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Communication

Biomarker Discovery for Detecting the Seed Ageing Degree and Priming Effect of Tobacco

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Abstract: Seed ageing is an important issue for the long-time seed storage of tobacco. Seed priming has been popularly applied in tobacco production. In this study, the development of molecular marker genes encoding proteins L-isoaspartyl methyltransferase NtPIMT1 and 8-oxoG DNA glycosylase 1 NtOGG1 to detect the degree of seed ageing and the effect of seed priming is conducted in tobacco. Quantitative real-time PCR (qRT-PCR) analysis reveals that relatively higher mRNAs of *NtPIMT1* and *NtOGG1* are observed in the dry and early germinating seeds. The expressions of *NtPIMT1* and *NtOGG1* are negatively correlated with the degree of seed damage in non-pelleted and pelleted seeds after accelerated ageing treatments. The early best effects of gibberellin (GA_3) priming on speed and uniform germination are observed in 33 h primed seeds, and relatively lower expressions of *NtPIMT1* and *NtOGG1* are observed in priming seeds. *NtPIMT1* and *NtOGG1* genes have potential for use as molecular markers in detecting the seed ageing degree and priming effect of tobacco.

Keywords: tobacco; seed ageing; seed priming; molecular marker

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1. Introduction

Tobacco (*Nicotiana tabacum* L.) is one of the most important crops in Yunnan Province in China. Seed ageing results in slow germination and poor germination uniformity, and eventually leads to the loss of seed viability [1,2]. Meanwhile, seed ageing tolerance is an important trait for the long-term storage of germplasm resources. Seed ageing is a critical issue to maintain seed viability during storage in tobacco. Therefore, the identification of molecular biomarkers for the evaluation of potential seed storability is important in tobacco production.

The accumulation of isoaspartyl (isoAsp) residues in seeds due to seed ageing has been indicated to adversely influence seed vigor and longevity [3–5]. The L-isoaspartyl methyltransferase (PIMT) is a protein-repairing enzyme that involves the conversion of abnormal L-isoaspartyl residues to normal L-aspartyl forms. It has been well reported that PIMT is involved in the regulation of seed vigor and longevity in plants. In *Arabidopsis*, the overexpression of *PIMT1* can reduce detrimental isoAsp residues in seed proteins, and then increase seed vigor and longevity [4]. In rice, the suppression of *OsPIMT1* causes the excessive accumulation of isoAsp and accelerates the loss of seed vigor [6]. However, little is known about the expression of tobacco *NtPIMT* during the course of seed germination and seed storage, particularly whether it can be used as a biomarker to evaluate the degree of seed ageing.

Reactive oxygen species (ROS) are major sources to induce DNA damages that cause seed ageing [7]. The repair of DNA damage during the initial imbibition is required for seed germination [8,9]. The 8-oxoG DNA glycosylase 1 (OGG1) protein is a bifunctional DNA glycosylase/DNA apurinic (AP) lyase that excises 8-oxo-G and cleaves DNA at the 3′-side

of the resulting AP site [10,11]. It has been revealed that the overexpression of *Arabidopsis AtOGG1* promotes seed longevity by reducing DNA damage in the dry seeds during storage, and by increasing the reparation of DNA lesions during germination [12]. Meanwhile, the expression of *MtOGG1* has also been reported to be involved in the oxidative stress response during seed imbibition in the model plant *M. truncatula*, when DNA repair is activated to withstand ROS injury [13]. The biochemical markers for detecting the degree of seed ageing have been proposed in plants [14–17]. However, the expressions of tobacco *NtOGG1* during seed germination and seed storage are still unclear, and it is interesting to reveal whether it can be used as a biomarker to evaluate the degree of seed ageing.

Meanwhile, seed priming is widely used to improve seed survival during storage [18] and seed vigor [19,20]. Seed priming is an important technology that allows seed imbibition while preventing radicle emergence [19]. Thus, the correct time-point of stop imbibition is an important issue to ensure priming effect. Usually, the assay of seed germination is used to evaluate the priming effect; however, the phenotype is largely influenced by the genotype, seed lot, and the germination conditions. The development of molecular biomarkers is helpful to choose the best time-point to stop priming [21]. To determine whether tobacco *NtPIMT1* and *NtOGG1* transcripts can be used as biomarkers for detecting the seed ageing degree and priming effect, their expressions are investigated during seed germination, seed ageing, and seed priming processes in this study. Our results provide novel insight into the practical applications of *NtPIMT1* and *NtOGG1* as molecular markers to determine the degree of seed ageing and the effect of seed priming in tobacco.

2. Materials and Methods

2.1. Plant Materials

The common cultivar 'Yunyan 87' of tobacco provided by Yuxi Zhongyan Seed Company Ltd. (Yuxi, China) was used in this study. The mature seeds were harvested and dried to a moisture content of approximately 4.5% in April of 2018, and then seeds were stored at 20 °C with 40% relative humidity conditions. After that, the seeds were used for the treatments of seed pelleting, accelerated seed ageing, and seed priming in June of 2021.

2.2. Accelerated Seed Ageing Treatment

The pelleted seeds were produced by Yuxi Zhongyan Seed Company Ltd. according to the Practice Rules of Tobacco Seed Pelleting in China (GB/T 24308-2019). To accelerate the effect of ageing treatment, the seeds were placed in a Petri dish with filter papers and 50 mL distilled water was added according to Konzen et al. [22] with minor modifications. Then, seeds were subjected to a standardized accelerated ageing treatment of 45 °C with 100% relative humidity for 1 and 3 days for pelleted seeds and 5 and 7 days for non-pelleted seeds. After that, seeds were dried to their original moisture content (approximately 4.5%) at 25 °C conditions. Unaged seeds were used as the control. Three biological replicates were conducted.

2.3. Seed Priming

Non-pelleted seeds were used for priming treatment according to the Practice Rules of Tobacco Seed Priming in China (GB/T 24308-2019). Seeds were placed in a container with 50 mg/L GA_3 and were incubated at 25 °C for 3, 9, 15, 21, 27, 33, and 39 h. After that, the imbibed seeds were harvested and dried to their original moisture content (approximately 4.5%). Unprimed dry seeds were used as the control. Three biological replicates were conducted.

2.4. Seed Germination

Thirty seeds per replicate were imbibed in Petri dishes (diameter 9 cm) with two filter papers and 10 mL distilled water was added at 25 °C for aged seeds and 15 °C for primed seeds for 12 days. Seeds were considered as germinated when the radicle protruded through the seed coat. The percentage of germinated seeds at 5 d was referred to as

germination potential. Germination index (GI) was calculated as follows: $GI = \Sigma\ (Gt/t)$, where Gt is the number of the germinated seeds on day t [23]. Three biological replicates were conducted.

2.5. Identification of NtPIMT1 and NtOGG1 in Tobacco

The protein sequences of PIMT and OGG1 of rice and *Arabidopsis* were obtained from NCBI (https://www.ncbi.nlm.nih.gov/; accessed on 1 February 2021) and TAIR (https://www.arabidopsis.org/; accessed on 1 February 2021), respectively. The genome of tobacco (*Nicotiana tabacum* L.) was searched through the blastp function of NCBI, and then the homologous candidate PIMT and OGG1 proteins of tobacco were obtained. After removing the redundant gene sequences and sequences without typical domains, we performed a Conserved Domains (CD) (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; accessed on 1 February 2021) and pfamscan (https://www.ebi.ac.uk/Tools/pfa/pfamscan/; accessed on 1 February 2021) search, and then the genes that were preserved were considered as tobacco PIMT and OGG1. The sequence similarities of amino acids among species were investigated using DNAMan. Evolutionary history was constructed by MEGA6. Briefly, all sequences were aligned by ClustalW with the default parameters, and the tree was created using the neighbour-joining method with the pairwise deletion option. Poisson correction and bootstrap analysis were conducted with 1000 replicates.

2.6. Expression Analysis

The non-pelleted seeds of each treatment were harvested in liquid nitrogen for RNA extraction. For pelleted seeds, they were firstly packed in a piece of gauze, and their pelleted materials were quickly washed and removed by water. Then, the seeds were harvested for RNA extraction in liquid nitrogen. Total RNA was extracted using the TransZol Plant kit (Transgen, www.transgen.com; accessed on 5 July 2021). The first-strand cDNA was synthesized with random oligonucleotides using the HiScriptII Reverse Transcriptase system (Vazyme Biotech Co., Ltd.). The qRT-PCR conditions were as follows: 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. The *NtActin* gene was used as internal control. All the primers were designed through the primer designing tool of NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast; accessed on 5 July 2021). Primers of *NtPIMT* (TGAGGGTACTCCCGCTTACA and TGTGGTCCAACCATCAGAGC), *NtOGG1* (TATTGTTGGCACGGTGGAGG and GAGCAATACATGCTGCGACC) and *NtActin* (TGAGATGCACCACGAAGCTC and CCAACATTGTCACCAGGAAGTG) were used. Normalized transcript levels were calculated using the comparative $2^{-\Delta\Delta C}{}_{T}$ method [24]. Three biological replicates were conducted.

2.7. Data Analysis

The significant differences between samples were compared using one-way analysis of variance (ANOVA) with the least significant difference (LSD) test based on the 1% level (p values < 0.01).

3. Results

3.1. Identification of NtPIMT and NtOGG1 in Tobacco

Two *NtPIMT* and one *NtOGG1* genes were identified from tobacco (Figure 1a). The ORF lengths of *NtPIMT1* and *NtPIMT2* were 894 and 693 bp, respectively, coding 293 and 231 amino acids (AA) with the molecular weights (MW) of 74.37 and 56.48 kDa. The ORF length of *NtOGG1* was 1176 bp, encoding 392 AA with the MW of 98.88 kD. The assay of amino acid sequence similarities showed that the sequence of NtPIMT1 was mostly similar to AtPIMT1 with 70.3% similarity (Figure 1b), and the sequence of NtOGG1 was mostly similar to AtOGG1 with 63.0% similarity (Figure 1d). The evolutionary analysis also confirmed that tobacco genes were more similar to *Arabidopsis* genes (Figure 1c,e). Meanwhile, a high similarity of amino acid sequences was observed between NtPIMT1 and NtPIMT2, and then the genes *NtPIMT1* and *NtOGG1* were focused on in the following investigation.

(a)

Name	Gene	ORF (bp)	AA	GC (%)	MW (kDa)	pI
NtPIMT1	LOC107790471	894	293	46.2	74.37	4.88
NtPIMT2	LOC107787075	693	231	46.46	56.48	5.00
NtOGG1	LOC107760687	1176	392	44.67	98.88	4.83

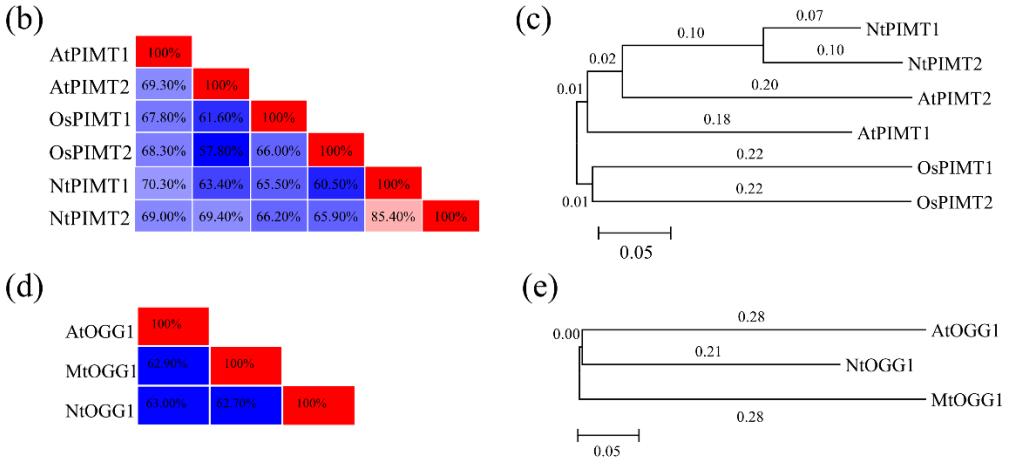

Figure 1. Characteristics of amino acid sequences of PIMT and OGG1 genes. (**a**) The basic information of *NtPIMT* and *NtOGG1* genes. Sequence similarity analysis of (**b**) PIMT and (**d**) OGG1 genes using DNAMan. Colour scale represents sequence similarity. Phylogenetic tree of (**c**) PIMT and (**e**) OGG1 genes using the neighbour-joining method with default parameters. Abbreviations: ORF—open reading frame; AA—amino acids; MW—protein molecular weight; pI—protein isoelectric point.

3.2. Expression Patterns of NtPIMT1 and NtOGG1 during Early Seed Germination

The radicle protrusion started as early as about 48 h at the imbibition stage in unaged and non-pelleted seeds of tobacco. In order to quickly detect the expressions of *NtPIMT1* and *NtOGG1*, their expression patterns were firstly investigated during the early germination stage (0–12 h) using unaged and non-pelleted seeds. qRT-PCR analysis showed that the expression of *NtPIMT1* gradually decreased during seed germination, and it had a relatively higher expression during 0 to 2 h imbibition stage (Figure 2a). The expression of *NtOGG1* was significantly induced at the initial imbibition stage (0 to 3 h) and decreased after 3 h imbibition with a relatively lower expression (Figure 2b). These results indicate that relatively higher mRNAs of *NtPIMT1* and *NtOGG1* exist in dry and early germinating seeds. Thus, the expressions of *NtPIMT1* and *NtOGG1* were mainly investigated in the dry or early germinating seeds subject to ageing conditions.

3.3. Expression Patterns of NtPIMT1 and NtOGG1 in Non-Pelleted Seeds after Artificial Ageing Treatments

To determine the relationships between *NtPIMT1* and *NtOGG1* expression and the degree of seed damage, the non-pelleted seeds were firstly used for artificial ageing treatments. Significantly lower germination potential and germination index were observed in seeds after 5 and 7 days of artificial ageing treatments compared to those in unaged seeds (0 d; CK) (Figure 3a–c). qRT-PCR results showed that no significant correlations between the expressions of *PIMT1* and *NtOGG1* in dry seeds and ageing degree were observed; however, the mRNA levels of *NtPIMT1* and *NtOGG1* in germinating seeds (9 h imbibition) gradually and significantly increased with the increasing degree of seed ageing (Figure 3d,e). These results suggest that the detection of *NtPIMT1* and *NtOGG1* expression in germinating seeds might be used to evaluate the degree of seed ageing in non-pelleted seeds.

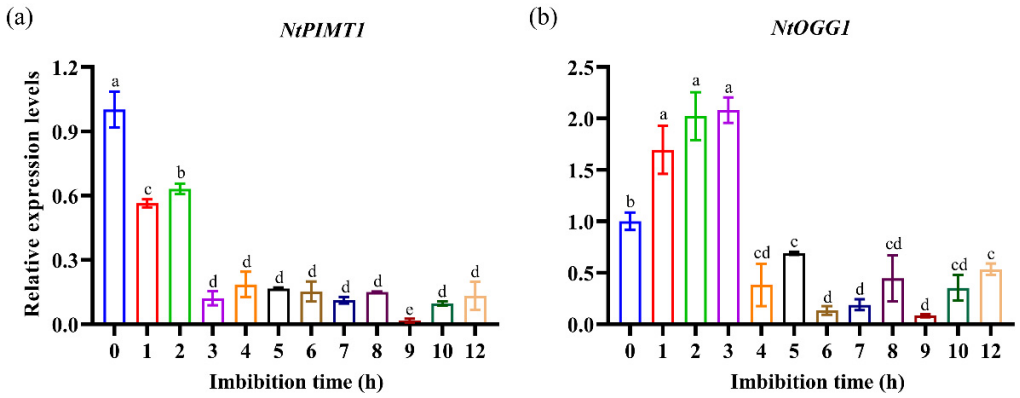

Figure 2. Characteristics of *NtPIMT1* and *NtOGG1* expression during seed germination in unaged and non-pelleted seeds of tobacco cultivar 'Yunyan 87'. Expression patterns of (**a**) *NtIPMS1* and (**b**) *NtOGG1* during early seed germination in 25 °C conditions. Data are means (±SD), $n = 3$. Different letters above the column indicate significant differences determined using ANOVA test: $p < 0.01$.

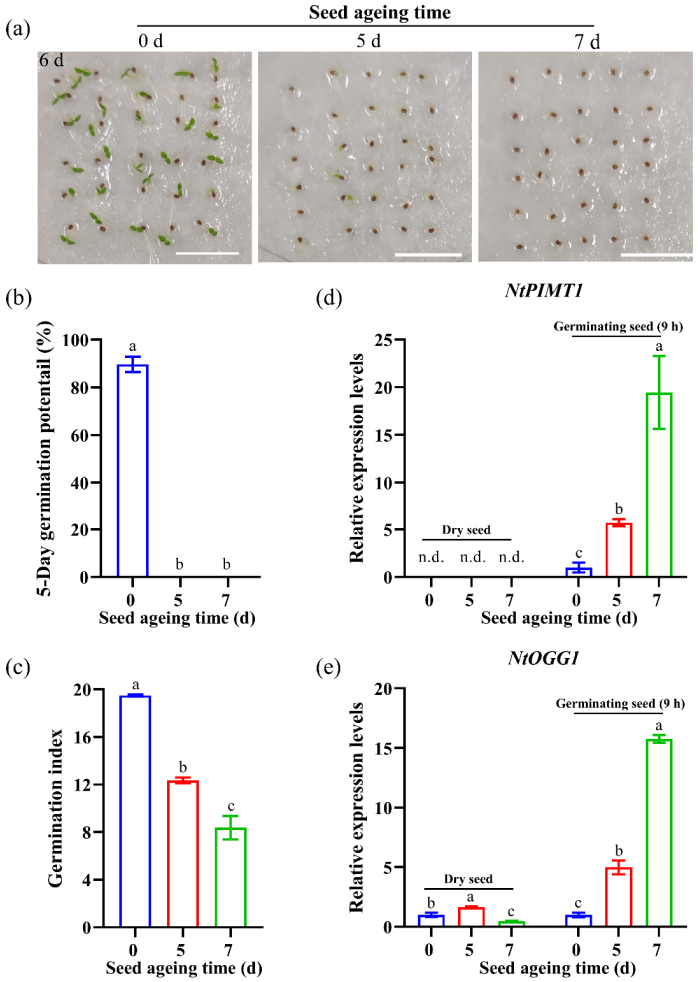

Figure 3. Characteristics of seed vigour and *NtPIMT1* and *NtOGG1* expression in non-pelleted seeds of tobacco cultivar 'Yunyan 87' after artificial ageing treatments. (**a**) Representative images of germination at 6 d under 25 °C conditions in seeds after 0- (CK), 5- and 7-day ageing treatments. Scale bars = 10 mm. (**b**) Germination potential; (**c**) germination index. Expression patterns of (**d**) *NtIPMS1* and (**e**) *NtOGG1* in dry and germinating (9 d) seeds. Data are means (±SD), $n = 3$. Different letters above the column indicate significant differences determined using ANOVA test: $p < 0.01$.

3.4. Expression Patterns of NtPIMT1 and NtOGG1 in Pelleted Seeds after Artificial Ageing Treatments

To further determine the correlations between *NtPIMT1* and *NtOGG1* expression and the degree of seed damage, the pelleted seeds were further used for artificial ageing treatments. Significantly lower germination potential and germination index were observed in seeds after 1 and 3 days of artificial ageing treatments compared to those in unaged seeds (0 d; CK) (Figure 4a–c). qRT-PCR results indicated that the mRNA levels of *NtPIMT1* and *NtOGG1* in dry seeds gradually and significantly increased with the increasing degree of seed ageing (Figure 4d,e). These results suggest that the detection of *NtPIMT1* and *NtOGG1* mRNAs in dry seeds might be used to test the degree of seed damage in pelleted seeds.

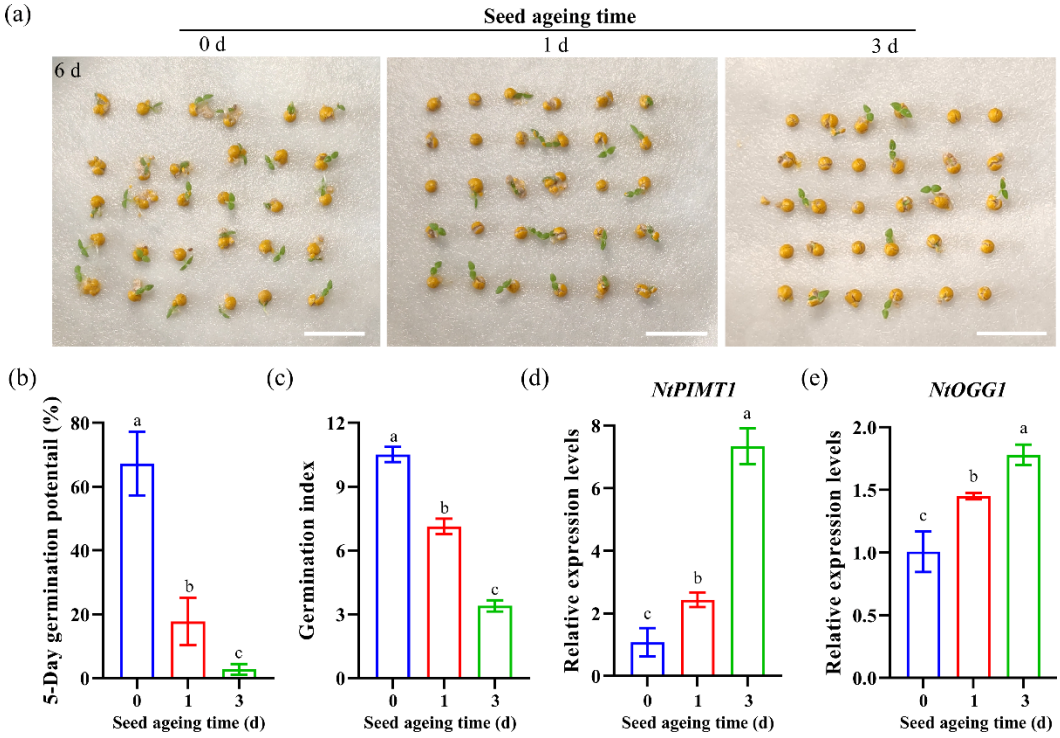

Figure 4. Characteristics of seed vigour and *NtPIMT1* and *NtOGG1* expression in pelleted seeds of tobacco cultivar 'Yunyan 87' after artificial ageing treatments. (**a**) Representative images of germination at 6 d under 25 °C conditions in seeds after 0- (CK), 1-, and 3-day ageing treatments. Scale bars = 10 mm. (**b**) Germination potential; (**c**) germination index. Expression pattern of (**d**) *NtPIMT1* and (**e**) *NtOGG1* in dry seeds. Data are means (±SD), $n = 3$. Different letters above the column indicate significant differences determined using ANOVA test: $p < 0.01$.

3.5. Expression Patterns of NtPIMT1 and NtOGG1 in Primed Seeds

To further determine whether *NtPIMT1* and *NtOGG1* expression can be used as molecular markers for detecting the priming effect in tobacco, the different duration of GA_3 priming treatments were conducted using non-pelleted seeds. Based on the traits of speed and uniform germination, i.e., germination potential and germination index, the early best priming effects were observed in seeds after 33 h priming treatment under 15 °C conditions (Figure 5a–c). qRT-PCR analysis showed that the relatively lower mRNA levels of *NtPIMT1* and *NtOGG1* were observed in seeds after 27 h priming (Figure 5d,e). These results suggest that the best stop time-point of seed priming might be at the 33 h stage when relatively lower *NtPIMT1* and *NtOGG1* expressions are detected in seeds.

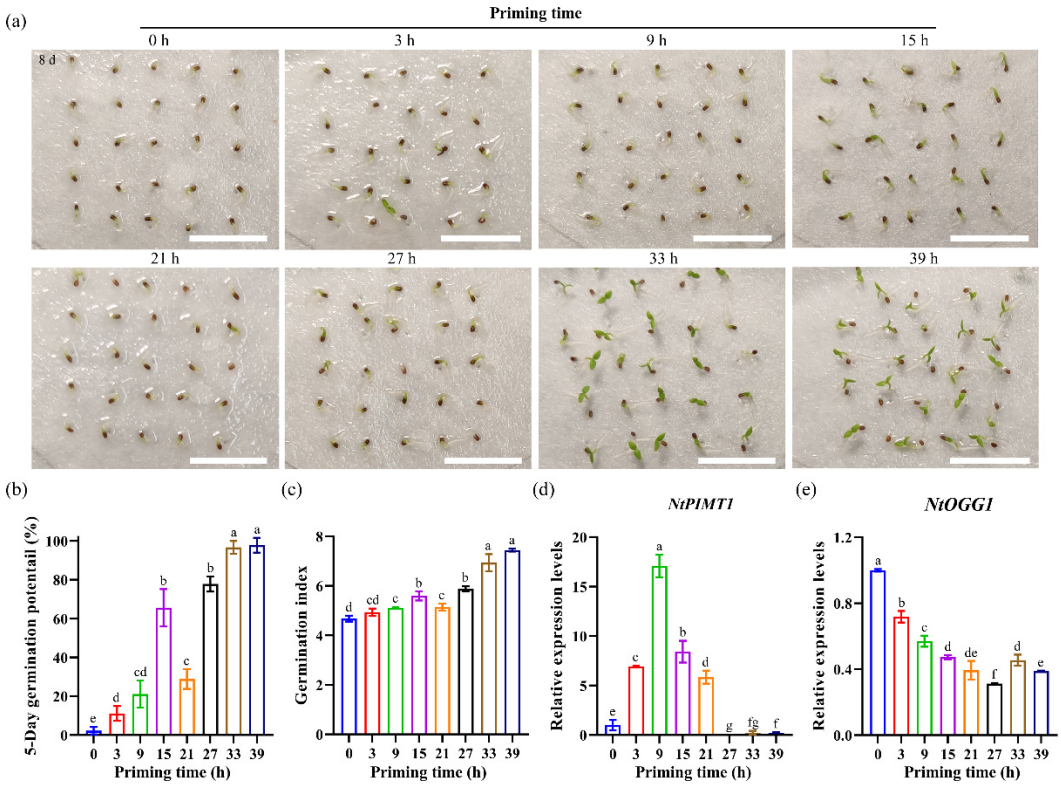

Figure 5. Characteristics of seed vigour and *NtPIMT1* and *NtOGG1* expression in non-pelleted seeds of tobacco cultivar 'Yunyan 87' after priming treatments. (**a**) Representative images of germination at 8 d under 15 °C conditions after 0 (CK), 3, 9, 15, 21, 27, 33, and 39 h GA_3 priming treatments. Scale bars = 10 mm. (**b**) Germination potential; (**c**) germination index. Expression pattern of (**d**) *NtPIMT1* and (**e**) *NtOGG1* in priming seeds. Data are means (±SD), $n = 3$. Different letters above the column indicate significant differences determined using ANOVA test: $p < 0.01$.

4. Discussion

Seed ageing usually occurs under high temperatures and humid conditions, which cause the loss of seed viability [1,25]. The rapid evaluation of seed ageing degree is important for seed storage in tobacco. Imbibition is the first step of seed germination, in which the quiescent embryonic cells shift into a metabolically active state [26]. The genes *PIMT* and *OGG1* have been used as biomarkers of seed quality in plants. In this study, we observed higher mRNAs of tobacco *NtPIMT1* and *NtOGG1* in the dry and early germinating seeds, suggesting these two genes might be used to test seed quality by detecting their mRNA levels in dry and early germinating seeds. Thus, the applications of *NtPIMT1* and *NtOGG1* as biomarkers for detecting the degree of seed ageing and the effect of seed priming were mainly investigated in this study.

PIMT is a protein-repairing enzyme that is involved in the seed vigour and longevity of *Arabidopsis* and rice [4–6]. The accumulated DNA damage during seed storage is repaired during seed imbibition [27,28]. The overexpression of *Arabidopsis OGG1* increases ageing tolerance via the decrease in 8-oxoG levels upon seed imbibition [12]. In this study, the biomarkers of *NtPIMT1* and *NtOGG1* for detecting the degree of seed damage after accelerated seed ageing treatments were investigated in tobacco. Using a rapid ageing treatment, we produced non-pelleted and pelleted seeds with different traits of seed vigour such as germination potential and germination index. The associations between the seed ageing damage and *NtPIMT1* and *NtOGG1* expressions in aged seeds were established; generally, high mRNA levels of *NtPIMT1* and *NtOGG1* were associated with lower seed vigour in aged seeds. These results suggest that the detection of *NtPIMT1* and *NtOGG1*

transcripts might be used as molecular markers for testing the degree of seed ageing in tobacco.

Molecular markers such as *OsIPMS1* have been explored to control the priming process in rice [29,30]. In this study, the genes *NtPIMT1* and *NtOGG1* as biomarkers to detect priming effect were investigated in tobacco. We produced seeds with different seed vigour by GA_3-priming treatments in tobacco. More rapid and uniform seed germination were observed in seeds after the GA_3 priming of tobacco in this study. Based on the phenotype of rapid and uniform seed germination, the best effects of GA_3 priming were observed early in seeds after 33 h priming treatment, when the relatively lower expressions of *NtPIMT1* and *NtOGG1* were observed. We assumed that the best stop time-point of seed priming was likely at the time when the relatively lower *NtPIMT1* and *NtOGG1* expression occurred in seeds during the priming process. The application of *NtPIMT1* and *NtOGG1* as biomarkers to determine the best time-point to stop priming needs to be further investigated by using more priming treatments in various varieties.

5. Conclusions

In summary, *NtPIMT1* and *NtOGG1* might be used as potential biomarkers to detect seed ageing degree and priming effect in tobacco. It is of great significance to rapidly and accurately evaluate seed ageing degree and priming effect by detecting the gene expression in the dry seeds or early germinating seeds in tobacco. Future studies should improve the reliability of rapid testing using different seed lots and varieties of tobacco.

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