

Cloning of *Lactate dehydrogenase* Gene and Effect on the Waterlogging Tolerance of *Brassica napus* L.

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Abstract: To investigate the mechanism on waterlogging tolerance in *Brassica napus*, 12 *B. napus* cultivars with different waterlogging tolerance were used in the research and Waterlogging Tolerance Index (WTI) was calculated by multiplying relative percentage germination and the relative seedling height. The results indicated that Lactate Dehydrogenase (LDH) enzyme activity rapidly increased at 24 h after waterlogging treatment and reached peak between 48-72 h. WTI was correlated with LDH enzyme activity at 24 h after water logging treatment and the correlation coefficient between them was 0.84. Transcription level of the BnLDH had significant difference in the 12 lines after waterlogging treatment. BnLDH expression level was very low before waterlogging treatment and induced by waterlogging treatment and arrived at peak at 48 h. Correlation analysis indicated correlation coefficient between WTI and BnLDH expression at 24 and 48 h after waterlogging treatment was 0.56 and 0.72, respectively. An LDH gene, denoted BnLDH-1, was cloned from oilseeds by the Rapid Amplification of CDNA Ends (RACE) from 12 materials and the results indicated all of them had same protein sequence.

Keywords: *Brassica napus* L., cloning, lactate dehydrogenase, waterlogging tolerance, waterlogging tolerance index lipase

INTRODUCTION

Oilseed rape (*Brassica napus* L.), is one of the most important oil crops in the world, especially in China, but it is generally susceptible to waterlogging stress. Water is critical for plants, but excess water is also harmful because it results in lower oxygen concentration in the soil and may block the exchange of CO₂ and O₂ between the soil and the atmosphere. Waterlogging may result in necrosis, stunting, defoliation, even plant death (Cornelious *et al.*, 2005), which can cause to yield loss. According to the reports, waterlogging led to 10-15% yield loss for rapeseeds. Generally, waterlogging leads to 10-15% yield loss for crops (Dennis *et al.*, 2000).

During waterlogging, oxygen dissolved in soil water reduced because of the slow diffusion rate of oxygen in water and its limited solubility (Sairam *et al.*, 2008) and resulted in hypoxia. Two-dimensional electrophoresis had showed that about more than 20 anaerobic proteins were expressed in maize roots under low oxygen conditions and most of them were involved in sugar metabolism and fermentation pathway (Sachs *et al.*, 1980; Dennis *et al.*, 2000). In the limiting oxygen conditions, the starting of fermentation metabolism was regarded as an adaptive phenomenon to maintain the

energy of ATP and the growing (Kennedy *et al.*, 1992; Good and Muench, 1993). And a number of enzymes including format dehydrogenase (FDH, EC 1.2.1.2.), alcohol dehydrogenase (ADH, EC 1.1.1.1), lactate dehydrogenase (LDH, EC 1.1.1.27) and pyruvate decarboxylase (PDC, EC 4.1.1.1) were produced in fermentation metabolism pathway (Kennedy *et al.*, 1992; Ricard *et al.*, 1994). Waterlogging tolerance of plants was closely related with ATP concentrations in plants and the induced expression level of *ADH* and *PDC* based on some reports (Waters *et al.*, 1991; Drew, 1997) because reduction in oxygen supply led to an 18-fold reduction in ATP production and plants had to compensated for this loss by accelerating sugar metabolism and glycolysis (Dolferus *et al.*, 2001).

During waterlogging, aerobic metabolism is transformed into anaerobic metabolism. According to Davis-Roberts pH-stat hypothesis, the primary response of plants to O₂ limitation is characterized by a burst of lactate production, which results in lower cytoplasm pH and activates the expression of *PDC* and *ADH* and inhibits the expression *LDH*. Then, *PDC* and *ADH* are active and ethanol metabolism is started (Agarwal and Grover, 2006).

In the research, LDH enzyme activity, gene expression and *LDH* sequence difference from different

waterlogging tolerance rapeseeds materials were compared and the correlation between *LDH* and WTI was studied.

MATERIALS AND METHODS

Plant materials: Oilseeds with different waterlogging tolerance ability, Zhongshuang 9, H305, Qing 8, 9558, H04, H1020, PH36, Huyou 17, GH01, Zhongshuang 6, 2021 and Zhongshuang 11 selected from more than 4000 lines based on 4 years waterlogging experiment from 2005-2009 in the field, were used as experiment materials. In 2010, the 12 cultivates were grown in experimental field of Chinese Academy of Agricultural Sciences, China. In the flowering stage, plants were treatment with waterlogging in the field. Roots treated with waterlogging and untreated were used as samples and preserved at -80°C. Gene cloning, enzyme activity and gene expression level analyzing were finished in Key Laboratory of Biology and Genetic Improvement of Oil Crops, Ministry of Agriculture of P.R. China.

Evaluation of waterlogging tolerance of 12 materials: One-hundred seeds were submerged in 10 mL of deionized water in tubes for 24 h in an incubator at 20°C (Ueno and Takahashi, 1997). After the treatment, the seeds were removed from the water and rinsed with distilled water. The seeds were placed on a moist filter paper in 9 cm Petri dishes and seeds without flooding treatment served as control. The germination tests were evaluated on the 7th day according to the ISTA Handbook of Vigour Test Methods. The percentage of germination and radicle length of germinated seeds were recorded. Relative germination rate, radical length, seedling height and fresh weight were expressed as a percentage relative of flooding treatment to control. The seed flooding tolerance was calculated by multiplying relative percentage germination and the relative seedling height (Zhang *et al.*, 2007).

Extraction of total genomic DNA and total RNA: Total genomic DNA was extracted from leaves of the 12 materials using Cetyl Trimethyl Ammonium Bromide (CTAB) method (Saghai-marooof *et al.*, 1984). Total RNA was isolated using a CTAB method (Jaakola *et al.*, 2001) and the contaminated DNA in RNA samples was removed by digestion with RNase-free DNase I (TaKaRa).

Cloning LDH from *B. napus*: 3' RACE and 5' RACE were carried out according to Rapid Amplification of DNA Ends (RACE) in terms of the user manual

(GeneRacer kit, Invitrogen, USA) with primers FBnLDH-31 (5'- CTCGACGTCAATGCTCAGGA TGT -3') and FBnLDH-32 (5'- CACTTC TTGGGCC ATTGG CTACTC -3') for 3' cDNA end(s) and primers RBnLDH-51 (5'- GAGTAGCCAATGGCCCA AGAA GTG -3') and RBnLDH-52 (5'- TGAGCATTGACG TCGAGATGATCTG -3') for 5' cDNA end(s). Primers FBnLDH (5'- CTCCTCTATAATAAGTACACTTCTC -3') and RBnLDH (5'- CTCCTCTATAATAAGTA CACTTCTC -3') were designed to amplify the cDNA and the corresponding genomic sequence of *BnLDH*. PCR products were analyzed by 1% agarose gel with GoldView/1×TAE electrophoresis and UV light visualization. DNA of target bands was recovered using a Gel Extraction Mini Kit (Watson Biotechnologies, Inc., China) and ligated to the pMD18-T vector. Positive clones were sequenced at Beijing Sun biotech co., Ltd, China. Sequence alignment, open reading frame translation and parameter calculation of the predicted protein were carried out on Vector NTI Advance 9.0. BLAST analyses of nucleotide and protein sequences were finished on NCBI website (<http://www.ncbi.nlm.nih.gov/>). Protein structure predictions were carried out on websites (<http://www.expasy.org> and [http:// www. softberry. com/berry.phtml](http://www.softberry.com/berry.phtml)).

Real time PCR characterization of *BnLDH* expression in root of 12 materials: Real time PCR was performed to detect the expression of *BnLDH* in roots of 12 materials according to the manual of SYBR[®] PrimeScript[®] RT-PCR Kit (TaKaRa). Aliquot of 0.5-μL total first strand cDNA from each sample was used as template in a 25-μL standard *Taq* PCR reaction with primer pair QFLDH (5'- ATTGTGTAA TGGCGAACACGG -3')/ QRLDH (5'- GAAGG TCAGGGATATGCTTCTTGA -3'). Annealed at 58°C, amplifications were carried out for 35 cycles. Primers FACT (5'- ATGACGGAGACCTTGGTGCA -3') and RACT (5'-TGTGCCTAGGACGACCAACA-3') were used as internal control.

LDH activity as saying in root of 12 materials after waterlogging treatment: (0.5 g) roots were grinded in an ice-cold mortar and LDH was extracted based on the method reported (Hanson and Jacobsen, 1984). The reaction mixture was 0.1 mol/L phosphate, pH 7.0, with 4 μmol/L NADH, 0.24 mmol/L pyruvate and started with extract (Mustroph and Albrecht, 2003).

RESULTS

Waterlogging Tolerance Index (WTI) of 12 lines: Waterlogging Tolerance Index (WTI) of 12 lines was

Table 1: WTI of 12 different waterlogging tolerances materials

Line	WTI
Zhongshuang 9	82.3
H1020	72.4
Zhongshuang 11	65.3
9558	62.3
Qing 8	56.2
Huyou 17	53.9
H04	40.7
H305	40.4
Zhongshuang 6	38.6
2021	37.3
PH36	13.6
GH01	13.2

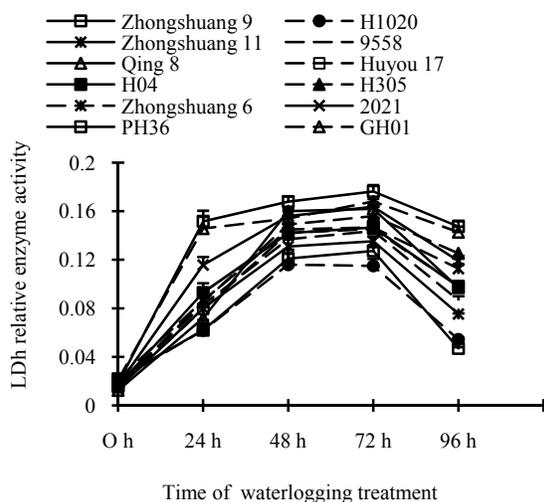


Fig. 1: BnLDH activity changes with time of waterlogging treatment

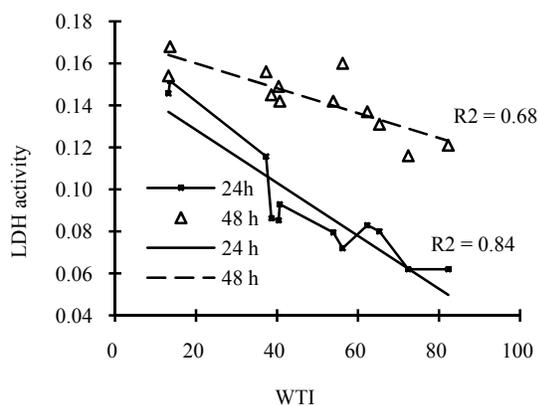


Fig. 2: Correlation between WTI and BnLDH activity at 24 and 48 h after waterlogging treatment

measured and the results were listed in Table 1. The results indicated WTI in 12 cultivates had significant difference and the WTI value changed from 13.2 to 82.3. They can be used as materials for waterlogging research.

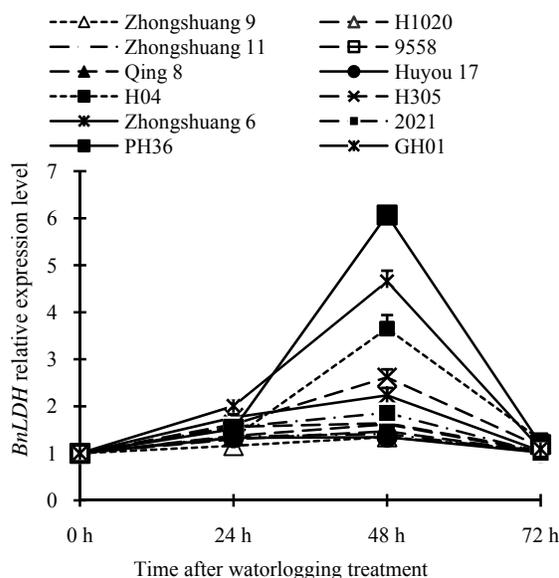


Fig. 3: BnLDH expression changes with time of the waterlogging treatment

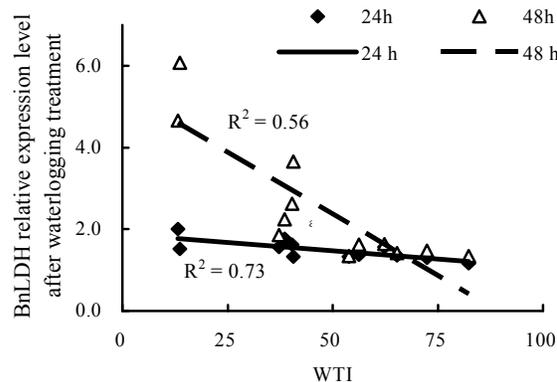


Fig. 4: Correlation between WTI and LDH expression level at 24 and 48 h after waterlogging treatment

BnLDH enzyme activity in 12 materials after waterlogging and correlated with WTI: Diurnal BnLDH enzyme activity pattern after waterlogging was listed in Fig. 1. The results showed that the BnLDH enzyme activity was induced by waterlogging from 0 to 48 h waterlogging treatment and reached the peak at 48 h. All lines had similar curves except the increasing speed. BnLDH enzyme activity increased quick in weak tolerance lines, for examples GH01. Correlation analyzing found the WTI was correlated with BnLDH enzyme activity (Fig. 2). Correlation coefficient between WTI and BnLDH activity at 24 and 48 h after waterlogging was 0.838 and 0.676, respectively. The result indicated that BnLDH activity was more related with waterlogging tolerance in earlier stage than in the later stage.

Transcription level of BnLDH in root of 12 materials: BnLDH expression level was analyzed by Real time PCR. The results suggested that transcription level of the BnLDH gene had significant difference in 12 cultivates after waterlogging treatment. BnLDH expression in PH36 and GH01 with lower WTI increased quickly after waterlogging treatment and slowly in some lines with higher WTI, for example, Zhongshuang 11 and Zhongshuang 9 (Fig. 3). But all the BnLDH expression in 12 lines was induced by waterlogging and reached their peak at 24 h after waterlogging treatment. Then the expression level decreased from 24 to 48 h.

Transcription level of BnLDH in roots of 12 materials was correlated with WTI: Correlation analysis indicated WTI was correlated with BnLDH expression level (Fig. 4). The correlation coefficients between WTI and BnLDH expression at 24 and 48 h waterlogging were 0.56 and 0.72, respectively.

DISCUSSION

Water is very important for plant growth, but both deficiency/drought and waterlogging can result in huge damage to plants. Because more waterlogging will lead to hypoxia, it is important to continue glycolysis metabolism to supply the energy for growth and hypoxia is one of the most important abiotic stresses encountered by most of plants. Some research had been done to isolate and characterize the genes involved in tolerance to hypoxia and to explore the molecular mechanism in crop plants.

The response of plant to hypoxia stress was first studied in maize roots by the method of two-dimensional electrophoresis. The results showed that about 20 kinds of proteins were synthesized during hypoxia stress (Sachs *et al.*, 1980), including sucrose synthase, phosphohexose isomerase, fructose-1,6-diphosphate aldolase, PDC, LDH and ADH (Chung and Ferl, 1999; Zeng *et al.*, 1999). The ADH mutant couldn't synthesize ADH or produce ethanol. But it can produce LDH and result in pH declining, so the mutant was very sensitive to waterlogging (Roberts *et al.*, 1984a, b; Sairam *et al.*, 2008). In this research, the results indicated that the WTI was negative correlation with the BnLDH enzyme activity and expression at 24 and 48 h after waterlogging treatment. It may be the high expression of *BnLDH* results in the pH declining quickly in the cells, which is harmful for the plants. The results also indicated that LDH pathway was not the main pathway during the waterlogging treatment and had other pathway to compensate the energy loss.

Based on some reports, aerenchyma formation and development of lateral roots may be the most important to plant tolerance to anoxic conditions (Colmer and Voesenek, 2009; Shabala, 2011). The aim of the method is to improve the utilizing efficiency of oxygen.

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