WY195, a new inducible promoter from the rubber powdery mildew pathogen, can be used as an excellent tool for genetic engineering

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Research

Keywords: WY195, inducible promoter, functional verification, GUS activity, monocotyledons, dicotyledons, hpaXm, hypersensitive response, TMV resistance

Posted Date: January 1st, 2020

DOI: https://doi.org/10.21203/rs.2.19804/v1

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Version of Record: A version of this preprint was published at Frontiers in Microbiology on December 21st, 2020. See the published version at https://doi.org/10.3389/fmicb.2020.610252.
Abstract

Background: In recent years, the rational use of inducible promoters and organ/tissue-specific promoters have become a focus of promoter research because these enable greater control of expression.

Results: The WY195 promoter in the genome of *Oidium heveae*, the rubber powdery mildew pathogen, was predicted using PromoterScan and its promoter function was verified by the transient expression of the b-glucuronidase (GUS) gene. WY195 drove high levels of GUS expression in dicotyledons (*Nicotiana tabacum*, *Hevea brasiliensis* Reyan 7-33-97 and *Hylocereus undatus*) and monocotyledons (*Oryza sativa* ssp. *japonica* cv. Nipponbare, *Hordeum vulgare* and *Zea mays*). qRT-PCR indicated that GUS expression regulated by the WY195 promoter was 17.54-fold greater than that obtained using the CaMV 35S promoter in dicotyledons (*N. tabacum*), and 5.09-fold greater than that obtained using the ACT1 promoter in monocotyledons (*O. sativa*). Furthermore, WY195-regulated GUS gene expression was induced under high-temperature and drought conditions. Soluble proteins extracted from WY195-*hpaXm* transgenic tobacco was bioactive. Defensive micro-HR induced by the transgene expression of *hpaXm* was observed on transgenic tobacco leaves. Disease resistance bioassays showed that WY195-*hpaXm* transgenic tobacco enhanced the resistance to tobacco mosaic virus (TMV).

Conclusions: WY195 is an excellent endogenous inducible promoter with great potential for development as a new tool for genetic engineering and should also promote *O. heveae* molecular research, which to date has been a challenge owing to the obligate parasitic nature of this pathogen.

Background

Promoters regulate the expression of genes. Microorganisms and plants are the two main sources of promoters used for plant genetic engineering. However, there are very few promoters that can efficiently express exogenous genes at a high level in both monocotyledons and dicotyledons. The constitutive promoter Cauliflower mosaic virus 35S (CaMV 35S) was first reported in 1985 [1]. Since then, the 35S promoter has become the most widely and frequently used promoter in plant biotechnology. Almost every genetically modified crop plant that is grown commercially carries a version of this promoter [2]. Even so, the 35S promoter is relatively less used in monocots [3] because the expression of exogenous genes regulated by the 35S promoter in monocots is much lower than that in dicots (expression has been reported to be up to 100-fold lower [4]. The rice Actin1 (ACT1) and the maize Ubiquitin1 (Ubi1) promoters are widely used in monocots, which are tens or even hundreds of times more efficient at regulating the expression of exogenous genes than the 35S promoter [4–8]. By contrast, in dicotyledons, the expression of exogenous genes regulated by the rice ACT1 or maize Ubi1 promoter is weak [5].

Over time, the deficiencies and defects of constitutive promoters have gradually emerged [9]. If exogenous genes are expressed in whole plants, a large amount of heterologous proteins or metabolites will accumulate in plants, upsetting the original metabolic balance of plants. In addition, some products may be toxic, hinder the normal growth of plants, and even lead to plant death [10]. By contrast, organ/tissue-specific promoters and inducible promoters can regulate gene expression more precisely in terms of time and space, thus making up for the deficiencies of constitutive promoters. Another advantage of developing organ/tissue-specific promoters and inducible promoters is that they are perceived to be a way of improving the safety of genetically
modified foods. In the transgenic crops, the organ/tissue-specific promoters can be used to control the expression of the exogenous genes in specific non-edible organs. The inducible promoters can be used to control the expression of exogenous genes only under specific induction conditions, but not in a normal growth environment or in a harvested environment. This will prevent the presence of exogenous proteins in our food, such increasing the safety of genetically modified foods.

To date, the most important biotechnology use of filamentous fungi is in the production of biological products, such as antibiotics, organic acids, and many commercial enzyme preparations [11]. However, filamentous fungi, which are lower eukaryotes, its endogenous promoters are being increasingly used to express endogenous genes or to express exogenous genes in plants or animals [12]. The most significant step forward in recent years has been the creation of multiple-protease-deficient expression hosts, which has resulted in gram/liter yields of proteins of human origin [13]. Filamentous fungi can express surprisingly high levels of endogenous genes, which indicates that endogenous genes are regulated by powerful promoters. This implies that filamentous fungi may be a good source of potential promoters that can be developed for biotechnological applications. There are few studies and reports on the use of endogenous promoters of filamentous fungi to express exogenous genes in filamentous fungi. Examples of inducible promoters that have been used to express exogenous genes of filamentous fungi include the Trichoderma reesei cbh1 promoter [14], the Aspergillus glaA promoter [15], and the Aspergillus nidulans alcA promoter [16]; examples of constitutive promoters include the Aspergillus nidulans gpdA promoter [17] and the Trichoderma pki1 promoter [18].

Obligate biotrophic fungi cannot be cultivated in vitro and, to date, obligate biotrophic fungal promoters have not been reported. As a result, research on all aspects of the genetic transformation and molecular biology of obligate biotrophic fungi are seriously lagging behind that of other filamentous fungi. O. heveae is the causal agent of powdery mildew, which is one of the most economically damaging diseases of rubber trees (Hevea brasiliensis), which are the main source of natural rubber. O. heveae is an obligate parasitic fungi that cannot be cultured in vitro [19], and molecular research of this pathogen is extremely backward [20]. To date, O. heveae endogenous promoters have not been reported.

Harpin proteins are ubiquitous in Gram-negative bacterial pathogens and are encoded by a hypersensitive response and pathogenicity (hrp) gene cluster that stimulates plants to produce a hypersensitive response (HR) class of protein elicitors. Harpin proteins not only induce a plant disease response when endogenously expressed but also induce plant disease resistance when applied externally [21]. HpaXm is a hrp gene obtained from Xanthomonas citri ssp. malvacearum and its protein is named hpaXm [22]. At present, hpaXm is classified as a harpin protein in the ‘others’ group. When applied exogenously, hpaXm induces plants to produce a HR and enhances the resistance of tobacco to Tobacco mosaic virus (TMV). Li Le et al. (2017) transformed hpaXm and its signal peptide fragment HpaXmΔLP into N. tabacum. Soluble protein extracted from hpaXm transgenic tobacco can stimulate a HR in tobacco. Endogenous expression of hpaXm can cause a micro-HR in tobacco, and can induce tobacco resistance to TMV [23].

The aim of this study was to provide a new tool for plant genetic engineering and to promote molecular research studies of O. heveae. PromoterScan online software (http://www-bimas.cit.nih.gov/molbio/proscan/) was used to predict the genome sequence of O. heveae, and the suspected promoter sequence WY195 was obtained. Promoter function verification and quantitative determination of expression levels showed that WY195 was a strong and efficient promoter. Furthermore, WY195 not only regulated the highly efficient
expression of exogenous genes in dicotyledons but also regulated the efficient expression of exogenous genes in monocotyledons and, hence, has great potential for development. In addition, a plant expression vector that regulates the expression of hpaxm by WY195 was constructed and transformed into N. tabacum to investigate the effect on the disease resistance of transgenic plants and demonstrate the practicality of the WY195 promoter.

## Results

### Promoter prediction

A TATA-box at 48476 bp and a TSS (transcription start site) at 48506 bp in one gene of the O. heveae genome were predicted using PromoterScan (Table 1), and the suspected promoter was named WY195 (BankIt2158358).

| Significant Signals | TFD # | Strand | Location | Sequence | Site | Reference |
|---------------------|-------|--------|----------|----------|------|-----------|
| junB-US2            | S01738| +      | 48464    | GGCCAAT  | junB-US2 | Nucleic Acids Res 19: 775–81 (1991) |
| NFI                 | S00281| +      | 48465    | GCCAATC  | NFI.2 | Mol Cell Biol 7: 3646-55 (1987) |
| CTF                 | S00780| -      | 48471    | GATTGG   | Hsp70.5 | Mol Cell Biol 7: 3646-55 (1987) |
| TFIID               | S00087| +      | 48477    | TATAAAA  | Ad2MLP U.S.5 | Cell 43: 165–75 (1985) |
| TFIID               | S00615| +      | 48477    | TATAWAW  | TATA-box-CS | Annu Rev Biochem 50: 349–83 (1981) |
| TFIID               | S01540| +      | 48477    | TATAAA   | TATA-box.2 | Nucleic Acids Res 14: 10009-26 (1986) |

## Amplification of WY195 and construction of plant expression vectors

WY195 was amplified by primers WY195F/R (CCCAAGCTTAACCAATAATTTTCACGAGGG/CGGGATCCTCTGCATGCTAGTGATTGTT), which generated a single clear PCR product of 251 bp (Fig. 1a). The
WY195 sequence was successfully introduced into pBI121 to construct the plant expression vector pBI121-WY195 (Fig. 1b).

**Verification of WY195 promoter function**

WY195 was transiently expressed in *N. tabacum* by performing ATMT. The WY195-regulated reporter gene GUS was successfully expressed in *N. tabacum*, producing β-glucuronidase, which decomposed X-Gluc to form a blue-colored substance. *N. tabacum* leaf discs expressing GUS regulated 35S promoter, which acted as a positive control, were also stained blue, whereas the wild-type *N. tabacum* leaf discs, which acted as a negative control for GUS expression, were not stained blue (Fig. 2). These results demonstrate that the WY195 sequence has a promoter function and is an endogenous promoter of *O. heveae*. At the same time, we can observe that WY195 staining was significantly better than 35S promoter (deeper blue). This indicates that the ability of WY195 to regulate GUS expression is much higher than 35S.

**WY195 expression range**

Histochemical staining for GUS activity revealed that WY195 drove the efficient expression of the exogenous gene (GUS) in transiently transformed dicotyledons (tobacco, rubber, and dragon fruit) and monocotyledons (barley, rice, and maize) (Fig. 3).

Relative GUS gene transient expression

qRT-PCR indicated that GUS expression regulated by the WY195 promoter was 17.54-fold greater (*P* ≤ 0.01; Fig. 4a) than that obtained using the CaMV 35S promoter in dicotyledons (*N. tabacum*), and it was 5.09-fold greater (*P* ≤ 0.01; Fig. 4b) than that obtained using the ACT1 promoter in monocotyledons (*O. sativa*).

**WY195 promoter type**

Using the ATMT method, GUS was stably expressed when regulated by WY195 (Fig. 5). PCR and Southern blot demonstrated that WY195 was successfully transformed into *N. tabacum* (Fig. 6) and regulated the expression of the GUS gene. However, GUS staining was not observed in the tissues or organs of the T1 generation of transgenic tobacco plants. Therefore, we concluded that WY195 is not a constitutive promoter or a organ/tissue-specific promoter but an inducible promoter.

PlantCARE analysis revealed that the WY195 sequence comprised nine copies of the TATA-box, five copies of the CAAT-box, a wound-responsive element, three elements related to the light response, one cis-acting regulatory element essential for anaerobic induction, one MYB cis-acting regulatory element, one binding site involved in drought-inducibility, and three cis-elements, the functions of which were unclear (Table 2).
Table 2
Sequence analysis of suspected promoter WY195 by PLANTCARE

| Cis-acting regulatory elements | Organism          | Position | Strand | Sequence       | Function                                      |
|-------------------------------|-------------------|----------|--------|----------------|-----------------------------------------------|
| TATA-box                      | Arabidopsis       | 87       | +      | TATA           | Core promoter element around − 30 of transcription start |
|                               | thaliana          | 223      | -      | TATACA         | Core promoter element around − 30 of transcription start |
|                               | Helianthus        | 132      | +      | TAATA          | Core promoter element around − 30 of transcription start |
|                               | annuus            | 227      | -      | TTTTA          | Core promoter element around − 30 of transcription start |
|                               | Glycine max       | 129      | -      | TATAA          | Core promoter element around − 30 of transcription start |
|                               | Lycopersicon      | 225      | +      | TATAAA         | Core promoter element around − 30 of transcription start |
|                               | esculentum        | 197      | +      | TTTTA          | Core promoter element around − 30 of transcription start |
|                               | Arabidopsis       | 128      | +      | ATTATA         | Core promoter element around − 30 of transcription start |
|                               | thaliana          | 130      | -      | TATA           | Core promoter element around − 30 of transcription start |
|                               | Arabidopsis       |           |        |                |                                               |
|                               | thaliana          |          |        |                |                                               |
|                               | Lycopersicon      |          |        |                |                                               |
|                               | esculentum        |          |        |                |                                               |
|                               | Brassica          |          |        |                |                                               |
|                               | napus             |          |        |                |                                               |
|                               | Arabidopsis       |          |        |                |                                               |
|                               | thaliana          |          |        |                |                                               |
| CAAT-box                      | Arabidopsis       | 2        | +      | CCAAT          | Common cis-acting element in promoter and enhancer regions |
|                               | thaliana          | 232      | +      | CAAAT          | Common cis-acting element in promoter and enhancer regions |
|                               | Brassica rapa     | 214      | +      | CCAAT          | Common cis-acting element in promoter and enhancer regions |
|                               | Arabidopsis       | 3        | +      | CAAT           | Common cis-acting element in promoter and enhancer regions |
|                               | thaliana          | 215      | +      | CAAT           | Common cis-acting element in promoter and enhancer regions |
|                               | Hordeum vulgar     |          |        |                |                                               |
|                               | Hordeum vulgar     |          |        |                |                                               |
| WUN-motif                     | Brassica oleracea | 177      | +      | TCATTACGAA     | Wound-responsive element                        |
| Chs-CMAla                     | Daucus carota     | 109      | -      | TTACTTTAA      | Part of a light responsive element              |
| Box Ⅱ                        | Pisum sativum     | 92       | -      | TTTCAAA        | Light responsive element                        |

(Line 171)
Based on the sequence analysis results, anaerobic induction, wound induction, strong light induction, and drought induction experiments were performed on the T1 generation of transgenic tobacco. On the other hand, due to the presence of cis-acting elements of unknown function in WY195, temperature induction, which is very common in the induction type, has also been carried out. GUS activity was detected in plants subjected to high temperature and drought conditions (Fig. 7), and not detected in plants subjected under other induced conditions.

WY195 regulated expression of hpaXm in tobacco

Using the ATMT method, hpaXm was stably expressed when regulated by WY195 (Fig. 8). The presence of hpaXm and WY195 in the T1 generation was verified by PCR (Fig. 9a). The WY195 primer amplified a band of the same size as WY195, which was about 250 bp. The hpaXm primer was amplified with hpaXm, generating the expected band of approximately 400 bp. The integration of the target genes was confirmed by Southern blot analysis (Fig. 9b). PCR products amplified in the transgenic T1 generation were hybridized with corresponding probes targeting WY195 and hpaXm to produce two unique bands of the corresponding size. This indicated that WY195 and hpaXm genes had been smoothly integrated into the genome of N. tabacum.

Soluble proteins produced by WY195- hpaXm transgenic tobacco elicited HR on tobacco leaves

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel patterns and bioactivity of soluble proteins from leaves of WY195-hpaXm transgenic tobacco, induced WY195-hpaXm transgenic tobacco, pBI121-hpaXm transgenic tobacco and N. tabacum wild type were compared. The molecular mass of hpaXm was estimated to be 13.3 kDa, and the putative GST–hpaXm protein was about 35 kDa [54]. The results suggested that the purified protein from the induced WY195-hpaXm transgenic tobacco was abundant among total proteins and had the same size as hpaXm from E. coli (Fig. 10). Same size protein as hpaXm were not obtained.
for uninduced WY195-hpaXm transgenic lines. Only proteins from induced WY195-hpaXm transgenic tobacco and pBI121-hpaXm transgenic tobacco were active, and the degree of protein-induced HR from the induced WY195-hpaXm transgenic tobacco was significantly stronger than that of the protein from pBI121-hpaXm transgenic tobacco and hpaXm prepared periodically from recombinant E. coli strain (Fig. 11), even though the proteins were boiled for 10 min. Instead, proteins isolated from uninduced WY195-hpaXm transgenic tobacco, pBI121 transgenic tobacco or N. tabacum wild type did not cause the HR (Fig. 11). It demonstrated that hpaXm was actively present in leaf tissues of induced WY195-hpaXm transgenic tobacco.

Defensive responses were induced in WY195- hpaXm transgenic tobacco

The T1 generation WY195-hpaXm transgenic tobacco was observed to determine whether defensive responses had been induced. As a result, no visible HR or micro-HR was induced on its leaf surface. However, dark blue micro-HR of scattered necrotic cell clusters were observed under the microscope after trypan blue staining, which significantly more severe than pBI121-hpaXm transgenic tobacco (positive control). And micro-HR was not induced in the N. tabacum wild type (negative control) (Fig. 12). The results showed that the expression of hpaXm which regulated by WY195 promoter produced defensive responses with partial hypersensitive cell death in transgenic tobacco leaves, and it is more severe than that in pBI121-hpaXm transgenic tobacco.

TMV resistance of WY195- hpaXm transgenic tobacco

First, the T1 generation WY195-hpaXm transgenic tobacco is induced correspondly, then challenged with TMV rub inoculation. As a result, hpaXm transgenic tobacco had significant TMV resistance compared to wild-type three-smoke (Fig. 13). The number of TMV lesions observed on wild-type N. tabacum after inoculation with TMV was significantly higher than that of the positive control 35S-hpaXm and WY195-hpaXm transgenic T1 plants (P ≤ 0.01; Table 3). Compared with wild-type N. tabacum, the average number of lesions observed on the positive control group was approximately 29.37% lower. Compared with wild-type tobacco and the positive control, the average number of lesions observed on the WY195-hpaXm transgenic T1 plants was approximately 66.44% and 52.48% lower, respectively.

Table 3 Resistance levels of WY195-hpaXm transgenic tobacco against TMV

| Plants                               | Plant leaf lesion number (X ± SE) | P ≤ 0.01 Significance level | Lesion reduction/ % |
|--------------------------------------|----------------------------------|----------------------------|--------------------|
| N. tabacum wild type                 | 47.67 ± 1.43                     | A                          |                    |
| T1 generation of 35S-hpaXm transgenic tobacco | 33.67 ± 2.33                     | B                          | 29.37              |
| T1 generation of WY195-hpaXm transgenic tobacco | 16.00 ± 2.31                     | C                          | 66.44              |

Preliminary analysis of the full length of WY195 by a series of deletion mutations

PROMO predicted that when the fault tolerance rate is set to the default of 15%, 558 transcription factors are obtained for WY195Q. However, this data is too cumbersome to analysis, and the error rate itself is higher. Thus,
the fault tolerance rate was reduced to zero, and 103 transcription factors were obtained. The 103 transcription factors were searched in the online non-redundant database JASPAR of the TFBS, and all databases of all species were selected. A total of 27 previously reported TFBS were retrieved. These 27 TFBS with known functions were marked on the WY195Q sequence (Fig. 14). Series deletion mutations of WY195Q were performed after avoiding the destruction of these TFBS. The plant expression vector pBI121-WY195Q, pBI121-WY195Q1, pBI121-WY195Q2 and pBI121-WY195Q3 were constructed for sequence WY195Q (2100 bp), WY195Q1 (1829 bp), WY195Q2 (1321 bp) and WY195Q3 (830 bp) (Table 4, Fig. 15a, Fig. 15b). The level of transient expression of the GUS gene regulated by these sequences in tobacco was measured by qRT-PCR (Fig. 15c). Among them, WY195Q2 regulates the highest expression of GUS gene. Thus the WY195 full length was preliminary analysed as 1321 bp.

Table 4  
Primers for WY195Q series deletion mutation

| Fragments | Upstream primers                      | Downstream primers                      |
|-----------|---------------------------------------|-----------------------------------------|
| WY195Q    | 195QF: CCCAAGCTTTTGATCTCGAGATCGTTTTTT | CGCGGATCCTCTGCATGCTAGTGATTGTT          |
| WY195Q1   | 195Q1F: CCCAAGCTTTTTCTCCTAATTTTCGTCAGTCC |                                      |
| WY195Q2   | 195Q2F: CCCAAGCTTTCCAGGCTTTAGAATATTTATG |                                      |
| WY195Q3   | 195Q3F: CCCAAGCTTTCTAAAGACGACATGTAATTATCC |                                      |
| WY195      | 195F: CCCAAGCTTTACCAATAATTTTCACGAGGG   |                                      |
| (Line 247) |                                      |                                      |

Discussion

Comparative genomics studies have shown that eukaryotic promoter sequences are more complex than prokaryotic promoter sequences, and that the mechanism of transcriptional regulation is more complicated [55]. In eukaryotes, the transcriptional regulation mechanisms of plants are more complex than those of animals and the study of plant promoters is currently lagging behind that of animal studies [55, 56]. In plant genetic engineering, microorganisms and plants are two main sources of promoters that used for regulating constitutive expression of genes. Virus-derived ones such as the 35S promoter and the FMV 34S promoter [57, 58], plant-derived ones such as rice ACT1 and maize Ubi1 promoter, and the like.

Filamentous fungi has a very potential promoter resource [59], but up to now, there have been fewer promoters of filamentous fungi that have been developed. Most of the filamentous fungal endogenous promoters that have been applied or developed are used to express endogenous genes or to express exogenous genes in their own, but rarely used to express exogenous genes in plants or animals. Although it has greatly developed the endogenous genes of filamentous fungi, the application of these excellent promoters is relatively limited. Among the filamentous fungal promoters, there are likely to be excellent promoters that can applied to various
organisms like the 35S promoter. In addition, filamentous fungal promoters that have been developed have relatively narrow sources in filamentous fungi. Due to the characteristics of obligate parasitism, the molecular research of obligate parasitic fungi is very backward, and its promoter has not been reported.

The WY195 promoter investigated in this study was derived from O. heveae. O. heveae is an obligate parasitic fungus, it obviously is a very big surprise discovery. Our results demonstrate that WY195 is a very efficient, promoter and that its ability to drive the expression of exogenous genes in N. tabacum is far superior to that of the 35S promoter (17.54 times). We can not only use WY195 to express its own endogenous gene in O. heveae, but also use WY195 to express a very useful exogenous gene in O. heveae. More importantly, WY195 can be used to express useful exogenous genes in plants, providing a new tool and method for plant genetic engineering. It has very good application potential.

Further research indicates that WY195 has a very wide range of transcriptional regulation. WY195 can also express the exogenous gene GUS in monocotyledon rice. And its ability to regulate GUS expression is also very good, 5.09 times of the ACT1 promoter. Few promoters that can efficiently express exogenous genes at a high level in both monocotyledons and dicotyledons. Classic as 35S, the expression of exogenous genes in monocotyledons is much lower than that in dicotyledons [4]. At the same time, the results of transient expression indicated that WY195 can also express the exogenous gene GUS well in dicotyledonous rubber and dragon fruit, as well as monocotyledonous barley and maize. WY195 has the advantage of a wide range of transcriptional regulation, making it a much broader application and value. The efficient regulation of the transient expression of exogenous genes in rubber by the WY195 promoter is particularly encouraging given that the obligate biotrophic nature of O. heveae has hampered research studies on this pathogen and, therefore, our understanding of the molecular basis of its pathogenesis is still limited.

Given that GUS staining was not observed in the tissues or organs of the T1 generation of transgenic tobacco plants, we concluded that WY195 is not a constitutive promoter or an organ/ tissue-specific promoter but an inducible promoter. Given that GUS activity was detected in tissues of plants subjected to high temperature or drought conditions, we inferred that WY195 is a high-temperature and drought-inducible promoter. We have confirmed that WY195 can regulate the expression of exogenous genes in major crops such as rice, com and barley. In crops, if WY195 is used rationally to regulate the expression of beneficial exogenous genes such as yield increase and disease resistance, the expression of these genes is stimulated by the induction factors such as high temperature and drought during the growing season of crops, and the stimulation of these factors is avoided during the harvest period of agricultural products. It can increase the yield and quality of crops, and can also reduce the safety risks of genetically modified foods as much as possible. The series of deletion mutations is only a preliminary analysis of the full length of the WY195 promoter. In the next work, we will perform functional verification on WY195’s unreported transcription factors, and then analyze the exact full length of WY195.

Lots of reports have confirmed that exogenous application of harpins can enhance the systemic disease resistance of plants, such as tobacco, cucumber, rice, Arabidopsis and so on [23, 60–63]. Similarly, hrp gene transgenic plants showed resistance to plants [23, 64, 65]. Results in our study supported the view, hpaXm also induced resistance when acting both intercellular and intracellular. HpaXm could confer defense responses without HR cell death against diverse plant pathogens when using exogenous application. And hpaXm expression regulated by WY195 promoter can enhanced more resistance of transgenic tobacco than that
regulated by 35S to TMV. There was no visible necrotic spots induced in WY195-hpaXm transgenic tobacco. This should be because WY195 is an inducible promoter. The hpaXm expression was very little or absent when the corresponding induction was not performed. When the corresponding induction was carried out, we observed a large amount of micro-HR on the transgenic tobacco leaves after trypan blue staining. This demonstrated that endogenous expression of hpaXm induced defensive responses in plants with cell death.

In short, our results demonstrate that the O. heveae endogenous promoter WY195 could potentially be developed to provide a new tool and method for genetic engineering of both monocotyledons and dicotyledons and also promote molecular research on rubber powdery mildew.

**Conclusion**

In a summary, we have predicted, screened, function verified and developed a new endogenous inducible promoter of O. heveae, which was named WY195. WY195 has driven high levels of GUS expression in monocotyledonous and dicotyledons. GUS expression regulated by the WY195 promoter was 17.54-fold greater than that obtained using the 35S promoter in dicotyledons (N. tabacum) and 5.09-fold higher than that obtained using the ACT1 promoter in monocotyledons (O.sativa). Furthermore, WY195-regulated GUS gene expression was induced under high-temperature and drought conditions. WY195 can regulate the hpaXm expression in tobacco, and the generated protein hpaXm induced the micro-HR. Disease resistance bioassays showed that WY195-hpaXm transgenic tobacco enhanced the resistance to TMV. On the one hand, WY195 can provide new methods and tools for genetic engineering. On the other hand, it should also promote O. heveae molecular research, which to date has been a challenge owing to the obligate parasitic nature of this pathogen.

**Materials And Methods**

**Plant and fungal materials**

Oidium heveae Steinm. strain HO-73 (provided by the Key Laboratory of Green Prevention and Control of Tropical Plant Diseases and Pests (Hainan University), Ministry of Education, China) was used in this study. The pathogen was cultured on young, bronze-stage leaves of the moderately susceptible rubber tree cultivar Reyan 7-33-97 [24]. The rubber plants were grown in a controlled growth chamber at 25 °C with a 16 h: 8 h, light:dark photoperiod. Tobacco (Nicotiana tabacum L.), dragon fruit (Hylocereus undatus (Haworth) Britton & Rose), rice (Oryza sativa L. ssp. japonica cv. Nipponbare), barley (Hordeum vulgare L.), and maize (Zea mays L.) were all grown in a controlled growth chamber at room temperature (25–30 °C) with a 16 h:8 h, light: dark photoperiod.

**Promoter prediction**

The whole genome sequence of O. heveae was analyzed and reported in a previous study by our laboratory [25]. PromoterScan was used to predict promoters in the O. heveae genome [26–29].

**Construction of plant expression vectors**

Based on the predicted sequence of the probable promoter WY195, primers were designed using Primer Premier 5.0 (Premier Biosoft International, CA, USA), and BamHI and HindIII restriction sites were introduced. The
primers were synthesized by the Beijing Genomics Institute. The WY195 gene was PCR-amplified from the whole genome of O. heveae (using primers WY195F/WY195R).

The pBI121 vector (TIANNZ, 60908-750y) contains the kanamycin resistance gene (KanR) and the reporter gene β-glucuronidase (GUS). GUS is regulated by 35S promoter. We digested the 35S promoter carried by the pBI121 vector using the restriction endonucleases HindIII (R0104S, New England Biolabs (NEB), Ipswich, MA, USA) and BamHI (R0136V, NEB) to use it as a backbone. The suspected promoter WY195 was recovered after the TA clone was correctly sequenced and then introduced into the site of the original 35S promoter to construct our recombinant vector.

**Verification of WY195 promoter function**

The recombinant vector PBI121-WY195 was transformed into Agrobacterium tumefaciens strain LBA4404 by triparental hybridization [30], and then transformed into N. tabacum using the Agrobacterium-mediated leaf disk method [31] for transient expression. Finally, the WY195 promoter function was verified by GUS staining [32].

**WY195 expression range**

After determining that WY195 can transiently regulate the expression of GUS in tobacco, we investigated whether WY195 can regulate the expression of exogenous genes in other plants. To investigate the expression range of WY195 regulation, we transiently expressed the GUS gene in young, bronze-stage rubber tree leaves and young pitaya (dragon fruit) stalks (dicotyledons), rice callus, young barley leaves and immature maize embryos (monocotyledons) using ATMT.

**Validation of relative gene transient expression using quantitative real-time-PCR**

The N. tabacum and O. sativa leaf discs in transient expression were partially used for GUS staining, and the remaining part was used to extract RNA and then reverse transcription. The cDNA that was generated was diluted 1000 times as a template and real-time fluorescence quantitative PCR was performed to verify the transient expression level of GUS.

RNAprep Pure Plant Kit (TIANGEN, DP441) was used for total RNA isolation, the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, K1621) was used for cDNA synthesis, and SYBR Premix Ex Taq II (TAKARA, RR820A) was used for qPCR. qRT-PCR was performed using a QIANGEN Rotor-Gene Q MDx RealTime PCR system with the following PCR condition: Step 1, 94 °C for 30 s; step 2, 45 cycles of 94 °C for 12 s, followed by 58 °C for 30 s and 72 °C for 30 s; step 3, 72 °C for 10 min. A-tubulin were used to normalize mRNA levels. The α-tubulin primers: α-tubulinF (TCTGAACCGACTTATTTAC) and α-tubulinR (CATGGACATCCTTTGCCACA). The GUS primers: GUSF (GTCGCGCAAGACTGTAACCA) and GUSR (TGGTTAATCAGGAACTGTTG). The relative expression level of the GUS gene was calculated using the 2-ΔΔCt method [33, 34]. Data were obtained from 3 sets of experiments and analyzed using SPSS version 16.0 software (SPSS Inc., Chicago, IL, USA).

**WY195 promoter type**

The recombinant vector pBI121-WY195 was transformed into N. tabacum using Agrobacterium tumefaciens-mediated transformation (ATMT) and stably expressed. Tissue culture was used to regenerate the explants.
The T0 generation seeds were collected and the T1 generation transgenic tobacco were planted. The leaves of the T1 generation transgenic tobacco (40 d) were collected for DNA extraction. PCR and a Southern blot were performed to verify whether the transformation had succeeded.

GUS staining was performed on the tissues or organs of transgenic tobacco plants that were successfully transformed. If the GUS staining result is positive, it would be determined based on the staining result that the promoter belongs to a constitutive promoter or an organ/ tissue-specific promoter. If the staining result is negative, the plant cis-acting regulatory element database PlantCARE35 would be used to analyze the sequence of WY195. Then, based on this, the corresponding induction would be carried out to analyze the type of the promoter.

Temperature induction was referenced and improved with methods of Baker et al. [35] and Éva et al. [36]: transgenic tobacco leaves were induced at 4 °C for 48 hours, and 42 °C for 48 hours. Drought induction was referenced and improved with methods of Jang et al. [37] and HUA et al. [38]: transgenic tobacco seedlings were introduced into a centrifuge tube containing 30% PEG 6000 for hours. Light induction was referenced and improved with methods of Xu et al. [39] and Albers et al. [40]: transgenic tobacco for continuous light incubation for 72 hours. Anaerobic induction was referenced and improved with methods of Niemeyer et al. [41] and Li et al. [42]: transgenic tobacco seedlings are placed in water for 2/48 hours. Wound induction was referenced and improved with methods of KesanaKurti et al. [43] and et al. [44]: transgenic tobacco leaves for 2/48 hour induction of puncturing wounds.

**Protein analysis and micro-HR observation**

The functional gene hpaXm which regulated by WY195 promoter was stably expressed in N. tabacum using ATMT. Soluble proteins were isolated from WY195-hpaXm transgenic tobacco leaves as Bollag et al. described with several modifications [45]. PMSF at 0.1 mol/L was added to protect proteins from destruction by proteases [46]. The bioactivity in aqueous solutions were tested. Purified proteins were resolved with SDS-PAGE. A portion of the aqueous solution of the protein preparation was heated in a boiling water bath for 10 min while the other portion was left untreated. And the biological activity of both was tested and resolved by native PAGE. Biological activity was boiled for 10 min [47]. Evaluated the tobacco HR response for it in comparision with hpaXm from E.coli strain BL21/pGEX-hpaXm.

Micro-HR can monitored by observing dead cells after leaves trypan blue staining [48–50]. Approximately 1 cm × 1 cm tobacco leaves were placed in lactophenol trypan blue solution, which contains 15.45% aqueous, 18.18% water saturated phenol, 17.82% glycerol, 18.18% distilled water and 27.27% trypan blue. Then the treated leaves were heated in a boiling water bath for 5–10 min and incubated at room temperature for 6–8 h, and were observed after decolorization. The protein of the pBI121-hpaXm with the same treatment was used as a positive control, and that of the empty vector (pBI121) transgenic tobacco and N. tabacum wild type were used as two different negative control. To determine if the two reactions spontaneously occurred in the transgenic lines, every three leaves on the plants were similarly studied at 10 day intervals until flowering.

**WY195-regulated expression of hpaXm in tobacco**

T1 generation transgenic tobacco were inoculated with TMV at 40 days after transplanting to soil. Inoculation was performed for TMV (100 µl; 18 µg/ ml of solution) by rubbing leaves using a finger in the presence of
abrasive diatomaceous [51, 52]. Inoculated tobacco were grown in a greenhouse maintained at room temperature (25°C– 28°C) and investigated for infection 7 d later [51, 53]. The number and size of lesions on leaves were determined. Disease severity (DS) was expressed as number of lesions per leaf for TMV. Correspondingly, resistance was given as percent decrease of DS in transgenic tobacco, relative to that in N. tabacum wild type or pBI121 vector transgenic tobacco, quantified using the formula 100 × (mean DS in control plants—mean DS in transgenic plants)/mean DS in the control [53].

**Analysis of WY195 promoter full length**

The 2000 bp upstream of the WY195 transcription start site plus the downstream 100 bp sequence was considered as the research object and was named WY195Q. The TFs (Transcription factor) of WY195Q were predicted by online database PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). Then the TFBS (Transcription factor binding site) of these TFs were searched in online database JASPAR (http://jaspar.genereg.net/). The function of these searched TFBS had been reported. And these TFBS were marked on the sequence of WY195Q. Series deletion mutations of WY195Q were performed after avoiding the destruction of these TFBS with known functions. Specific primers were designed, and the plant expression vectors were constructed after TA cloning. Transient expressed it in N. tabacum, and the expression levels were determined by qRT-PCR. The expression levels to each other were compared to initially determine the effective full length of the promoter.

**Declarations**

Compliance with Ethical Standards

**Disclosure of Potential Conflicts of Interest** No potential conflict of interest was reported by the authors.

**Research Involving Human Participants and/or Animals** This article does not contain any studies with human or animals subjects performed by any of the authors.

**Informed Consent** not applicable.

Availability of Data and Materials

All data and materials were available within the manuscript and supporting informations

Funding

This study was supported by the National Natural Science Foundation of China (No. 31660033), the Innovation Team of Hainan Natural Science Foundation of China (2016CXTD002), the National Key Basic Research Plan of China (No.2011CB111612), the National Natural Science Foundation of China (No. 31560495, No.31760499), China Agriculture Research System (No.CARS-34-BC1), the key development plan of Hainan (No. ZDYF2016208) and the scientific research beginning project of Hainan University (No. kyqd1535). The funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript.

Acknowledgements

Not applicable.
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**Figures**

**Figure 2**

Construction of plant expression vector. a Amplification of WY195. (M) Marker2000; (CK-) Negative control with ddH2O as template; (A1) and (A2) WY195. b Map of recombinant vector pBI121-WY195.

**Figure 4**

Histochemical staining for GUS activity in transiently transformed leaf discs of N. tabacum. (A1)–(A6) CK−, negative control, leaf discs of wild-type N. tabacum; (B1)–(B6) CK+, positive control, GUS activity in leaf discs regulated by the 35S promoter; (C1)–(C6) GUS activity in leaf discs regulated by the WY195 promoter.
Histochemical staining for GUS activity in transient transformed dicotyledons and monocotyledons. (A1) to (A3) GUS activity in dicotyledons regulated by the WY195 promoter; (B1) to (B3) CK+, positive control, GUS activity regulated by the 35S promoter; (C1) to (C3) CK–, negative control, GUS activity in wild-type.

Figure 8
Transient expression of GUS regulated by WY195 promoter. a Transient expression in dicotyledons (N. tabacum); CK+, 35S. b Transient expression in monocotyledons (O. sativa); CK+, ACT1 promoter. Different lowercase letters indicate a significant difference (P <0.05), and different uppercase letters indicate a very significant difference (P <0.01).

Figure 10

Stages of Agrobacterium-mediated tobacco transformation. (A) Tobacco leaf discs for co-cultivation with Agrobacterium inoculum. (B) Callus of N. tabacum. (C) Adventitious buds. (D) 14-day-old plantlets that survived on Kanamycin selection media transplanted to a jar. (E) Mature plants transplanted to soil.

Figure 12

Southern blot analysis of WY195 in the genome of transgenic N. tabacum. (M) DNA molecular weight marker (DIG-labeled); (A) WY195 promoter; (CK−) N. tabacum wild-type.
Figure 14

Histochemical staining for GUS activity in a T1 generation of WY195 transgenic tobacco grown under different conditions. (A) High temperature induced WY195-GUS transgenic tobacco; (B) Drought-induced WY195-GUS transgenic tobacco; (CK+) 35S-GUS transgenic tobacco; (CK-) wild-type N. tabacum.

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Figure 16

Map of the recombinant vector pBI121-WY195-hpaXm.

Figure 18

Molecular verification of WY195-hpaXm transgenic tobacco plants. a PCR amplification. (M) Marker2000; (A) WY195-hpaXm transgenic tobacco plants, primers: WY195F/R; (B) WY195-hpaXm transgenic tobacco plants, primers: hpaXmF/R; (C) CK+, template: wild-type O. heveae, primers: WY195F/R; (D) CK+, template: wild-type X. citri, primers: hpaXmF/R; (E) CK+, template: recombinant vector pBI121-WY195-hpaXm, primers: WY195F/R; (F) CK+, template: recombinant vector pBI121-WY195-hpaXm, primers: hpaXmF/R; (G) CK+, template: 35S-hpaXm transgenic tobacco plants, primers: 35SF/R; (H) CK+, template: 35S-hpaXm transgenic tobacco plants, primers: hpaXmF/R; (I) CK-, template: wild-type N. tabacum, primers: WY195F/R; (J) CK-, template: wild-type N. tabacum, primers: hpaXmF/R; (K) CK-, template: ddH2O, primers: WY195F/R; (L) CK-, template: ddH2O, primers: hpaXmF/R. b PCR-Southern blot analysis. (M) DNA molecular weight marker (DIG-labeled); (CK–) N. tabacum wild-type; (A) WY195 promoter; (B) hpaXm.
Figure 19

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) patterns of soluble protein preparations. (A) Soluble protein preparations (1μL; 308ng) including hpaXm from induced T1 generation WY195-hpaXm transgenic tobacco. (B) Soluble protein preparations (1μL; 361 ng) from N. tabacum wild type; (C) Soluble protein preparations (1μL; 366.5ng) from pBI121-hpaXm transgenic plant; (D) Soluble protein preparations (1μL; 301ng) including hpaXm from uninduced T1 generation WY195-hpaXm transgenic tobacco. (E) Soluble protein preparations (1μL; 325ng) including hpaXm associated with a GST mark.

Figure 21
Soluble proteins of hpaXm from WY195-hpaXm transgenic tobacco could stimulate HR. Bioactivity assays of proteins from leaf tissues, compared with the preparations of GST-hpaXm from E. coli. (A) GST-hpaXm (325 ng/μL) (CK+); (B) N. tabacum wild type (CK-) (361 ng/μL); (C) pBI121 transgenic tobacco (344 ng/μL); (D), (E) and (F) induced T1 generation WY195-hpaXm tobacco (308 ng/μL); (G) and (H) pBI121-hpaXm transgenic tobacco (366.5 ng/μL) (CK+); (I) PBS buffer (1 μL); (J), (K), (L) and (M) uninduced WY195-hpaXm tobacco (288 ng/μL); (N) ddH2O (CK-).

Figure 24

Trypan blue staining of WY195-hpaXm transgenic tobacco leaves. Micro-HRs were shown as areas stained blue. (A1) and (A2) induced WY195-hpaXm transgenic N. tabacum; (B1) and (B2) pBI121-hpaXm transgenic N. tabacum; (CK+); (C1) and (C2) with exogenous application of the hpaXm proteins (CK+); (D1) and (D2) pBI121 transgenic N. tabacum (CK-); (E1) and (E2) uninduced WY195-hpaXm transgenic N. tabacum; (F1) and F2) N. tabacum wild type (CK-).
Figure 26

Leaves of transgenic N. tabacum plants inoculated with TMV (1.8 μg). Leaves transformed with (A) hpaXm or (B) vector pBI121, and (C) wild type. Lesions are present on leaves susceptible to TMV. Similar results were obtained from five sets of experiments: a total of 30 plants of each genotype were inoculated.
Figure 28

Detailed distribution of transcription factor binding sites on the WY195Q sequence.

Figure 30

Series deletion mutations of WY195Q. a Schematic diagram of fusion of the 5’ series deletion fragment of WY195Q with the GUS gene. b PCR verification of the WY195 promoter series deletion mutant vector. c WY195Q series deletion mutant fragment drives the transient expression of GUS gene in N. tabacum.