nitrate and ‘off’ in presence of ammonium and glutamine. This nitrate-inducible promoter was used to express the transcription factor AflR and its cofactor AflS that were responsible for activating eight sterigmatocystin (precursor for aflatoxin B1) promoters for sterigmatocystin production in Aspergillus nidulans20. Further, this genetic circuit was used to produce β-carotene in Aspergillus nidulans from the carotenoid gene cluster of Fusarium fujikuroi21. Moreover, these BDPs are regulated by activators, transcription factors and enhancers that facilitate binding of the protein complexes driving expression of the gene pair. Modification of histones, positioning of the nucleosomes and binding of the transcription factors regulating BDP activity have been studied in model fungal systems such as Aspergillus nidulans and Saccharomyces cerevisiae22-25.

Fusarium oxysporum species complex are common soil pathogens that infect most crop plants causing wilt disease. It enters through the root system and eventually reaches the vascular tissues26. During infection, it releases an array of extracellular enzymes that degrade the plant cell wall for its progression. These enzymes are triggered in the presence of the inducer compound most probably derived from the host plant. Xylan is an important component of plant cell wall and the virulent organisms possess enzymes to break xylan as one of the virulence factors. In this study, we have identified an intergenic region shared by divergent genes encoding a putative beta-xylosidase and arabinoxylan arabinofuranohydrolase placed head to head. This intergenic region was able to drive the expression of reporter genes GUS and EGFP demonstrating bidirectional activity of this DNA fragment. Moreover, this region was inducible and displayed differential transcription of the two reporter genes. This is the first study that reports the isolation of a BDP from an economically important phytopathogen Fusarium oxysporum.

Methods

Primers used in this study. The primers used in this study are listed in Table 1.

| Primer name | Primer sequence |
|-------------|-----------------|
| Pxy Fw      | 5′ CCACGGCCGCGCTTTGGAGGCCGTGAAGACTAAAG 3′ |
| Pxy Rv      | 5′ TCTGACTAGCCTGAGGAATGTTCTCGAAATAAGC 3′ |
| EGFP Fw     | 5′ TTGCTAGCAGTGGTACGAAGGCGGAGG 3′ |
| EGFP Rv     | 5′ CAACCCGGGCTGCTGCTCTCTACACG 3′ |
| GUS Fw      | 5′ TCTCAGATCTTTAGTGTGTGCTCCTCTGGCTG 3′ |
| GUS Rv      | 5′ TTACGGCCGCGATGTAAGCTGCTGTGAAGAAAACC 3′ |
| GAt Fw      | 5′ TCTGACGCTGCTGCTGCTCTCTACACG 3′ |
| GAt Rv      | 5′ CAACAGCTGCTCATGCTGCTGCTCGGATG 3′ |

Table 1. Primers used in this study.

Culture conditions. The strain used in this study, Fusarium oxysporum f. sp. cubense (Foc) race 1 was kindly provided by Dr. T. R. Ganapathi from Bhabha Atomic Research Centre, Mumbai and grown in potato dextrose medium. For conidial preparation, Foc was grown in potato dextrose agar (PDA) medium incubated at 30 °C for 8–10 days whereas for liquid cultures potato dextrose broth (PDB) was used at medium and cultures shaken at 160–200 rpm at 30 °C.

Isolation and in silico analysis of the bidirectional promoter from Foc. The sequence from Foc race 1 contig1365 whole genome shotgun sequencing database with GenBank accession no AMGP01001365 was identified wherein this intergenic sequence was located between two genes involved in xylan metabolism. This intergenic region was isolated from Foc genomic DNA using primer set PxyFw/PxyRv (Table 1) and sequenced. The sequence was analyzed for promoter elements using online software such as PLANT Care, PlantPAN 2.0 and PLACE.
Construction of the vector for Foc transformation. The intergenic region (Pxy) was amplified from the genomic DNA of Foc using primers PxyFw/PxyRv (Table 1) and digested using NotI and Nhel sites. EGFP along with the glucoamylase gene terminator was amplified using primers EGFPFw/EGFPRv (Table 1) from pCSN44-eGFP vector gifted by Prof. N. S. Punekar (IIT-B, Mumbai) and digested using Nhel and Xmal. These two digested fragments were inserted into the vector backbone pCSN44 digested previously using NotI and Xmal in a three-way ligation reaction to give pCSN44Pxy-eGFP vector. The pCSN44Pxy-eGFP vector was digested using NotI and SacI and used for further cloning. The β-glucuronidase gene was amplified from the vector pBI121 (AF485783.1) using the primers GUSfw/GUSRv (Table 1) and digested by NotI and BglII. The glucoamylase gene terminator was amplified from pCSN44eGFP using primers GAtw/GEFPRv (Table 1) and digested using BglII and SacI restriction enzymes. These two fragments were cloned into the digested pCSN44Pxy-eGFP vector to form the pCSN44Pxy-EGFP-GUS vector. This newly constructed vector was purified and sequenced.

Foc transformation and selection of the transformants. Foc spheroplasts were used for transformation. The spheroplasts were prepared by first growing the Foc culture in PDA at 25 °C for 8–12 days. Conidia were harvested from this plate using Tween 80 solution (0.005% v/v) and separated from the mycelia by filtering it through the absorbent cotton bed. Approximately, 107 spores/mL were inoculated into PDB and incubated at 30 °C and shaken at 200 rpm for 22–23 hours. The following day, mycelia were separated using four layers of cheese-cloth and washed several times with chilled distilled water followed by chilled osmotic medium (0.27 M CaCl2 and 0.6 M NaCl). The washed mycelia were transferred to a flask containing osmotic medium, lysing enzyme [900 mg/mL] (Novozyme, USA) and bovine serum albumin fraction V [60 mg/mL] (Sigma, USA) and mixed to form a homogenous slurry. This mixture was incubated in a water bath at 37 °C for 2–3 hours. The reaction mixture was swirled vigorously to release protoplast from the mycelial debris. This reaction mix was filtered through mira cloth (Calbiochem, USA) to separate the spheroplasts from the mycelial debris. The spheroplasts were pelleted by centrifuging at 18,111 g in a swinging bucket rotor for 10 min. The pellet was washed with 1X STC solution (1.2 M D-sorbitol, 50 mM CaCl2 and 35 mM NaCl). The supernatant was discarded and the pellet was re-suspended in 140 µl of sterile 1X STC. Five micrograms of linearized plasmid DNA along with equal volume of 2X STC and 50 µl 50% w/v PEG (t) (2X STC: 50% w/v PEG 8000:1:1) was added to the spheroplasts and incubated on ice for 30 min. After incubation, 1 µl of PEG(t) was added to the mixture and incubated at room temperature for 30 min. This mixture was spread on to PDA medium with 27.38% sucrose and incubated at room temperature. After 16–18 h, the plates were overlaid with PDA medium supplemented with hygromycin-B (50 µg/mL) and incubated at 30 °C for 2–3 days till the appearance of transformants on the plates. The putative transformants were grown on PDA medium supplemented with hygromycin and screened by PCR using primers Pxyfw/EGFPfw and GAtw/GUSRv (Table 1). Since the growth seemed more comparable with the wild-type (WT) transformant T17 was chosen for further analysis.

Histochemical β-glucuronidase (GUS) assay. The histochemical GUS assay was performed as described by Jefferson and co-workers28. Foc WT and T17 conidia were inoculated in PDB medium and shaken for 72 h at 160 rpm/30 °C. The mycelia were filtered and washed with sterile distilled water to remove remnants of the PDB medium. Equal amount of mycelia were inoculated into minimal medium supplemented with different carbon sources such as 1% xylan from beechwood (SRL, India), 1% arabinogalactan from larch wood (Sigma, USA), 1% pectin from citrus peel (Sigma, USA), 1.5% soluble starch (Sigma-Aldrich, USA), 1.35% D-xylene (Himedia, India) and 3% sucrose (Calbiochem, USA). The mycelial samples were collected after 3, 8, 12 and 24 h and incubated in GUS buffer overnight at 37 °C. Post-incubation the mycelial samples were clarified using 70% ethanol and observed for indigo coloration.

4-Methyl-umbelliferyl-β-D-glucuronde (MUG) assay. The WT and T17 Foc strains were grown in PDB medium for 72 h at 30 °C in an orbital shaker. The mycelia were separated using cheese cloth and washed thoroughly with sterile distilled water to remove traces of the medium. The washed mycelia were inoculated in minimal medium supplemented with one of the carbon sources (xylan, arabinogalactan and pectin). The flasks were incubated for 12 h at 30 °C in an orbital shaker and used for MUG assay28. The mycelia were again separated, weighed and crushed using liquid nitrogen to fine powder. This powder was suspended in chilled 1X extraction buffer (10 mM EDTA, 10 mM β-mercaptoethanol, 0.1% sodium n-lauryl sarcosine, 0.1% Triton X-100 prepared in 50 mM sodium phosphate buffer, pH 7.0). The suspension was centrifuged at 18,407 rpm for 20 min. The supernatant was removed and the pellet was resuspended in 1X STC solution (1.2 M D-sorbitol, 50 mM CaCl2, and 35 mM NaCl) and incubated at 40 °C for 3 hours. The suspension was centrifuged at 18,407 g in a swinging bucket rotor for 10 min. The pellet was re-suspended in 140 µl of sterile 1X STC. Five micrograms of linearized plasmid DNA along with equal volume of 2X STC and 50 µl 50% w/v PEG (t) (2X STC: 50% w/v PEG 8000:1:1) was added to the spheroplasts and incubated on ice for 30 min. After incubation, 1 µl of PEG(t) was added to the mixture and incubated at room temperature for 30 min. This mixture was spread on to PDA medium with 27.38% sucrose and incubated at room temperature. After 16–18 h, the plates were overlaid with PDA medium supplemented with hygromycin-B (50 µg/mL) and incubated at 30 °C for 2–3 days till the appearance of transformants on the plates. The putative transformants were grown on PDA medium supplemented with hygromycin and screened by PCR using primers Pxyfw/EGFPfw and GAtw/GUSRv (Table 1). Since the growth seemed more comparable with the wild-type (WT) transformant T17 was chosen for further analysis.

Fluorescence microscopy for enhanced green fluorescent protein (EGFP) detection. For EGFP detection, the WT and T17 Foc strains were grown in PDB and inoculated in minimal medium supplemented with different carbon sources as described in section 2.5. The mycelia were observed under oil immersion 100X objective Nikon Eclipse Ni fluorescence Upright microscope (Nikon, Japan) 3, 8, 12 and 24 h post-inoculation. A 480/30 wavelength light obtained through an FITC filter set was used for EGFP excitation. The images were captured using the Nikon DS-Q2 monochrome camera (Nikon, Japan).
EGFP estimation using fluorimetry. The WT and T17 Foc strains were grown in PDB medium for 72 h, at 30 °C in orbital shaker and mycelia were separated using cheese cloth. This mycelia were washed with sterile distilled water and inoculated in minimal media containing different carbon sources such as xylan, arabinogalactan, pectin, xylose, starch and sucrose as described previously. After growing them for 12 h, at 30 °C in an orbital shaker, 2 ml of the growth medium containing the mycelia was used for measuring the EGFP fluorescence, with an excitation of 488 nm and emission of 509 nm using respective medium as blank. For the zero-hour WT and T17 time point samples, PDB medium was used as blank. The mycelial suspension after measuring fluorescence intensity was harvested at 21,130 \( g \) for 10 min and the wet weight was measured. The EGFP expression was calculated as the FI per mg of wet weight of mycelia. Each measurement was done in triplicates and the experiment was repeated twice.

Statistics. Two-way ANOVA Tukey’s multiple comparison test using GraphPad Prism 8.0.2. (\(* P < 0.05; ** P < 0.01; **** P < 0.0001\) ) was conducted for all experimental values.

Results

In silico analysis and isolation of the bidirectional promoter from Foc. The 1063 bp Pxy promoter (in contig1365 with GenBank accession no AMGP01001365) sequence analysis depicts presence of putative cis-acting elements in both orientations determined by PLACE (https://www.dna.affrc.go.jp/PLACE/?action=newplace) and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) online software.

Figure 1. In silico identification of core and cis-regulatory elements in Pxy sequence. The 1063 bp Pxy promoter (in contig1365 with GenBank accession no AMGP01001365) sequence analysis depicts presence of putative cis-acting elements in both orientations determined by PLACE (https://www.dna.affrc.go.jp/PLACE/?action=newplace) and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) online software.
sequence alignment of these three sequences was carried out using MultAlin (http://multalin.toulouse.inra.fr/multalin/) online software (Supplementary Fig. S1). Further when an open blast was performed, Pxy homologs were identified in different formae speciales of *Fusarium oxysporum* with 100% query cover and up to 95% identity. These FASTA sequences were retrieved and used for multiple sequence alignment using MultAlin online software (Supplementary Fig. S2).

**Genetic modification of *Foc***. Seventeen transformants were obtained in hygromycin supplemented medium and these were phenotypically comparable with the WT strain (Fig. 2B). These transformants were sub-cultured for seven generations in the medium containing hygromycin. Of these, three transformants (T1, T8 and T17) were screened by genomic DNA PCR. To check for the quality of the genomic DNA extracted from WT and all the transformants, PCR was performed using TEF1α primers (Table 1) to amplify 251 bp region (Fig. 2C). Further, the amplicons of 1983 bp corresponding to Pxy promoter plus EGFP coding sequence along with glucoamylase gene terminator and 2012 bp corresponding to β-glucuronidase coding sequence and glucoamylase gene terminator was amplified from T8 and T17 transformants (Fig. 2D,E). However, the T1 transformant showed no amplification of the 2012 bp fragment and was therefore not used for further analysis. Both these PCR amplicons were absent in the WT genomic DNA sample. [NTC-non template control, +ve ctrl-positive control, ladder-1 Kb gene ruler].

**β-glucuronidase analysis.** In the histochemical staining method, the enzyme GUS reacts with 5-Bromo-4-chloro-3-indolyl-β-D-Glucuronide (X-Gluc) substrate to 5-Bromo-3-chloro-indole which upon oxidative dimerization becomes indigo. The WT and T17 transformant grown on different carbon source containing media for 3, 8, 12 and 24 h were individually incubated in GUS buffer overnight at 37 °C. The WT strain grown in different carbon sources containing medium did not show any indigo coloration, indicating that there is no GUS activity. Additionally, no GUS activity was observed in the T17 protein lysate samples collected at zero-hour time point. The mycelia of T17 grown in xylan, arabinoxylan and pectin showed indigo coloration at all four time points tested, demonstrating the presence of GUS enzyme (Fig. 3). This indicated that the promoter was inducible with xylan, arabinoxylan and pectin thereby transcribing the GUS gene resulting in the functional enzyme. It may be noted that, there was no β-glucuronidase activity observed in the T17 mycelia grown in the medium supplemented with xylose, sucrose and starch. Further, to assess the amount of β-glucuronidase activity, MUG assays at 0 and 12 hours for all the samples was performed. In the MUG assays, 4-Methyl-umbelliferyl-β-D-glucuronide is converted to β-glucuronide and 4-Methyl-umbelliferone (4-MU) by the action of β-glucuronidase enzyme. The 4-Methyl-umbelliferone is a fluorochrome with an excitation and emission at 365 nm and 465 nm, respectively.
The fluorescence intensity was measured by individually mixing the protein lysates from WT and T17 mycelia with MUG substrate for varying time periods (10 to 70 min). The \( \beta \)-glucuronidase specific activity was calculated in terms of the amount of 4-MU produced per milligrams of total protein per minute. There was no significant \( \beta \)-glucuronidase activity observed in WT grown in medium containing xylan, arabinogalactan and pectin in protein lysates from both 0 and 12 h time points. Activity was also not observed in protein lysates from the T17 transformant at 0 h time point. However, \( \beta \)-glucuronidase activity was significantly induced in the T17 transformant grown in medium supplemented with xylan followed by lesser activity in pectin and least in arabinogalactan (Fig. 4). This indicated that the \( \beta \)-glucuronidase activity is triggered by xylan, arabinogalactan and pectin.

**EGFP analysis.** The *Foc* WT and T17 mycelia grown in minimal medium supplemented with xylan, arabinogalactan, pectin, starch, sucrose, xylose as carbon source for different time points (0, 3, 8, 12 and 24 h) was observed under fluorescence microscope using FITC filter having excitation at 488 nm and emission at 509 nm. The EGFP expression was seen in the mycelia grown in medium containing xylan, arabinogalactan and pectin; but, not in sucrose containing medium (Fig. 5). This clearly indicates that the Pxy promoter is triggered by all these carbon sources except sucrose. The EGFP fluorescence that corresponds to the amount of EGFP expression was quantitated using fluorimeter. After measuring the fluorescence intensity, the mycelial wet weight was determined and expressed as FI/mg of mycelia. The T17 transformant showed maximum fluorescence in xylan-containing medium followed by starch, arabinogalactan, pectin and xylose in decreasing order (Fig. 6). This indicates that the Pxy promoter is regulated by carbon sources that are an important component of the plant cell wall.

**Discussion**

Plant pathogenesis is a complex series of events that are triggered when a pathogen encounters its host plant. During this interaction, the pathogen secretes an array of proteins, enzymes and toxins that prevent host defense response and favors pathogen survival and multiplication culminating into a disease\(^30,31\). The protein coding genes are triggered by a signaling cascade which perceive plant molecules as signals to initiate pathogenesis. Thus, it is likely that all those genes required during pathogenesis are regulated at the promoter level by a few signaling molecules\(^32\). The regulation of the pathogenicity genes can be economized if there is a single regulatory sequence.
that controls the expression\(^9,33\). However, such a phenomenon is quite common in prokaryotes where the operon system is established\(^{35-36}\). Nonetheless, in eukaryotes, at least two genes can be controlled by a single regulatory sequence (called as intergenic region) that controls the expression of two flanking genes.

In the present study, we identified such an intergenic region of 1063 bp that lies between two genes coding for putative beta-xyllosidase (accession number: ENH64116) and arabinoxylan arabinofuranohydrolase (accession number: ENH64117). These genes belong to the glycosyl hydrolase family 43 and are known to play an important role in the breakdown of xylan which is an important component of plant cell wall. Moreover, xylanases play a significant role in both penetration and horizontal expansion of phytopathogenic fungi in infected plants\(^{37,38}\).

Thus, a solitary signaling molecule from the host plant can simultaneously trigger the expression of these two genes, involved in xylan metabolism.

Bidirectional promoters are well studied in prokaryotes\(^3\), humans\(^4,39,40\), plants\(^41\), yeasts\(^5,42\) and insects\(^7\). However, there are only two reports on identification of bidirectional promoters from filamentous fungi. The \(\text{niiA}\) and \(\text{niiA}\) genes of \textit{Aspergillus nidulans} coding for nitrate and nitrite reductases are divergently transcribed and are separated by a 1200 bp intergenic region. These genes are under the control of the positively acting \(\text{NirA}\) transcription factor, which mediates nitrate utilization\(^52\). Three penicillin biosynthesis genes \(\text{pcbAB}\), \(\text{pbc}\), and \(\text{penDE}\) are arranged as a cluster in \textit{Penicillium chrysogenum}, \textit{Aspergillus nidulans} and \textit{Penicillium nalgiovense}. Among these three genes, the expression of \(\text{pcbAB}\) and \(\text{pbc}\) genes are controlled by a 1.16 kb bidirectional promoter region regulated by nutritional and developmental factors\(^{43,44}\). This indicates that the genes involved in a common metabolic pathway are clustered together and a maximum of two genes are regulated by an intergenic region. The Pxy promoter isolated in this study is comparable to the two promoters isolated from \textit{Aspergillus} and \textit{Penicillium}. Further, the Pxy promoter controls the expression of genes required for xylan metabolism and is an important element in pathogenesis.

For characterization, the Pxy promoter sequence was cloned in pCSN44 vector in between two reporter genes (\(\beta\)-glucuronidase and EGFP) and the bidirectionality of the intergenic sequence was tested by assaying the gene products. This strategy was also adopted in plants such as rice and \textit{Arabidopsis}, wherein two reporter genes (GUS and GFP) were cloned across the intergenic region\(^{45,41}\). In the current study, Pxy promoter sequence was inducible as there was no expression noticed in the zero hour time point irrespective of the media in which the transformant is grown. The reporter signals were observed after 3 h of incubation in the medium containing different carbon sources. Since this promoter controls the expression of gene products involved in xylan metabolism, we tested xylan and xylose as the most probable inducers. However, \(\beta\)-glucuronidase and EGFP expression was highest in the xylan-containing medium, but only EGFP signals were observed in xylose medium. This indicated that the Pxy promoter sequence differentially regulated the reporter gene pair. Moreover, other plant sugars such as starch, arabinogalactan, pectin and sucrose were further tested as inducers for Pxy sequence. \(\beta\)-glucuronidase activity was maximum in pectin followed by arabinogalactan, whereas no activity was seen in starch and sucrose. The EGFP fluorescence was observed in pectin, starch and arabinogalactan in a decreasing order of intensity. Since, EGFP fluorescence was differentially expressed with starch and xylose than that of the \(\beta\)-glucuronidase activity, the Pxy sequence appeared to differentially regulate the reporter gene pair. The in planta Pxy promoter activity was detected by infecting the susceptible cultivars of banana (cv. Rasthali) with WT and T17 strains. The RNA was extracted from the infected roots after 18 h, converted into cDNA and used as template for quantitative real-time PCR. The \(\beta\)-glucuronidase and EGFP expression (using TEF1\(\alpha\) as normalization gene control) was 9- and 7-fold, respectively in the T17 transformant as compared to the WT strain in \textit{ planta} (Supplementary Fig. S3). These results suggested that Pxy promoter sequence is activated during infection. Bidirectional promoters are known to be regulated in specific tissues at particular developmental stage or triggered during stress of hormonal stimulation\(^5,44\).
In synthetic biology and genetic engineering, the strength of the promoter is crucial to drive the expression of the heterologous protein. The promoter strength can be fairly determined by studying the binding affinity of the transcription factors to its sequence or assessing the expression level of the gene driven by the promoter. In our

Figure 5. Enhanced green fluorescent protein (EGFP) visualization using fluorescence microscopy. Expression of EGFP gene at different time point was determined using fluorescence microscopy of *Fusarium oxysporum f. sp. cubense* (Foc) grown in medium containing different carbon sources. Fluorescence was seen in Foc T17 strain grown in medium containing xylan, arabinogalactan, starch, xylose and pectin, whereas no change was seen in WT strain grown under all conditions and in T17 strain grown in sucrose containing medium. Bar represents 50 µm.
study, we determined the expression levels using quantitative real-time PCR, and found that the promoter Pxy is directionally biased. The relative expression of the EGFP and GUS genes showed no difference when xylan was used as an inducer (data not shown). Such bidirectional regulatory sequences find applications in genetic and metabolic engineering where the need for cumulative co-expression and stacking of two genes arises.

Wiemann and co-workers used an inducible niaD promoter sequence to drive the expression of sterigmatocystin Zn(II)2Cys6 transcription factor-encoding gene aflR and its cofactor aflS. These two factors regulate the expression of Aspergillus nidulans endogenous secondary metabolites genes to produce sterigmatocystin. Thus, a single promoter was used to regulate 8 genes from the sterigmatocystin gene cluster. Therefore, if the regulatory sequence is an inducible one, it’s highly valuable for expressing heterologous proteins. The Pxy promoter sequence isolated in this study is a compact and inducible promoter which is regulated by plant sugars and can be readily used for expressing heterologous proteins in filamentous fungi.

Received: 17 June 2019; Accepted: 16 January 2020; 
Published online: 11 February 2020

References
1. Wei, W., Pelechano, V., Järvelin, A. I. & Steinmetz, L. M. Functional consequences of bidirectional promoters. Trend. Genet. 27, 267–276 (2011).
2. Wakano, C., Byun, J. S., Di, L. J. & Gardner, K. The dual lives of bidirectional promoters. Biochimica et Biophysica Acta -Gene Regulatory Mechanisms. 1819, 688–693 (2012).
3. Christoffersen, C. A., Brickman, T. J. & McIntosh, M. A. Regulatory architecture of the iron-regulated fepD-ylbD bidirectional promoter region in Escherichia coli. J Bacteriol. 183, 2059–2070 (2001).
4. Trinklein, N. D. et al. An abundance of bidirectional promoters in the human genome. Genome Res. 14, 62–66 (2004).
5. Xu, Z. et al. Bidirectional promoters generate pervasive transcription in yeast. Nature. 457, 1035–1037 (2009).
6. Nel, H. et al. Widespread bidirectional promoters are the major source of cryptic transcripts in yeast. Nature. 457, 1038 (2009).
7. Behura, S. K. & Severson, D. W. Bidirectional promoters of insects: genome-wide comparison, evolutionary implication and influence on gene expression. J. Mol. Biol. 427, 521–536 (2015).
8. Liu, X. et al. Synthesis of seed-specific bidirectional promoters for metabolic engineering of anthocyanin-rich maize. Plant Cell Physiol. 59, 1942–1955 (2018).
9. Tan, S. Z. & Prather, K. L. Dynamic pathway regulation: recent advances and methods of construction. Curr. Opin. Chem. Biol. 41, 28–35 (2017).
10. Peremarti, A. et al. Promoter diversity in multigene transformation. Plant Mol. Biol. 73, 363–378 (2010).
11. Vogl, T. et al. Engineered bidirectional promoters enable rapid multi-gene co-expression optimization. Nat Commun. 9, 3589 (2018).
12. Dhadi, S. R., Krom, N. & Ramakrishna, W. Genome-wide comparative analysis of putative bidirectional promoters from rice, Arabidopsis and Populus. Gene. 429, 65–73 (2009).
13. Wang, Q. et al. Searching for bidirectional promoters in Arabidopsis thaliana. BMC Bioinform. 10, S29 (2009).
14. Banerjee, J., Sahoo, D. K., Dey, N., Houts, R. L. & Maiti, I. B. An intergenic region shared by At4g35985 and At4g35987 in Arabidopsis thaliana is a tissue specific and stress inducible bidirectional promoter analyzed in transgenic Arabidopsis and tobacco plants. PLoS ONE. 8, e79622 (2013).
15. Yang, J., Wang, X., Hasi, A. & Wang, Z. Structural and functional analysis of a bidirectional promoter from Gossypium hirsutum in Arabidopsis. Int. J. Mol. Sci. 19, 3291 (2018).
16. Araceli, O. A., Alfredo, C. R., Javier, M. M. & Luis, H. E. A phosphate starvation-driven bidirectional promoter as a potential tool for crop improvement and in vitro plant biotechnology. Plant Biotechnol J. 15, 558–567 (2017).
17. Nevalainen, H. & Peterson, R. Making recombinant proteins in filamentous fungi- are we expecting too much? Front. Microbiol. 5, 75 (2014).
18. Bergh, K. T., Litzka, O. & Brakhage, A. A. Identification of a major cis-acting DNA element controlling the bidirectionally transcribed penicillin biosynthesis genes acvA (pcbAB) and ipnA (pcbC) of Aspergillus nidulans. J Bacteriol. 178, 3908–3916 (1996).
19. Martin, J. F. Molecular control of expression of penicillin biosynthesis genes in fungi: regulatory proteins interact with a bidirectional promoter region. J. Bacteriol. 182, 2355–2362 (2000).
20. Muro-Pastor, M. L., Strauss, J., Ramón, A. & Sczaczecchio, C. A paradoxical mutant GATA factor. Euksyrot Cell. 3, 393–405 (2004).
21. Wiemann, P. et al. CoIN: co-inducible nitrate expression system for secondary metabolites in Aspergillus nidulans. Fungal Biol. Biotechnol. 5, 6 (2018).

Figure 6. Fluorescence intensity determination of Enhanced green fluorescent protein (EGFP) expressed in Fusarium oxysporum f. sp. cubense (Foc). Quantitative analysis of EGFP expression in Foc strains at 12 h time point in minimal medium containing xylan, arabinogalaetan, starch, xylose and pectin as sole carbon source using fluorimeter. Two-way ANNOVA Tukey’s multiple comparison test was performed using GraphPad Prism 8.0.2. (****P < 0.0001).
22. García, I., González, R., Gómez, D. & Scanzocchio, C. Chromatin rearrangements in the prnD-prnB bidirectional promoter: dependence on transcription factors. *Eukaryot Cell.* 3, 144–156 (2004).

23. Reyes-Dominguez, Y. et al. Nucleosome positioning and histone H3 acetylation are independent processes in the *Aspergillus nidulans* prnD-prnB bidirectional promoter. *Eukaryot Cell.* 7, 656–663 (2008).

24. Nützmann, H. W. et al. Bacteria-induced natural product formation in the fungus *Aspergillus nidulans* requires Saga/Ada-mediated histone acetylation. *Proc. Natl. Acad. Sci. USA.* 108, 14282–14287 (2011).

25. Elsson, G. L., Xue, Y., Song, R. & Acar, M. Insights into bidirectional gene expression control using the canonical GAL1/GAL10 promoter. *Cell Rep.* 25, 737–748 (2018).

26. Ghag, S. B., Shelkawat, U. K. & Ganapathi, T. R. Fusarium wilt of banana: biology, epidemiology and management. *Int. J. Pest Manag.* 61, 250–263 (2015).

27. Varadarajalu, L. P. & Punekar, N. S. Cloning and use of sC as homologous marker for *Aspergillus niger* transformation. *J. Microbiol. Methods.* 61, 219–224 (2005).

28. Jefferson, R. A., Kavanagh, T. A. & Bevan, M. W. GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6, 3901–3907 (1987).

29. Asai, S. et al. High-quality draft genome sequence of *Fusarium oxysporum* f. sp. cubense strain 160527, a causal agent of Panama disease. *Microbiol. Resour. Announc.* 8, e00654–19 (2019).

30. Presti, L. L. et al. Fungal effectors and plant susceptibility. *Ann. Rev. Plant Biol.* 66, 513–545 (2015).

31. Rodríguez-Moreno, L., Ebert, M. K., Bolton, M. D. & Thomma, B. P. Tools of the crook-infection strategies of fungal plant pathogens. *Plant J.* 93, 664–674 (2018).

32. Tan, K. C. & Oliver, R. P. Regulation of proteinaceous effector expression in phytopathogenic fungi. *PLoS Pathog.* 13, e1006241 (2017).

33. Zhang, J. et al. Spatial clustering and common regulatory elements correlate with coordinated gene expression. *PLoS Comput. Biol.* 15, e1006786 (2019).

34. Price, M. N., Huang, K. H., Arkin, A. P. & Alm, E. J. Operon formation is driven by co-regulation and not by horizontal gene transfer. *Genes Immun.* 15, 809–819 (2015).

35. Freyre-Gonzalez, J. A. & Trevino-Quintanilla, L. G. Analyzing regulatory networks in bacteria. *Nat. Educ.* 3, 24 (2010).

36. Junier, I. & Rivoire, O. Conserved units of co-expression in bacterial genomes: an evolutionary insight into transcriptional regulation. *PLoS ONE.* 11, e0155740 (2016).

37. Nguyen, Q. B., Itoh, K., Van Vu, B., Tosa, Y. & Nakayashiki, H. Simultaneous silencing of endo-β-1,4-xylanase genes reveals their roles in the virulence of *Magnaporthe oryzae*. *Mol. Microbiol.* 81, 1008–1019 (2011).

38. Yu, C. et al. Deletion of endo-β-1,4-xylanase *Vmxyl1* impacts the virulence of *Valsa mali* in apple tree. *Front. Plant Sci.* 9, 663 (2018).

39. Davies, G. E. et al. Identification of bidirectional promoters in the *human* KIR genes. *Genes Immun.* 8, 243 (2007).

40. Ardakani, F. B. et al. Integrative analysis of single-cell expression data reveals distinct regulatory states in bidirectional promoters. *Epigenetics Chromatin.* 11, 66 (2018).

41. Wang, R. et al. Isolation and functional characterization of bidirectional promoters in rice. *Front. Plant Sci.* 7, 766 (2016).

42. Punt, P. J. et al. The intergenic region between the divergently transcribed *nita* and *nida* genes of *Aspergillus nidulans* contains multiple *Nia* binding sites which act bidirectionally. *Mol. Cell. Biol.* 15, 5688–5699 (1995).

43. Mertens, S., Walz, M. & Kück, U. Expression studies with the bidirectional *pAB-ppcb* promoter region from *Acremonium chrysogenum* using reporter gene fusions. *Appl. Microbiol. Biotechnol.* 42, 57–66 (1994).

44. Kourmpetli, S. Isidore et al. Bidirectional promoters in seed development and related hormone/stress responses. *BMC Plant Biol.* 13, 187 (2013).

45. Higo, K., Ugawa, Y., Iwashita, M. & Korenaga, T. Plant cis-acting regulatory DNA elements (PLACE) database: 1999. *Nucleic Acids Res.* 27, 297–300 (1999).

46. Lescoat, M. et al. PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for *in silico* analysis of promoter sequences. *Nucleic Acids Res.* 30, 325–327 (2002).

Acknowledgements
Authors thank Prof. N. S. Punekar (IIT-B, Mumbai) for providing the fungal transformation vector and Dr. T. R. Ganapathi (BARC, Mumbai) for providing *Foc* culture used in this study. S.G. acknowledges the grant received from Department of Science and Technology, India under the DST-INSPIRE Faculty scheme (DST/INSPIRE FACULTY AWARD/IFA15-LSPA32) for this study.

Author contributions
S.G. conceived the study; S.G. and J.D. designed the research; A.D., V.G. and S.G. performed the research; S.G., J.D. and A.D. analyzed the data; and S.G., A.D. and J.D. wrote the paper.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-59159-0.

Correspondence and requests for materials should be addressed to S.B.G.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/

© The Author(s) 2020