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Full Length Article

Changes in Viability of Lincang Hulled Wheat Seeds under Different Storage Temperatures and Changes in *Lipoxygenase* (*LOX*) Gene Activity and Expression Levels during Germination

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Abstract

This study investigated the changes in the viability indices (such as germination force, germination percent, weight germination index, seedling vigor index) of Lincang hulled wheat seeds after storage at different temperatures for different periods and determined the variations in the *lipoxygenase* (*LOX*) activity and the relative expression level (REL) of *LOX* gene. Lincang hulled wheat seeds were sealed in aluminum foil bags (10 cm ×15 cm) and then stored in the gene bank at low-temperature below 0°C (-7.5±2.5°C, LT1), at low-temperature above 0°C (7.5±2.5°C, LT2), at the indoor temperature (ambient temperature, AT) and at high temperature (40±1°C, HT) for 63, 125, 189, 210, 252 and 365 days. The viability indices including the *LOX* activity and the REL of *LOX* genes were then measured through germination test, spectrophotometer and quantitative real time polymerase chain reaction (qRT-PCR), respectively. The ANVOA, relativity and stepwise regression in SPSS software were carried out to analyze the data. The results indicated that viability indices of Lincang hulled wheat seeds after storage at LT1 and LT2 for 365 days showed no significant drop compared with seeds after storage at LT1 and LT2 for 252 days. The viability indices of seeds after storage at AT for 365 days were decreased significantly. After storage at HT for 14 d, all viability indices were significantly dropped compared with that were stored for 7 days. The *LOX* activity of germinated Lincang hulled wheat seeds as well as the relative expression level of *LOX* genes in leaves had a significant influence on the storage period of seeds. A higher *LOX* activity and a higher relative expression level of *TaLOX2-5DL* gene could lead to a short time storage of seeds at LT1; a higher relative expression level of *TaLOX3-4A* led to a shorter time storage at LT2 and AT; and a higher *LOX* activity led to a shorter storage period of seeds at HT.

Keywords: Lipoxygenase activity; Hulled wheat; Relative expression level; Viability indices

Introduction

Lipoxygenases (LOXs) are traditionally accepted as seed storage proteins in plants, although they have been reported to be involved in pathogen defense of plant seeds. LOXs play an important role in grain storage, seed vigor and bread-making (Feng *et al.*, 2012). As key enzymes, they can catalyze the oxidation of polyunsaturated fatty acids (Dong *et al.*, 2015) with molecular oxygen, resulting in the formation of unstable hydroperoxides (Leenhardt *et al.*, 2006) and accumulate in the seeds (Dong *et al.*, 2015). Therefore, LOX proteins constitute an important class of lipid-hydrolyzing enzymes (Feng *et al.*, 2010).

Lipid degradation has been reported to be one of the reasons causing seed deterioration during storage and finally leading to embryo death. Seed viability is a very important criterion for long-term storage of seed or germplasm resources in plant (Dong *et al.*, 2015; Sun *et al.*, 2017). A negative relationship between LOX activity and seed longevity has been observed in maize and rice (Li *et al.*, 2007; Long *et al.*, 2013; Dong *et al.*, 2015). After storage at 42°C under relative humidity of 84% for 15 days, the maize varieties which lacked *LOX-1* and *LOX-2* genes showed a slight change in germination, but those with *LOX-1* and *LOX-2* genes showed a significant decline in germination, suggesting that *LOX-1* and *LOX-2* are a decisive factor in influencing seed longevity (Li *et al.*, 2007; Dong *et al.*, 2015). Moreover, the influence of expression levels of *LOX* genes on seed longevity has been observed in rice. The results indicated that rice lines over-expressing *LOX2* gene

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showed lower seed viability, while RNAi of *LOX2* enhanced seed longevity after accelerated aging (Huang *et al.*, 2014). A decrease in seed germination rate was observed to be accelerated in rice with over-expression of *LOX3* genes when the seeds were routinely stored (Long *et al.*, 2013).

LOX is one of the most important factors affecting the storage of wheat grain (Zhang *et al.*, 2015). Low *LOX* activity or *LOX* gene deletions can effectively decrease lipid oxidation, thereby reducing grain oxidative deterioration and extending the storage time (Leenhardt *et al.*, 2006; Zhang *et al.*, 2015). The *LOX* activity varies among different wheat species. For instance, the *LOX* activity is 3 times lower in einkorn (*Triticum monococcum* ssp. *monococcum)* than in durum wheat (*T. turgidum* ssp. *durum*, AABB); the *LOX* activity was 2.5-times lower in durum wheat than that in bread wheat (*T. aestivum* ssp. *aestivum*, AABBDD) (Leenhardt *et al.*, 2006). Compared with wild-type, three transgenic lines, namely *LOX* RNAi-4, *LOX* RNAi-7 and *LOX* RNAi-9, exhibited drastic decrease in the levels of *LOX* transcripts during grain development; the *LOX* activity was significantly lower and the germination rate was substantially higher (Dong *et al.*, 2015).

Gluten and dough quality are also influenced by *LOX* activity. A negative correlation was found between specific *LOX* activity and dough extensibility, which explains the ambivalent role of this enzyme in gluten and dough quality (Permyakova *et al.*, 2010; Zhang *et al.*, 2015). *LOX* activity is negatively correlated with flour strength (Zhang *et al.*, 2015). These findings showed that *LOX* activity not only affects the color of flour and flour products but also affects gluten strength and dough rheological properties (Zhang *et al.*, 2015).

The *LOX* loci *Lpx-A1*, *Lpx-B1* and *Lpx-D1* were found on chromosome 4A, 4B and 4D, while *Lpx-A2*, *Lpx-B2* and *Lpx-D2* were found on chromosome 5A, 5B and 5D in wheat, respectively (Carrera *et al.*, 2007; Zhang *et al.*, 2008; Garbus *et al.*, 2009; Feng *et al.*, 2012). In durum wheat, three *LOX* loci, namely *Lpx-1*, *2* and *3*, were found on both 4A and 4B chromosomes (Carrera *et al.*, 2007; Zhang *et al.*, 2008; Garbus *et al.*, 2009; Feng *et al.*, 2010). Among them, three copies of *Lpx-B1* locus, namely *LpxB1.1*, *Lpx-B1.2* and *Lpx-B1.3*, were identified (Carrera *et al.*, 2007; Verlotta *et al.*, 2010), while only one *LOX* gene was found in *Lpx-A1* locus (Garbus *et al.*, 2013). In addition, deletion of *Lpx-B1.1* was found to be associated with a sharp decrease in *LOX* activity and improved pasta color (Carrera *et al.*, 2007; Feng *et al.*, 2010).

In common wheat, three *LOX* genes *(TaLOX1, TaLOX2 and TaLOX3)* were isolated and assigned to the short arm of chromosome 4D, the long arm of chromosome 5D and chromosome 4A, respectively (Feng *et al.*, 2010, 2012). *TaLOX1 and TaLOX2* were expressed during seed development (Feng *et al.*, 2010). The transcript level of *TaLOX3* increased significantly during the development of wheat grains and genotypes with high *LOX* activity were found to be associated with higher gene expression level (Feng *et al.*, 2012). Molecular markers of *Lpx-B1*alleles in durum wheat, such as *Lpx-B1.1a*, *Lpx-B1.1b*, *Lpx-B1.1c*, *Lpx-B1.2* and *Lpx-B1.3*, were found (Verlotta *et al.*, 2010) and applied (Zhang *et al.*, 2014). Among the three *Lpx-B1.1* genotypes, the varieties with *Lpx-B1.1b* had significantly higher *LOX* activity than those with *Lpx-B1.1a* and *Lpx-B1.1c* and the varieties with *Lpx-B1.1c* allele had the lowest *LOX* activity. The *LOX* activity in *Lpx-B1.3-harboring* genotypes was significantly higher than that in *Lpx-B1.2-carring* genotypes (Zhang *et al.*, 2014).

In bread wheat, two QTLs of *LOX* activities, *i.e*., *QLpx.caas.1AL* and *QLpx.caas-4B*, are closely linked with the SSR loci Xwmc 312 and Xgwm 251, accounting for 13.4–25.2% of the phenotypic variance for the 1AL locus and 14.3–27.0% for the 4B locus, respectively (Geng *et al.*, 2011). The marker combination Xwmc312/Xgwm251 is efficient and reliable for evaluating *LOX* activity and can be used in marker-assisted selection (MAS) for targeting flour color attributes in noodle and other wheat-based products (Geng *et al.*, 2011). On chromosome 4BS, two complementary functional markers of *LOX* gene (designated as *TaLox-B1*), *LOX16* and *LOX18*, were developed based on the single nucleotide polymorphism (SNP) of two alleles at the *TaLox-B1* locus, amplifying 489- and 791-bp fragments in bread wheat cultivars with higher and lower *LOX* activities, respectively. Furthermore, two functional markers were closely linked to simple sequence repeat (SSR) locus Xgwm251 on chromosome 4BS, with a genetic distance of 1.8 cM (Geng *et al.*, 2012). Similarly, *Lox-B23* was developed as a co-dominant marker of two novel genes (*TaLox-B2* and *TaLox-B3*) on chromosome 4BS, which has been proved to be significantly associated with *LOX* activity. *TaLox-B2* has two alleles, *i.e*., designated *TaLox-B2a* and *TaLox-B2b*. Among the five allelic combinations of *LOX* genes at *TaLox-B1, TaLox-B2* and *TaLox-B3* loci, wheat cultivars with a combination of *TaLox-B1a/TaLox-B2a/TaLox-B3a* exhibited the highest *LOX* activity, whereas those with the combination of *TaLox-B1a/TaLox-B2b/TaLox-B3b* showed significantly lower *LOX* activity. *TaLox-B3a* gene can significantly increase the *LOX* activity in bread wheat (Zhang *et al.*, 2015). Recently, QTL associated with the activity of seed soluble *LOX* has been identified on the short arm of chromosome 4D in hexaploid wheat, while the two regions which are responsible for the activity of soluble *LOX* in the leaves are found on the short arm of chromosome 2D (Permyakova *et al*., 2017).

The main objective of this research was to study changes in activity and expression levels of *LOX* genes in germinated Lincang hulled wheat seeds after storage at different temperatures for different period and to explore the correlation among the activity and the expression level of LOX genes and viability indices of seeds. The results provided theoretical basis for effectively conserving, protecting and reusing special and rare Yunnan hulled wheat germplasm resources.

Materials and Methods

Plant Materials and Storage Experiment of Seeds

The experimental material, Lincang hulled wheat, was collected from Lincang District of Yunnan Province from the Late 70s to the Early 80s in the 20th century, which belongs to *Triticum aestivum* ssp. *yunnanense* King (AABBDD). It is a local variety or landrace with no excoemum or awn, characterized by white hull and red grain (var. *ankoncum* King). The number of preservation in the Yunnan Province and national gene bank for wheat is Yun 0006 and XM0927, respectively.

The seeds of Lincang hulled wheat with germination rate of 91% were sealed in × aluminum foil bags (10 ×15 cm), with 12 g per bag and then stored at -7.5±2.5℃ (LT1), at 7.5±2.5℃ (LT2), indoor temperature (ambient temperature (AT) -5 to 31℃) and high temperature (HT) 40±1℃, respectively. Considering the little changes in the viability of seeds stored at LT1, LT2 and AT for short storage time, only five bags of seeds were stored at these temperatures since January 4, 2016, and other 10 bags of seeds were stored at high temperatures since July 11, 2016.

Viability Indices of Lincang Hulled Wheat Seeds after Storage at Different Temperatures for Different Storage Periods

The viability indices of seeds stored at different temperatures for different storage period were tested. The viability indices of seeds stored at HT were tested every 7 days and the viability indices of seeds stored at the other 3 temperatures were tested every 2, 3 months. Three samples were taken from one bag, each containing 100 seeds and then put into a petri dish without sterile filter paper. After adding deionized water to cover half of the seeds, the petri dish was placed in a light incubator at 20±1℃ for7 days in darkness. The number of germinated seeds was recorded every 24 h after soaking.

The height of 10 seedlings per duplication (sprout length plus root length, cm) was measured at the end day of germination test. Then, germination force (GF), germination percent (GP), weighted germination index (WGI) and seedling vigor index (SVI) were calculated as:

GF = number of seeds germinated within 3 days/N×100%,

GP= number of seeds germinated within 7 days/N×100%,

WGI = (7×N1 + 6×N2 + … + 1×N7)/(7×N) (Zhang *et al.*, 2014; Barrero *et al.*, 2015) and SVI=GI×S

Where N= the total number of seeds used for germination test. N1, N2, N3 … N7 are the number of seeds germinated at the first day, the second day, the third day, till the seventh day; GI is the germination index; S is the average length of the stems.

LOX Activity in Germinated Grains

After germinating for 7 days, 0.5 g of germinated grains with radicle and embryo being removed were put into the mortar. Then, l mL phosphate buffer (0.1 mol.L^{-1}, pH=7.5) was added before being fully mixed into homogenate and stored at 4ºC. After that, homogenate was extracted for 2 h, centrifuged for 10 min at 12000 rpm and finally the supernatant was kept as the crude enzyme solution.

Linoleic Acid Substrate

A 0.5 mL of Tween-20 was dissolved in 10 mL of boric acid buffer (0.05 mol. L^{-1}, pH 9) and then 0.5 mL of linoleic acid was added in before fully mixing. Subsequently, 1.3 mL of NaOH (1 mol. L^{-1}) solution was added to clarify the mixed solution. Then, 90 mL of boric acid buffer (0.05 mol. L^{-1}, pH9) was added, and the pH was adjusted to 7 with HCl. Finally, the substrate was made up to 200 mL.

Reaction System

A 0.15 mL of linoleic acid substrate and 30 μL of as-prepared crude enzyme liquid were added in the 4.75 mL of sodium acetate buffer (0.05 mol. L^{-1}, pH=5.6). A unit of *LOX* is defined as that activity which produces an optical density of 1, at 234 nm in 1 min in a total volume.

Expression Analysis of the *LOX* Genes

The total RNA of the leaves grown for 7 days after seed germinating was isolated with Mini BEST Universal RNA Extraction Kit (TaKaRa Biotechnology, Dalian, China). The cDNA was synthesized with PrimeScript^{Tm}II1st Strand cDNA Synthesis Kit, and preserved at -20℃.

Using the SYBR® *Premix Ex Taq*™ (TIiRNaseH Plus kit), qRT-PCR was performed in 20 µL of reaction mixtures containing 10 μL of SYBR® *Premix Ex Taq* (TIiRNaseH Plus) (2×), 0.4 μL of PCR forward and reverse primer (10 μmol.L^{-1}), 0.4 μL of ROX Reference Dye II (50×), 2 μL of DNA and 6.8 μL of ddH_2O. The amplifications were conducted for 30 s at 95ºC, 40 cycles of 5s at 95ºC, 34 s at 60ºC.

Two pairs of primers for the target gene and one pairs of primers for reference gene were used to amplify cDNA from Lincang hulled wheat (Table 1). Based on the parallelism and inflection points in the amplified curves, the peak in the melting curve, and the Tm of amplified products of the target and reference genes, the PCR primers that were suitable for researching the relative expression level of Lincang hulled wheat *LOX* gene were selected, which were the primers of reference gene*26SrRNA*, the primers of the target gene*TaLOX2-5DL, TaLOX2* and the primers of the target gene *TaLOX3-4A, TaLOX3*. The primers of the target gene *TaLOX1-4DS* were eliminated, because they are not suitable for amplifying the cDNA from Lincang hulled wheat (Feng *et al.*, 2012).

Statistical Analysis

All measurements were performed among three biological replicates and data were analyzed by the IBM SPSS statistics version 19.0 software package (SPSS Inc., Chicago, Illinois, USA). The ANOVA and multiple pairwise comparisons (LSR) method were used to determine the significant differences between means. The bivariate correlation analysis function was used to estimate correlation coefficients. The relative expression levels of *LOX* genes were calculated using the $2^{-\triangle Ct}$ method, where $\triangle$Ct (target gene) = Ct (target gene) - Ct (26S rRNA, reference gene). The REL was used to estimate correlation coefficients between *LOX* activity and viability indices.

Results

Viability Indices of Hulled Wheat Seeds after Storage at Different Temperatures

The viability indices (such as GF, GP, WGI and SVI) of Lincang hulled wheat seeds after storage at different temperatures showed similar values with an increase of storage period (days, storage period, SP). The variations of GF (Fig. 1), GP (Fig. 2), WGI (Fig. 3) and SVI (Fig. 4) of seeds stored at LT1 (-7.5±2.5ºC) and LT2 ($p > 0.05$) had no significant difference, although both showed fluctuating values with an increase of SP. Except WGI, the variations of GF, GP and SVI of seeds stored at AT showed significant differences with an increase in SP, of which GF dropped from 86 to 45.3% as SP increased from 252 to 365 days; GP dropped from 96.7 to 77% as SP increased from 125 to 365 days; SVI declined from 548 to 397.3 as SP increased from 189 to 365 days . When the seeds were stored in high temperature (40±1ºC, HT), the values observed of viability indices GF, GP, WGI and SVI have declined gradually from maximum 62%, 91%, 0.6 and 605.6 of 7 day to 33%, 66%,0.4 and 305.9 of 70 day, respectively.

Under the condition of identical storage period, the GF, GP, WGI and SVI of seeds had changed significantly as the temperature increased from LT1 to AT (Fig. 1–4). With storage period of 365 d, GF of seeds stored at LT1, LT2 and AT were 57.7, 63.3 and 45.3%, respectively; GP were 86.7, 94.0 and 77% at LT1, LT2 and AT, respectively; WGI were 0.617, 0.662 and 0.511, respectively; SVI were 486.7, 516.1 and 397.3, respectively. In terms of the average value of each viability index, GF was 63, 62.9, 71 and 36% as the temperature increased from LT1 to HT, respectively; GP was 92.2, 92.4, 89.9 and 71.7%, respectively; WGI was 0.641, 0.647, 0.613 and 0.445, respectively; SVI was 468.2, 467.9, 4 66.6 and 333.4, respectively. It can be seen that the viability indices of seeds stored at HT were significantly lower than those at other three temperatures.

***LOX* Activity of Lincang Hulled Wheat Seeds after Stored at Different Temperatures**

With an increase in SP, the *LOX* activity of germinated Lincang hulled wheat seeds increased firstly and then decreased (Fig. 5). The *LOX* activity increased firstly from 2.651 $U.g^{-1}$ min^{-1} of 63 day to 3.406 $U.g^{-1}$ min^{-1} of 189 day and then dropped to 2.553 $U.g^{-1}$ min^{-1} of 365 day for seeds stored in LT1, from 2.525 $U.g^{-1}$ min^{-1} of 63 day to 4.019 $U.g^{-1}$ min^{-1} of 189 day and then to 2.448 $U.g^{-1}$ min^{-1} of 365 day for seeds stored in LT2, from 3.192$U.g^{-1}$ min^{-1} of 63 day to 3.748 $U.g^{-1}$ min^{-1} of 189 day and then to 2.519 $U.g^{-1}$ min^{-1} of 365 day for seeds stored in AT, from 3.441$U.g^{-1}$ min^{-1} of 7 day to 3.719 $U.g^{-1}$ min^{-1} of 28 day and then declined to 2.134 $U.g^{-1}$ min^{-1} of 70 day for seeds stored in HT, respectively. However, the *LOX* activities of seeds stored at and after 35 day d in HT were lower than others three temperatures.

The Changes in the Relative Expression Levels of *LOX* Genes *TaLOX2-5DL* and *TaLOX3-4A*

After storage at different temperatures, changes in the relative expression levels (REL) of *LOX* genes, *TaLOX2-5DL* and *TaLOX3-4A*, both gradually decreased with the increase of SP.

Where, the REL of *LOX* genes were $2^{-\Delta Ct}$.

For example, when the seed was stored at LT1, the REL of *TaLOX2-5DL* decreased from maximum value of 63 day to minimum value of 365 day; when the seed was stored at LT2, except for the storage period of 365 day, the REL of *TaLOX2-5DL* decreased from maximum value of 63 day to minimum value of 252 day; at the temperature of AT, except for the storage period of 63 day, the REL of *TaLOX2-5DL* decreased from maximum value of 125 day to minimum value of 365 day; while at HT, except 70 day, the REL of *TaLOX2-5DL* decreased from maximum value of 7 day to minimum value of 63 day (Fig. 6). The change in the REL of *TaLOX3-4A* was similar to that of *TaLOX2-5DL*. The REL of *TaLOX3-4A* decreased from maximum value of 63 day to minimum value of 365 day for seeds stored in LT1 and LT2, respectively., The REL decreased from maximum value of 125 day to a minimum value of 365 day for seeds stored in AT and declined from maximum value of 7 day to minimum value of 63 day for seeds stored under HT (Fig. 7).

Correlations between Observed Traits, the Stepwise Regression Analysis of Germination Percent and Storage Period Versus Other Traits

After the Lincang hulled wheat seeds were stored at different temperatures, the positive correlation among viability indices such as GF, GP, WGI, SVI were significant (Table 2). At LT1, the simple correlation coefficient between WGI with GF, GP and SVI was 0.874 ($p<0.01$), 0.750 ($p<0.01$) and 0.675 ($p<0.05$), respectively, and that between SVI and GF was 0.647 ($p<0.05$). At LT2,

Table 1: Primers and their sequence used to study the expression levels of LOX genes of Lincang hulled wheat

Gene	Types	Name	Forward(F)/ reverse(R) Sequence (5'–3')	reference
TaLOX2-5DL	Target gene	TaLOX2	TCACCACGGGCGAGAACAAG/AGAGTTGGCGACGAAGACG	Feng *et al*., 2012
TaLOX3-4A	Target gene	TaLOX3	GGGAAGAACAAGCAG GCGTG/CGCTGACGAGGTGGAAGATG	Feng *et al*., 2012
26S rRNA	Reference gene	26S rRNA	GAAGAAGGTCCCAAGGGTTC/TCTCCCTTTAACACCAACGG	Feng *et al*., 2012

Table 2: Correlation coefficient among observed traits

Temperature	Observed traits	SP	WGI	GF	GP	SVI	LOX activity	REL of T*aLOX2-5DL*
	WGI	0.329						
	GF	0.225	0.874**					
LT1	GP	-0.166	0.750**	0.477				
	SVI	0.244	0.675*	0.647*	0.462			
N=12	LOX activity	-0.214	0.346	0.178	0.463	0.102		
	REL of *TaLOX2-5DL*	-0.918**	-0.178	0.003	0.201	0.012	0.006	
	REL of *TaLOX2-4A*	-0.862**	-0.114	0.093	0.167	0.062	0.098	0.964**
	WGI	0.517						
	GF	0.373	0.732**					
LT2	GP	0.371	0.782**	0.349				
	SVI	0.539	0.809**	0.397	0.507			
N=12	LOX activity	-0.209	0.109	-0.145	-0.148	0.398		
	REL of *TaLOX2-5DL*	0.142	-0.106	-0.075	0.016	-0.125	-0.238	
	REL of *TaLOX2-4A*	-0.820**	-0.300	-0.102	-0.150	-0.461	-0.118	-0.474
	WGI	-0.481						
	GF	-0.381	0.856**					
AT	GP	-0.543*	0.909**	0.805**				
	SVI	-0.523*	0.866**	0.718**	0.793**			
N=15	LOX activity	-0.214	0.074	-0.081	0.018	0.234		
	REL of *TaLOX2-5DL*	-0.681**	0.398	0.616*	0.611*	0.447	0.040	
	REL of *TaLOX2-4A*	-0.732**	0.487	0.690**	0.646**	0.548*	0.056	0.976**
	WGI	-0.509**						
	GF	-0.498**	0.941*					
	GP	-0.487**	0.945**	0.804**				
HT	SVI	-0.505**	0.927**	0.921**	0.818**			
N=30	LOX activity	-0.795**	0.345	0.360	0.353	0.360		
	REL of *TaLOX2-5DL*	-0.079	0.409*	0.440*	0.309	0.488**	0.204	
	REL of *TaLOX2-4A*	0.320	0.129	0.144	0.090	0.204	0.063	0.499**

Note: LT1:-7.5±2.5°C; LT2: 7.5±2.5°C; AT: ambient temperature; HT: 40±1°C; WGI: weighted germination index; GF, germination force; GP: germination prevent; SVI: seedling vigor index; SP: storage period; REL: relative expression level; *, Significant correlation at 0.05 levels (two-sided test); **, Significant correlation at 0.01 levels (two-sided test)

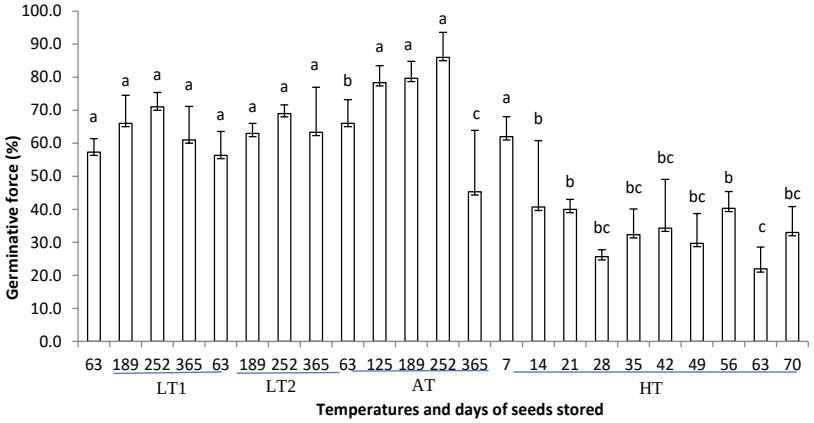

Fig. 1: The changes trend of the germination force with the increase of the temperature and storage period (days)
LT1: -7.5±2.5°C; LT2: 7.5±2.5°C; AT: ambient temperature; HT: 40±1°C; under identical temperature while with different storage period, the different letters show significant difference at 0.05 levels, the same below

simple correlation coefficient between WGI with GF, GP and SVI was 0.732 (p<0.01), 0.782 (p<0.01) and 0.809 (p<0.01), respectively. At the temperature of AT, the simple correlation coefficient between WGI with GF, GP and SVI was 0.856 (p<0.01), 0.909 (p<0.01) and 0.866 (p<0.01), respectively; that between GF with GP, SVI was 0.805 (p<0.01) and 0.718 (p<0.01) and that between GP and SVI was 0.793 (p<0.01). At HT, the simple correlation coefficient

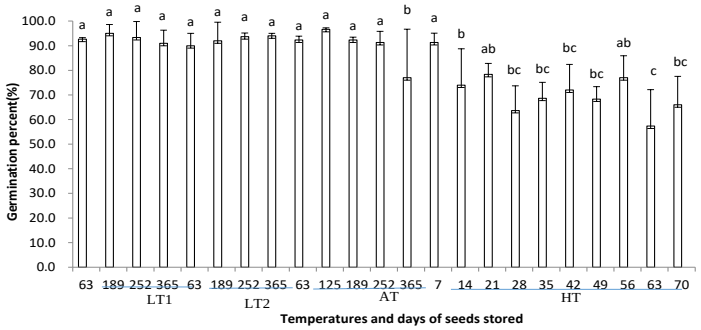

Fig. 2: The changes trend of the germination percent with the increase of the temperature and storage period (days)

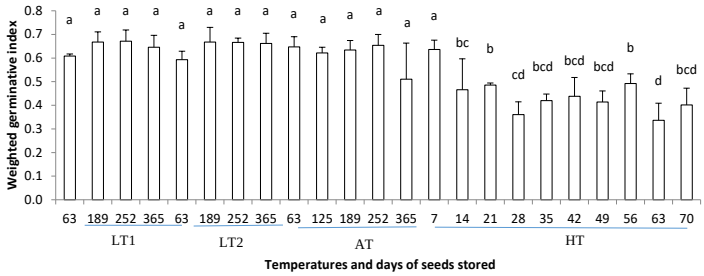

Fig. 3: The changes trend of the weighted germnative index with the increase of the temperatures and storage period (days)

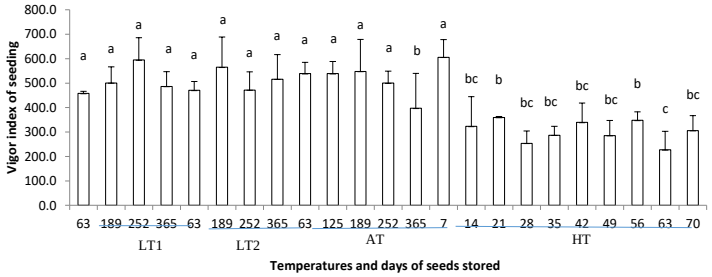

Fig. 4: The changes trend of the seeding vigor index with the increase of the temperature and storage period (days)

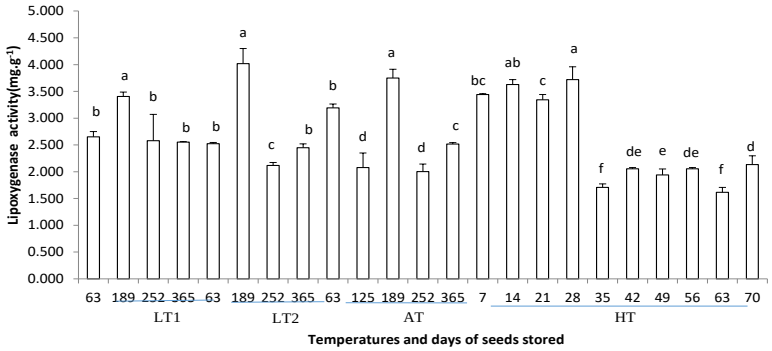

Fig. 5: The changes trend of the *lipoxygenase* activity with the increase of the temperatures and storage period (days)

between WGI with GF, GP and SVI was 0.941 (p<0.01), 0.945 (p<0.01) and 0.927 (p<0.01), respectively, that between GF with GP, SVI was 0.804 (p<0.01) and 0.921 (p<0.01) and that between GP and SVI was 0.818 (p<0.01).

The storage period (SP) of seeds had a significant negative correlation with the relative expression levels (REL, $2^{-\triangle Ct}$) of the *LOX* genes *TaLOX2-5DL* and *TaLOX3-4A* at LT1, with the correlation coefficient being -0.918 and

-0.862, respectively. In addition, the correlation coefficient between REL of *TaLOX2-5DL* and that of *TaLOX3-4A* was strong (p<0.01). At LT2, SP had a negative correlation with the REL of T*aLOX2-4A* (r=-0.820, p<00.1) only. At AT, the SP, GF and GP had a significant negative or positive correlation with the RELs of *TaLOX2-5DL* and *TaLOX2-4A*, with the correlation coefficient being -0.681 (p<0.01), -0.732 (p<0.01), 0.616 (p<0.05), 0.690 (p<0.01) and 0.611 (p<0.05) and 0.646 (p<0.01), respectively. In addition, there was a significant positive correlation between SVI and the REL of *TaLOX2-4A* (r=0.548, p<0.05) as well as an extremely significant positive correlation between the REL of *TaLOX2-5DL* and *TaLOX2-4A* (r=0.946, p<0.01). At HT, there was a strong negative correlation between SP and the viability of seed, with the correlation coefficient was -0.509, -0.498, -0.487 and -0.505, respectively; there was a strong negative correlation between SP and *LOX* activity. The positive correlations between the REL of *TaLOX2-5DL* with WGI (r=0.409, p<0.05), GF (r=0.440, p<0.05), SVI (r=0.488, p<0.01) were significant. In addition, positive correlation between the RELs of *TaLOX2-5DL* and *TaLOX2-4A* was strong (r=0.499, p<0.01) as well.

In general, there was a positive correlation among viability indices. However, the correlation between viability indices at AT and HT was negative. The correlation between *LOX* activity and SP was very significantly negative at HT, but was not so significantly negative at the other three temperatures. In addition, the correlation between *LOX* activity and viability indices was also not significant at all temperatures. There was a very significant negative correlation between the REL of *TaLOX2-5DL* and SP at LT1 and AT, as well as a very significant negative correlation between the REL of *TaLOX3-4A* and SP, excepting at HT. The correlation between the REL of *LOX* genes and viability indices was non-significant at LT1 and LT2. Therefore, it can be concluded that the *LOX* activity

Table 3: Parameters of model for stepwise regression analysis

Temperatures	dependent variable	R	R^2	R^2 adjusted	estimated standard error	equations	
						F	Sig.
LT1	GP	0.970	0.940	0.918	1.198	41.918	0.000
	SP	0.981	0.963	0.949	25.788	68.951	0.000
LT2	GP	0.782	0.611	0.572	2.794	15.731	0.003
	SP	0.820	0.672	0.639	68.457	20.489	0.001
AT	GP	0.949	0.900	0.883	3.539	53.974	0.000
	SP	0.732	0.536	0.500	76.908	15.028	0.002
HT	GP	0.979	0.959	0.956	2.541	317.968	0.000
	SP	0.932	0.868	0.853	7.842	57.059	0.000

Note: LT1:-7.5±2.5°C; LT2: 7.5±2.5°C; AT: ambient temperature; HT: 40±1°C; GP: germination prevent; SP: storage period

Table 4: Coefficient of stepwise regression analysis

Temperature	dependent variable	components	Non standardized coefficient		standardized coefficient	t	Sig.
			B	standard error			
	GP	constant	20.332	6.841		2.972	0.018
		WGI	163.588	17.761	1.710	9.211	0.000
LT1		SP	-0.019	0.003	-0.525	-5.679	0.000
		GF	-0.458	0.092	-0.900	-5.002	0.001
	SP	constant	457.718	72.160		6.343	0.000
		REL of TaLOX2-5DL	-29282.365	2172.614	-0.920	-13.478	0.000
		SVI	0.416	0.102	0.279	4.074	0.004
		LOX activity	-63.165	18.248	-0.237	-3.461	0.009
LT2	GP	constant	48.195	11.179		4.311	0.002
		WGI	68.322	17.226	0.782	3.966	0.003
	SP	constant	397.179	44.391		8.947	0.000
		REL of TaLOX3-4A	-17136.086	3785.704	-0.820	-4.527	0.001
AT	GP	constant	24.288	6.950		3.495	0.004
		WGI	96.580	12.153	0.791	7.947	0.000
		REL of TaLOX2-5DL	588.081	197.480	0.296	2.978	0.012
	SP	constant	336.365	39.723		8.468	0.000
		REL of TaLOX3-4A	-11238.632	2899.142	-0.732	-3.877	0.002
HT	GP	constant	4.448	3.147		1.414	0.169
		WGI	206.723	14.350	1.659	14.405	0.000
		GF	-0.689	0.105	-0.758	-6.582	0.000
	SP	constant	63.781	7.932		8.041	0.000
		LOX activity	-17.238	1.881	-0.700	-9.164	0.000
		REL of TaLOX3-4A	3127.257	524.324	0.434	5.964	0.000
		SVI	-0.061	0.014	-0.342	-4.395	0.000

Note: LT1:-7.5±2.5°C; LT2: 7.5±2.5°C; AT: ambient temperature; HT: 40±1°C; GP: germination prevent; SP: storage period; WGI: weighted germination index; GF: germination force; REL: relative expression level; SVI: seedling vigor index

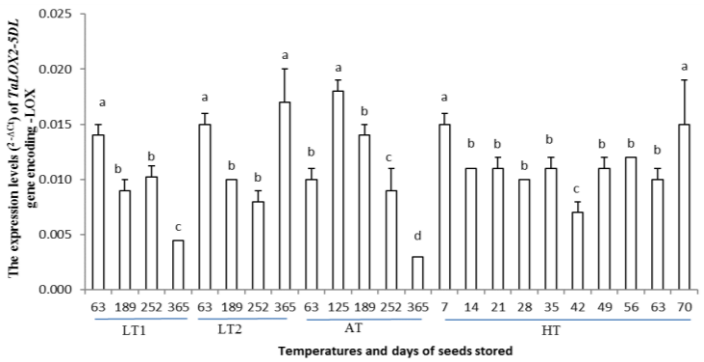

Fig. 6: The changes trend of the expression levels ($2^{-\Delta Ct}$) of *LOX* gene *TaLOX2-5DL* with the increase of the temperatures and storage period (days)

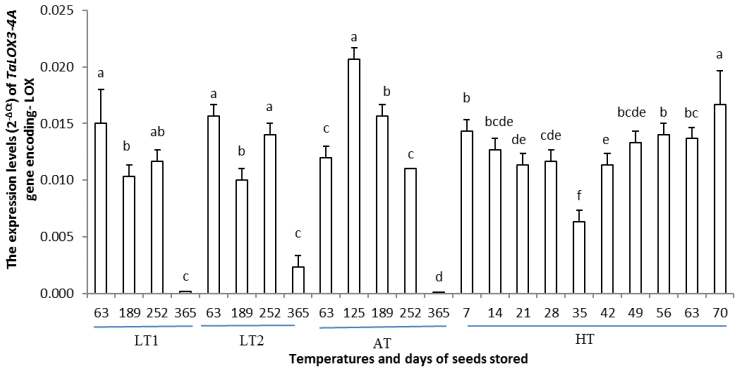

Fig. 7: The changes trend of the expression levels ($2^{-\Delta Ct}$) of *LOX* gene *TaLOX3-4A* with the increase of the temperature and storage period (days)

and the REL of *LOX* genes have different impacts on SP and viability indices at different temperatures. The GP and SP were reported to be associated with the seed longevity in some study previously. Therefore, the stepwise regression analysis of the GP, SP versus other observed traits were carried out.

By regarding GP and SP as dependent variables and regarding other observed traits as independent variables, the F value of the gained equation was between 15.731 and 317.968 (Table 3) and the significant value was from 0.000 to 0.003, suggesting that gained equation was significant. However, only GP was associated with viability indices of seeds after storage at all temperatures except AT (Table 4). The *LOX* activity was associated with SP at LT1 and HT, with the standardized correlation coefficient being -0.237 and -0.700, respectively, which were consistent with the simple correlation coefficient (-0.214 and -0.795). The relative expression levels (REL) of *TaLOX2-5DL* was associated with SP at LT1. Moreover, the standardized correlation coefficient (-0.920) between them was also agreed with the normal correlation coefficient (-0.918). In contrast, the REL of *TaLOX3-4A* was associated with SP at all temperatures, except temperature LT1. The standardized correlation coefficient between the REL *of TaLOX3-4A* and SP was 0.820 and -0.732 at LT2 and AT, respectively, which were agreed with the normal correlation coefficient (-0.820 and -0.732). The contribution of the REL of *TaLOX3-4A* to SP at temperature HT was not consistent with the actual value. Therefore, after storage at different temperatures, the *LOX* activity of germinated Lincang hulled wheat grains and the REL of *LOX* genes in leaves had association with SP rather than GP, except for REL of *TaLOX2-5DL* for seeds stored in AT. Moreover, the association such as the SP was shorter if the *LOX* activity was higher for seeds stored in LT1 and HT, the SP was shorter if the REL of *TaLOX2-5DL* was higher for seeds stored in LT1, and the SP was shorter if the REL of *TaLOX2-4A* was higher for seeds stored in LT2 and AT were found.

Discussion

In this study, after the Lincang wheat hulled seeds were stored at different temperatures, the *LOX* activity of germinated grains first increased and then decreased with the storage period. This result is consistent with the results previously reported in the dry seeds of wheat (Chen *et al.*, 2015), but not in line with the trend for brown rice. In brown rice, *LOX* activity increased with the storage period (Kim *et*

al., 2007), indicating that the change in the *LOX* activity of wheat seeds with storage periods is different from that of rice. High temperature has been reported to induce a rise of *LOX* activity in growing root of barley (Tamás *et al.*, 2009) and in leaves of winter wheat seedlings (Babenko *et al.*, 2014). In this study, the *LOX* activity of seeds stored at temperature of HT with storage period of 7 to 28 d was higher significantly than those at other three temperatures (except for *LOX* activity with storage period of 189 d). This indicated that high temperature (40±1℃) enhanced the *LOX* activity of germinated Lincang hulled wheat seeds due to the accumulation of lipids, which can be taken as an indicator of ecological plasticity (Babenko *et al.*, 2014).

In a previous study, the viability indices of seeds, especially GP, have been regarded as an indicator of seed longevity and influence of *LOX* activity on seed longevity has been reported. For example, transgenic wheat lines with lower *LOX* activity had substantially higher germination rate than wild type (Dong *et al.*, 2015). Maize varieties with lower *LOX* activity or lacking *LOX*-1 or -2 genes had insignificant change of germination rate, while varieties with *LOX*-1 or -2 genes showed a significant decline in germination rate, suggesting that *LOX*-1 or -2 genes may be a definitive factor influencing maize seed life span (Li *et al.*, 2007). Therefore, the *LOX* activity is negatively correlated with seed longevity in maize (Li *et al.*, 2007; Long *et al.*, 2013; Dong *et al.*, 2015).

In this study, the *LOX* activity of germinated Lincang hulled wheat seeds had no significant correlation with GP or other viability indices. Moreover, stepwise regression analysis to GP indicated that the *LOX* activity of germinated Lincang hulled wheat seeds was not associated with GP. However, when storage period of seed was considered as seed longevity, the *LOX* activity of germinated Lincang hulled wheat seeds was negatively correlated with SP (-0.795) of seeds under high-temperature condition (HT, 40±1ºC). This indicates that higher the *LOX* activity was, the shorter the SP of seed was under this high temperature. In addition, the *LOX* activity of germinated Lincang hulled wheat seeds had no significant negative correlation with SP (-0.214) of seeds under low-temperature condition (LT1, -7.5±2.5℃). Further, the standardized regression coefficient (-0.237 and -0.700) between the *LOX* activity of germinated Lincang hulled wheat seeds and SP was consistent with the simple correlation coefficient (-0.214 and -0.795) at LT1 and HT, indicating that the *LOX* activity of germinated Lincang hulled wheat seeds had a negative effect on SP for seeds stored in LT1 and HT.

It has been reported that the expression level of *LOX* genes has an impact on germination rate or seed longevity. Up-regulated or over-expressed level of *LOX* genes is harmful to seed longevity. Rice lines with over-expressing *LOX2* gene showed lower seed viability, and RNAi of *LOX2* could enhance the longevity of aged seeds (Huang *et al.*, 2014). Rice over-expressing *LOX3* gene showed an enhanced decrease in seed germination ability when the seeds were routinely stored, demonstrating that *LOX3* gene has a negative effect on seed longevity (Long *et al.*, 2013).

In this study, there was a very significant negative correlation between REL of *LOX* genes in leaves and storage period (seed longevity), except at temperature of HT. The standardized correlation coefficient gained by the stepwise regression analysis indicated that there was a negative correlation between the REL of *TaLOX2-5DL* and SP of seeds stored at LT1, as well as a negative correlation between the REL of *TaLOX3-4A* and SP of seeds at LT2 and AT. However, the correlation between them was positive at HT. Therefore, it can be known that the main factors influencing SP are *LOX* activity and REL of *TaLOX2-5DL* at LT1 and are mainly REL of *TaLOX3-4A* at LT2 and AT. Further study is needed to demonstrate the correlation between the REL of *TaLOX3-4A* and SP at HT.

Conclusion

Influence of *LOX* activity of germinated Lincang hulled wheat seeds and REL of *LOX* gene on storage period of seeds under different temperature were diverse. At -7.5 ± 2.5^{o}C, the factors influencing storage period of seeds were *LOX* activity and REL of TaLOX2-5DL; at 7.5±2.5^{o}C and ambient temperature, the factors was mainly REL of TaLOX3-4A; while at high temperature (40±1^{o}C) the main factor was *LOX* activity.

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