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Uracil walking primer PCR: An accurate and efficient genome-walking tool

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ABSTRACT

Genome walking PCR has been extensively used to acquire unknown genomic regions bordering known DNAs. However, non-target amplification challenges the efficacy of existing genome-walking PCRs. Herein, we conceived a new genome-walking method termed Uracil walking Primer PCR (UP-PCR). The UP-PCR features introducing an uracil base at the penultimate position of arbitrary walking primer (AWP) 3′ end. A UP-PCR set comprises three nested amplification steps, which are performed by an AWP sequentially coupling a set of three nested site-specific primers, respectively. Prior to secondary UP-PCR, primary UP-PCR product is processed with uracil DNA glycosylase to destroy the carried AWP. As a result, only target primary product is exponentially amplified in the next UP-PCR(s), as it is the only product with binding sites for the both primers. The performance of UP-PCR has been validated by walking three selected genes. The walking experiments showed that each secondary or tertiary UP-PCR generated one to two amplicon ranging in size from 0.2 to 5.0 kb, while with a negligible non-target background; and the amplicons of the secondary UP-PCRs were all correct, indicating that tertiary UP-PCR is generally unnecessary. These findings suggested that UP-PCR has a satisfactory walking ability, specificity, and speed. Collectively, the proposed UP-PCR is a potential candidate method for genome walking.

1. Background

Genome walking, also known as chromosome walking, is a molecular biology research technique for amplifying unknown genomic regions bordering known DNAs.[1] Modern genome-walking techniques are generally random PCR-based.[2] A random PCR-based genome-walking technology has shown broad application prospects in studying mechanisms of gene expression and regulation,[3–4] identifying transgenic sites,[5] and filling gaps in genome sequencing.[6]

The documented PCR-based genome-walking technologies can be clustered into types i and ii, based on whether involving pretreatment of genomic template. Type i technology requires prior genomic template pretreatment to PCR.[7–8] Alu-PCR,[9] vectorette PCR,[10] and cassette PCR[11] belong to type i. Type ii technology, namely random PCR, achieves walking by randomly annealing arbitrary walking primer (AWP) to unknown flank, thus omitting the template pretreatment prior to PCR. Therefore, type ii has become the mainstream genome-walking technology due to its simplicity and low cost.[12] Fork PCR,[13] thermal asymmetric interlaced PCR,[14] and wristwatch PCR[15] belong to type ii.

To implement so-called walking, random PCR requires at least one low annealing-temperature (generally 25–30 °C) cycle in primary PCR, so as to facilitate AWP randomly positioning to some site(s) on unknown flank.[16] This positioning directs the extension primed by AWP towards known region, thus generating a target amplicon that has AWP at the 5′ end and known region at the 3′ part. This target amplicon can be exponentially boosted in the following high annealing-temperature cycles. However, three categories of non-target amplicons are also synchronously produced. Category i non-target amplicon is produced by site-specific primer (SP) alone; category ii is produced by both SP and AWP; and category iii is produced by AWP alone.[17–18] Categories i and ii

Abbreviations: PCR, Polymerase Chain Reaction; UP-PCR, Uracil base Primer PCR; AWP, Arbitrary Walking Primer; SP, Site-specific Primer; oSP, outmost SP; mSP, middle SP; iSP, innermost SP; UNG, Uracil DNA glycosylase.

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non-targets can be easily diluted by the inner SP(s) in the next PCR(s), but how to dilute category iii has become an urgent issue to be solved in random PCR.[19–20]

To overcome category iii non-target amplicon prevailing in traditional random PCRs and thus improve walking specificity, we herein conceived a new method termed UP-PCR. The UP-PCR features adopting AWP with an uracil base at the penultimate position of the 3′ end. The primary UP-PCR product is processed with uracil DNA glycosylase (UNG) prior to secondary UP-PCR. This UNG processing removes the AWP attached to primary UP-PCR products. The category iii non-target, a stubborn product in traditional random PCRs, is thus readily diluted in the next nested UP-PCR(s); however, the amplification of target product can be still proceeded, because it has the exact binding sites for the both primers. The feasibility of UP-PCR was validated by exploring unknown flanks of several selected genes.[21–22]

2. Materials and methods

2.1. Preparation of genomic DNAs

The genome of *Levilactobacillus brevis* CD0817[22–26] was extracted from the cultured cells following the recommended protocol of TIANGEN Bacterial DNA Kit (TIANGEN Biotechnology Co., Ltd., Beijing, China). The genome of *Oryza sativa* was given by Dr. Peng Xiaojue at Nanchang University (Nanchang, China).

2.2. Design of primers

Three nested SPs (outmost SP, oSP; middle SP, mSP; and innermost SP, iSP) were sequentially chosen from each gene in the direction of 5′ to 3′. Three AWPs, having an uracil base at the penultimate position of the 3′ end, were also invented. All these primers showed a melting temperature of 60 – 65 °C, without an obvious structure of hairpin or self-dimer. And each primer pair did not form an obvious inter-primer dimer. The primers used in this experiment are given in Table 1.

2.3. UP-PCR procedure

In this study, a walking experiment included three parallel UP-PCR sets. An UP-PCR set included three sequential rounds of nested amplifications (primary, secondary, and tertiary UP-PCRs), which were performed by AWP pairing with a SP set of three SPs, respectively.

Primary UP-PCR, employing genome as template, was executed by AWP and oSP. Primary UP-PCR solution (25 μL) included 1 × T3 Super PCR Mix, 0.2 μM oSP, 0.2 μM AWP, and 10–100 ng bacterial genome or 100–1000 ng *Oryza sativa* genome. A three-stage cycling program was adopted for this UP-PCR amplification (Table 2).

Prior to secondary UP-PCR, 1 μL the primary UP-PCR product was treated with 1 U of UNG according to the protocol recommended in the UNG kit (Takara), to remove the attached AWP. Then the reaction solution was incubated at 95 °C for 10 min to inactivate UNG.

Table 2
PCR cycling conditions of UP-PCR.

UP-PCR round	Thermal conditions	Cycles
Primary	95 °C, 3 min	
	95 °C, 15 s; 60 °C, 15 s; 72 °C, 1 min	5 cycles
	95 °C, 15 s; 25 °C, 15 s; 72 °C, 1 min	1 cycle
	95 °C, 15 s; 60 °C, 15 s; 72 °C, 1 min	30 cycles
	72 °C, 5 min	
Secondary	95 °C, 3 min	
	95 °C, 15 s; 60 °C, 15 s; 72 °C, 1 min	About 30 cycles
	72 °C, 5 min	
Tertiary	95 °C, 3 min	
	95 °C, 15 s; 60 °C, 15 s; 72 °C, 1 min	About 30 cycles
	72 °C, 5 min	

Secondary UP-PCR, employing the UNG-treated product as template, was executed by AWP and mSP. Secondary UP-PCR solution (25 μL) included 1 × T3 Super PCR Mix, 0.2 μM mSP, 0.2 μM AWP, and 1 μL the UNG-treated product. The secondary UP-PCR cycling program consisted of thirty 60 °C (annealing temperature) cycles (Table 2).

Tertiary UP-PCR, employing the secondary UP-PCR product as template, was executed by AWP and iSP. Tertiary UP-PCR solution (25 μL) included 1 × T3 Super PCR Mix, 0.2 μM iSP, 0.2 μM AWP, and 1 μL the 100-fold diluted secondary UP-PCR product. The tertiary UP-PCR cycling program consisted of thirty 60 °C cycles (Table 2).

2.4. Analysis of UP-PCR product

The UP-PCR products were detected with agarose gel (1.0 %) electrophoresis as the method reported by Wang et al.[15] Then the gel was visualized with ChemiDoc XRS + imaging system (Bio-Rad Laboratories, Inc., California, USA). The clear secondary/tertiary UP-PCR DNA bands were extracted from the gel using TaKaRa agarose gel DNA extraction kit (Takara Bio Inc., Beijing, China). The extracted UP-PCR products were directly sequenced at Sangon Biotech Co., Ltd. (Shanghai, China). The sequencing data were analyzed with the function of "By Cluster W Method" in the MegAlign software.

3. Results

3.1. Principle and process of UP-PCR

The principle and process of UP-PCR is schematically illustrated in Fig. 1. An UP-PCR set includes three successive nested UP-PCRs. The key to UP-PCR method is the use of AWP containing an uracil base at the penultimate position of the 3′ end.

Primary UP-PCR is executed by AWP and oSP. The initial 60 °C cycles exclusively allow oSP to synthesize several target first strand DNAs. The subsequent 25 °C cycle encourages AWP to randomly anneal to genome and the target first strand, thus synthesizing many non-target DNAs and target second strand. This target second strand has AWP at the 5′ end and oSP complement at the 3′ end, thus can be exponentially boosted in the

Table 1
Primers designed in this work.

Target gene	SPs	AWPs
gadA	oSP: GGATGCTGCCTTCGGTGGGTTATTT	AWP1: CCGATGCCACTGCTGAGCTCT*UG*
	mSP: TGGTCACAAGTACGGCATGGTTTAC	AWP2: CCTGACCGCCTTCTACACCTGCA*UA*
	iSP: ACGGTTGACTCCATTGCCATTAACT	AWP3: TTCATCTCTCTTTCGCTGGCAC*UC*
gadR	oSP: ATCTCCATTTCCATAGGTTGCTCCA	
	mSP: GGATACTGGCTAAAATGAATTAACTCGGAT	
	iSP: TAGCCAACCGTAAACCTGCGTAAAA	
hyg	oSP: ATACACATGGGGATCAGCAATCGCG	
	mSP: CATCCATAGCCTCCGCGACC	
	iSP: CACGGCGGGAGATGCAATAGGT	

Note: An AWP is universal to the three sequential rounds of nested amplifications in an UP-PCR set. The three AWPs perform three parallel UP-PCR sets in a genome-walking experiment.

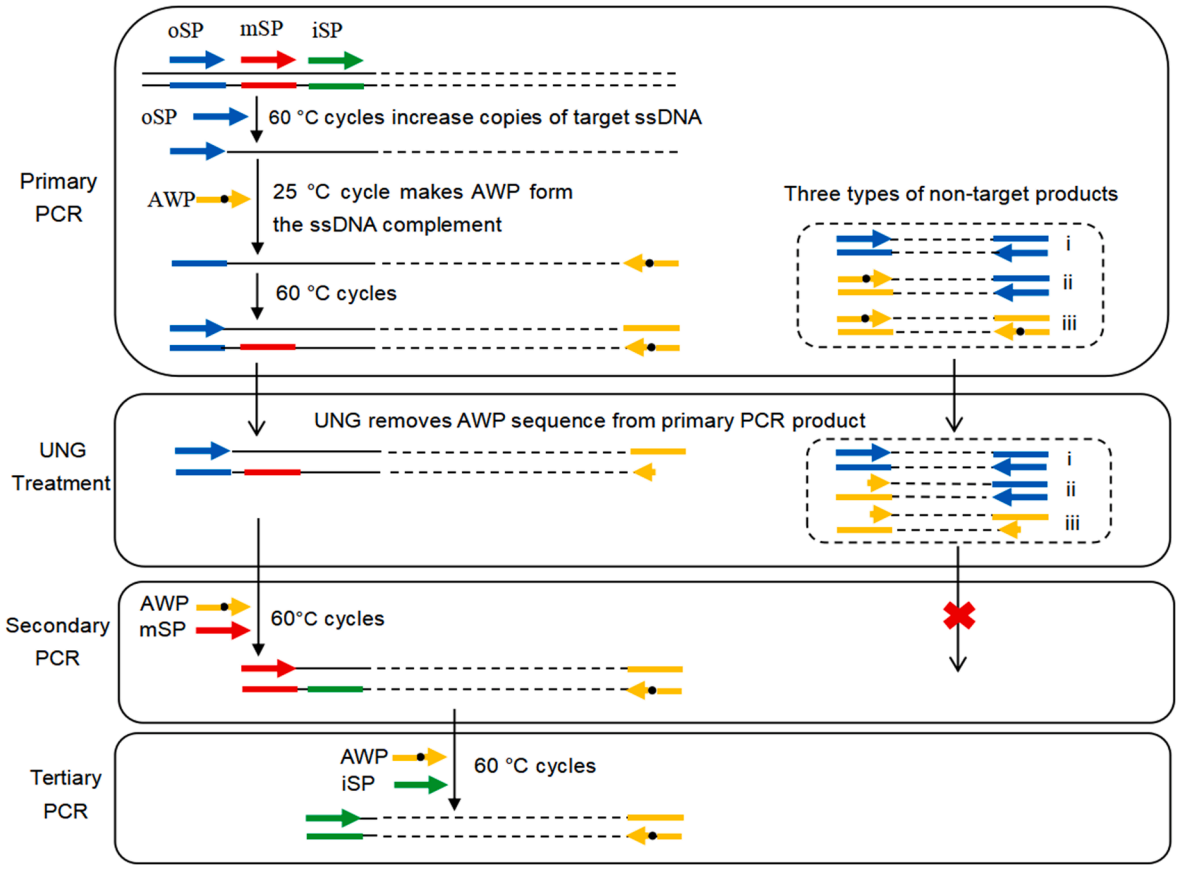

Fig. 1. Schematic diagram of UP-PCR. Thin solid line and thin dashed line represent known and unknown sequences, respectively. Colored arrows and bold lines represent primers and primer complements, respectively. Small black dot in AWP represents the uracil base. AWP, arbitrary walking primer; oSP, outmost site-specific primer; mSP, middle site-specific primer; iSP, innermost site-specific primer; and ssDNA, single-stranded DNA.

subsequent 60 °C cycles; any non-target DNA is bereft of chance being further amplified, owing to lacking an authentic site for any primer. However, three categories of non-targets will still be produced in this PCR, because of the 25 °C cycle plus the high complexity of genome. These non-targets include category i non-target produced by oSP alone, category ii produced by oSP plus AWP, and category iii produced by AWP alone. Among which, the target second strand, the second strand of category ii non-target, and the two strands of category iii non-target have the 5′ AWP.

Afterwards, UNG treatment is imposed on the primary UP-PCR product. After UNG treatment, the aforementioned four DNA strands lose the 5′ AWP. Conversely, the other DNA strands, including the target first strand, the first strand of category ii non-target, and the two strands of category i non-target, are not affected by UNG treatment.

Secondary UP-PCR with about thirty 60 °C cycles is executed by AWP and mSP, employing the UNG-treated product as template. The unaffected target first DNA strand is exponentially amplified, because it has the perfect binding sites for the two primers. Any strand of categories i and ii non-target DNAs cannot be amplified because it at least lacks the perfect binding site for mSP. Category iii non-target cannot be amplified either, as each strand of it is deprived of the 5′ AWP. Clearly, only the target primary product can be amplified in secondary UP-PCR.

Tertiary UP-PCR, like secondary UP-PCR, further selectively enriches the target product.

3.2. *Effects of diluting primary UP-PCR product on secondary UP-PCR*

To explore the effects of diluting primary UP-PCR product on secondary UP-PCR, the *gadR* gene was selected for the experiment. The primary UP-PCR product of *gadR* was diluted 0, 10, 100, 1000, and 10,000 times, respectively. Next, these dilutions were individually treated with UNG. The UNG-treated products were then used as templates of the secondary UP-PCRs, respectively. The secondary UP-PCR outcomes in Fig. 2 show that these dilutions exhibited almost the same amplification pattern, although the DNA band strength was

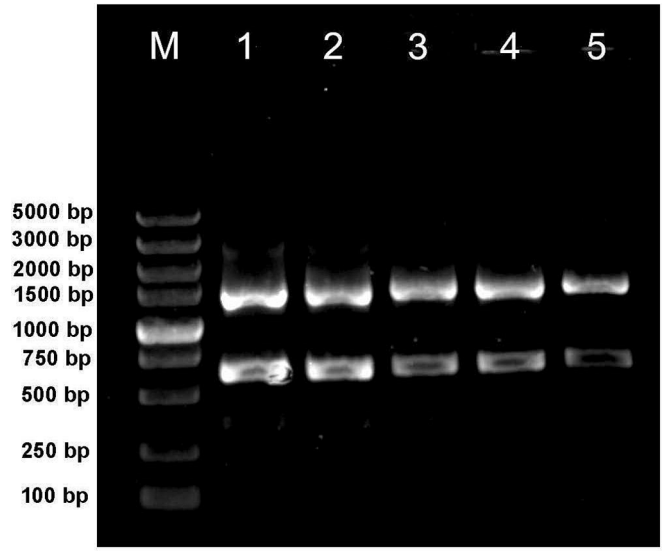

Fig. 2. Effects of dilution multiple of primary UP-PCR product on secondary UP-PCR. Lanes 1–5 are the secondary UP-PCR results of the 0, 10, 100, 1000, and 10,000 times diluted primary UP-PCR products, respectively.

gradually weakened with dilution multiple.

3.3. Suitable cycle number of secondary UP-PCR

The UNG-treated product of *gadR* underwent secondary UP-PCRs with twenty, twenty-five, thirty, thirty-five, and forty cycles, respectively. The secondary UP-PCR results are shown in Fig. 3. As shown, secondary UP-PCR amplification was somewhat insufficient when the cycle number was below 30, but more cycles could not further enhance the amplification after 30 cycles. Therefore, about 30 cycles are suggested to be performed in secondary UP-PCR.

3.4. Effects of diluting secondary UP-PCR product on tertiary UP-PCR

The secondary UP-PCR product of *gadR* was diluted 0, 10, 100, 1000 and 10,000 times, and then used as templates of the tertiary UP-PCRs, respectively, aiming to determine a dilution of secondary product suitable for tertiary amplification. Thirty cycles were carried out for each tertiary UP-PCR. As shown in Fig. 4, two DNA bands were obtained when the secondary product was diluted within 100 times, while only the small band was obtained when the secondary product was diluted 1000 times or more. A 0–100-fold dilution of secondary UP-PCR product is thus suggested to be used as template of tertiary UP-PCR.

3.5. Suitable cycle number of tertiary UP-PCR

To determine the suitable cycle number of tertiary UP-PCR, the 100-fold diluted secondary UP-PCR product was used as templates of the tertiary UP-PCRs with twenty, twenty-five, thirty, thirty-five, and forty cycles, respectively. The results in Fig. 5 show that almost the same amplification pattern was obtained with different cycle numbers. When the cycle number was less than 30, the UP-PCR amplification increased as cycle number; and when the cycle number reached 30, its further increase showed a marginal effect on the amplification. Therefore, about 30 thermal cycles were proper for tertiary UP-PCR.

According to the above data, the optimized UP-PCR process is given as follows: (i) primary UP-PCR begins with five 60 °C cycles, followed by one 25 °C cycle and about thirty 60 °C cycles; (ii) secondary UP-PCR is performed for about thirty 60 °C cycles, using 0–10,000-fold diluted UNG-treated primary UP-PCR product as template; and (iii) tertiary UP-PCR is performed for about thirty 60 °C cycles, using 0–100-fold diluted secondary UP-PCR product as template.

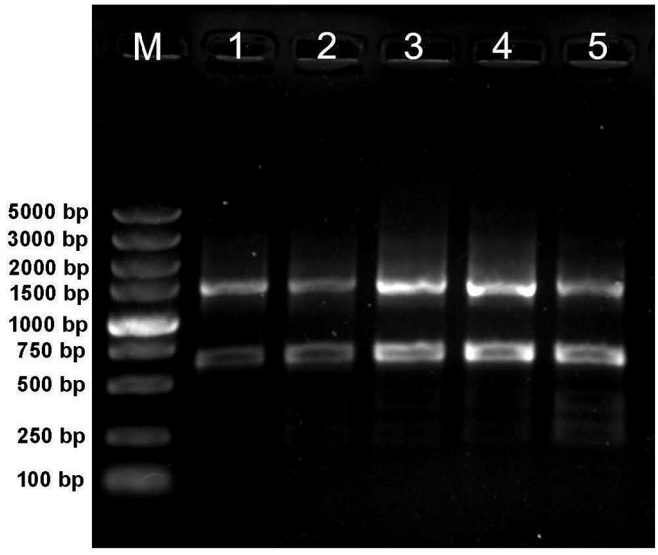

Fig. 3. Effects of cycle number on secondary UP-PCR. Lanes 1–5 are the results of the secondary UP-PCRs performing twenty, twenty-five, thirty, thirty-five, and forty cycles, respectively.

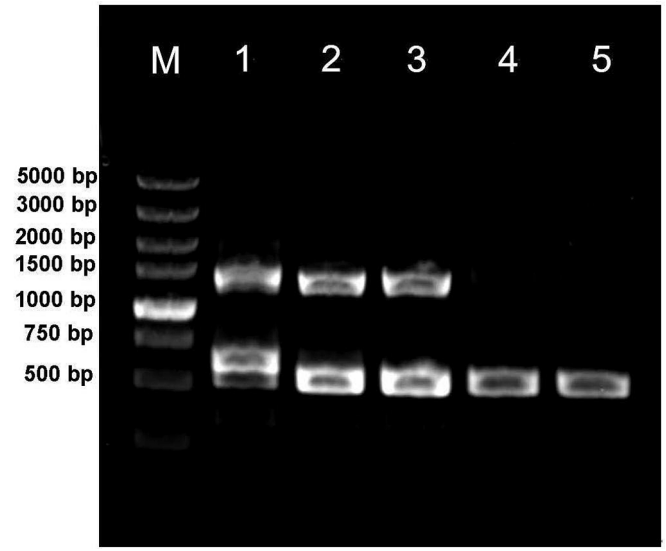

Fig. 4. Effects of secondary UP-PCR product dilution multiple on tertiary UP-PCR. Lanes 1–5 are the tertiary UP-PCR results of the 0, 10, 100, 1000, and 10,000 times diluted secondary UP-PCR products, respectively.

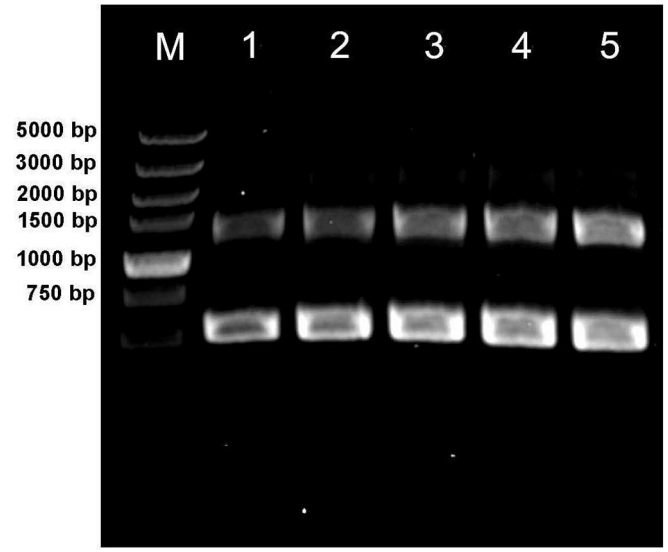

Fig. 5. Effects of number of cycles on tertiary UP-PCR. Lanes 1–5 are the results of the tertiary UP-PCRs performing twenty, twenty-five, thirty, thirty-five, and forty cycles, respectively.

3.6. Validation of UP-PCR

To test the performance of UP-PCR, it was used to walk the genes *gadA* (Fig. 6A) and *gadR* (Fig. 6B) in *L. brevis* CD0817 and gene *hyg* (Fig. 6C) inserted into *Oryza sativa*. Three nested SPs were chosen from each gene as described in Table 1 and Fig. 1. The three AWPs (Table 1) were individually paired with each SP set to perform three parallel sets of UP-PCRs. The UP-PCR products of these genes were separated with 1.0 % agarose gel electrophoresis, followed by being visualized with the gel imaging system. As shown in Fig. 6, one to two DNA band of prominence was produced in each secondary or tertiary UP-PCR. The DNA bands ranged in size from 200 to 5,000 bp. In addition, all the secondary/tertiary UP-PCRs showed a negligible background. These DNA bands were recovered and sequenced. The sequencing data

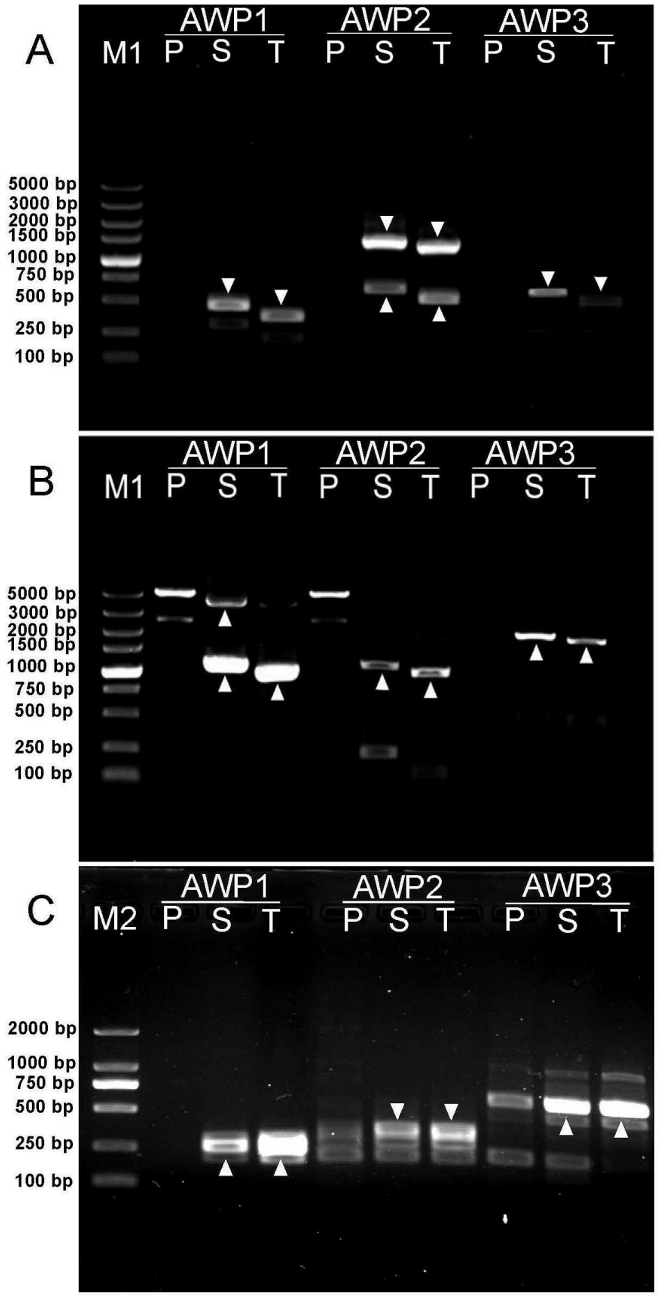

Fig. 6. Validation results of UP-PCR walking on *gadA* (A) and *gadR* (B) of *L. brevis* CD0817 and *hyg* (C) of *Oryza sativa*. AWP1, AWP2, and AWP3 denote the three parallel sets of UP-PCRs in a walking experiment. Lanes P, S, and T stand for primary amplification, secondary amplification, and tertiary amplification in an UP-PCR set, respectively. The clear target amplicons are indicated by white arrow heads. Lane M1 is TaKaRa DL 5,000 DNA Marker, Lane M2 is TaKaRa DL 2,000 DNA Marker.

indicated that the unknown flanks of these genes were successfully captured by UP-PCR.

4. Discussion

Genome walking has always been an important molecular tool in biology researches. A random PCR-based genome-walking technology is particularly preferred by scientific research communities, attributed to its straightforwardness and low cost.[27] With the in-depth research and continuous innovation to genome walking, various random PCR tools have been proposed, such as wristwatch PCR,[28] bridging PCR[17] and thermal asymmetric interlaced-PCR.[14]

To realize the walking mediated by AWP, these random PCRs have to perform at least one low annealing-temperature cycle in primary amplification reaction.[29] Because of this low annealing-temperature cycle plus the complexity of genome, three categories of non-target amplicons will be formed in primary amplification reaction, including those formed from SP alone (i), from SP plus AWP (ii), and from AWP alone (iii).[19,28,30] The former two categories of non-targets will be easily excluded by the inner SP(s) in the next nested PCR(s), as they lack a perfect annealing site for this primer.[18] The real challenge in a random PCR arises from the accumulation of category iii non-target.[28] The amplification specificity of existing random PCRs, however, basically depends on the differential amplification between target DNA and category iii non-target DNA. This differential amplification implies that non-target amplification is unavoidable in these PCRs.[31]

In order to fundamentally overcome category iii non-target product, we herein proposed a new genome-walking tool UP-PCR. In this tool, we introduce an uracil base at the penultimate position of AWP 3′ end. After UNG treatment, the AWP, being attached to category iii non-target product, is broken.[29–33] Clearly, the UNG-treated category iii non-target cannot be amplified in the next nested PCR(s). In this study, the amplification specificity of UP-PCR has been well demonstrated by walking the three selected genes.[21–22] As shown in Fig. 6, the non-target background was negligible in UP-PCR. However, the non-target background of an existing random PCR is typically not negligible as it depends on the aforementioned differential amplification.[34–36] Therefore, UP-PCR has a higher amplification specificity than the existing random PCRs.

Our genome-walking results also showed that two successive rounds of nested UP-PCRs suffice to get a positive outcome, possibly attributed to the effective elimination of category iii non-target product by UNG.[37] However, many traditional PCRs require three rounds of amplifications to get a positive outcome. As stated above, category iii non-target DNA is inevitably amplified in each round of PCR in a traditional PCR. Therefore, to obtain a sufficient amplification specificity, a traditional method has to increase PCR rounds.[38–39] The recently reported uracil base PCR uses uracil base instead of thymine base as one of the extension substrates, but still uses a traditional AWP.[37] Conversely, UP-PCR uses the four normal bases as the extension substrates, but uses AWP containing an uracil. Accordingly, UP-PCR omits the two experimental steps mandatory in uracil base PCR. These two steps are the single-cycle amplification and the subsequent purification of the resultant amplification product.[37] Therefore, UP-PCR shows a simpler experimental process than those traditional PCRs.[16–17]

The successful random annealing of AWP to unknown flank is the prerequisite for succeeding genome walking. A random PCR generally adopts a low annealing-temperature cycle to realize such an annealing.[2,30] The current method contains one 25 °C cycle in primary UP-PCR. At 25 °C, we suspect that AWP will easily find out a place(s) suitable for its partial annealing on unknown region, and then extend towards known region, thus synthesizing a target DNA strand.[40–41] This strand is a perfect template for UP-PCR amplification. UP-PCR can generally succeed once such a target DNA strand is synthesized. From this point of view, UP-PCR shows an equal success rate to most random PCRs, such as wristwatch PCR,[25] fork PCR,[13] and thermal asymmetric interlaced-PCR.[14]

Resemble to other random PCRs, the walking ability of UP-PCR is generally unpredictable because it depends on the random annealing of AWP on unknown flank.[35–36] Due to this unpredictability, a random genome-walking method typically adopts a strategy of parallel PCRs to ensure walking ability.[38–39] In specific, these parallel PCRs are performed by AWPs with different sequences, respectively. The AWPs can randomly anneal to different positions on unknown flank, and thus generate DNA bands with different sizes. In practical applications, only the largest DNA band needs to be considered.[36,38] UP-PCR also adopts the strategy of parallel PCRs. In this study, the largest DNA band

obtained by UP-PCR reached up to 5.0 kb, a walking ability equal to that of the existing methods.

5. Conclusion

Herein, an efficient but accurate UP-PCR genome-walking approach was constructed. The UP-PCR mainly relies on AWP containing an uracil base and the UNG treatment after primary PCR amplification. As a result, the amplification of non-target products associated with AWP is inhibited, ensuring the amplification specificity of this approach. In general, secondary UP-PCR amplification can generate a positive walking outcome. The performance of UP-PCR has been confirmed by walking three selected genes. Overall, the proposed UP-PCR is a promising candidate method for performing genome walking. However, the current UP-PCR still includes an enzymatic cleavage step. In the future, efforts should be made to construct a random PCR genome-walking approach that is completely free of enzymatic cleavage step.

6. Data and code availability

The datasets about the genes *gadA* and *gadR* in *L. brevis* CD0817 and gene *hyg* inserted into *Oryza sativa* presented in this study can be found online at: https://www.ncbi.nlm.nih.gov/genbank/, AYM03982.1; https://www.ncbi.nlm.nih.gov/genbank/, KF206149.

CRediT authorship contribution statement

Hong Chen: Writing – review & editing, Writing – original draft, Visualization, Investigation. **Bingkun Tian:** Visualization, Investigation. **Rongrong Wang:** Data curation. **Zhenkang Pan:** Formal analysis. **Dandan Gao:** Writing – review & editing, Conceptualization. **Haixing Li:** Writing – review & editing, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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