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Research Article

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

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Abstract: A *Formate dehydrogenase* (FDH) gene, named *BnFDH-1*, was cloned from oilseeds (*Brassica napus* L.) by the Rapid Amplification of cDNA Ends (RACE) method. The Complementary DNA (cDNA) consisted of 1488 bp and had an open reading frame of 1140 bp encoding a polypeptide of 384 amino acids with a molecular weight of 42.3 kD and an isoelectric point of 8.17. *BnFDH-1* showed high homology to known *FDH* genes, especially *FDH* from *Arabidopsis thaliana*. Conserved domain search and motif characterization identified BnFDH protein as Rossmann-fold NAD (P) (+)-binding proteins. NetPhos 2.0 search predicted 18 significant phosphorylation sites. To investigate the effect of *BnFDH* on waterlogging tolerance of *B. 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. *BnFDH-1* was cloned from the 12 materials also, but the results indicated all of them had same protein sequence. *BnFDH* expression level in 12 *B. napus* cultivates with different waterlogging tolerance after waterlogging treatment was measured. The results indicated expression level of *BnFDH* in 12 cultivates had significant difference. But correlation analysis proved that *BnFDH* expression level was uncorrelated with Waterlogging tolerance index (WTI). So it was not the *BnFDH* that was key gene for the waterlogging tolerance of *B. napus*.

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

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 CO_2 and O_2 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 reports, waterlogging leaded to 10-15% yield loss for rapeseeds and wheat and about 11% yield loss for cotton in the world.

Oxygen dissolved in soil water reduced and resulted in hypoxia because of the slow diffusion rate of oxygen and limited solubility in water during waterlogging (Sairam *et al*., 2008). Two-dimensional electrophoresis found that more than 20 anaerobic proteins over expressed in maize roots under low oxygen conditions and most of them were involved in sugar metabolism and fermentation (Dennis *et al*., 2000; Sachs *et al*., 1980). 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 (Good and Muench, 1993; Kennedy *et al*., 1992). And a number of enzymes including *Formate 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 induced during waterlogging (Kennedy *et al*., 1992; Ricard *et al*., 1994). Waterlogging tolerance of plants was closely related with ATP concentrations and the induced expression levels of *ADH* and *PDC* based on some reports (Drew, 1997; Waters *et al*., 1991), because reduction in oxygen supply leaded to an 18-fold reduction in ATP production and plants had to

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compensate for the loss by accelerating sugar metabolism and glycolysis (Dolferus *et al*., 2001).

As waterlogging, arerobic metabolism was transformed into annerobic metabolism. According to Davis-Roberts pH-stat hypothesis, the primary response of plants to O_2 limitation was characterized by a burst of lactate production, which resulted in lower cytoplasm pH and activated the over expression of *PDC* and *ADH* and inhibited the expression *LDH*. Then *PDC* and *ADH* were active and ethanol metabolism started (Agarwal and Grover, 2006).

FDH induced by one-carbon metabolites, such as methanol, formaldehyde and formate and *FDH* transcripts increased quickly and strongly in response to various stresses (Li *et al*., 2001). In the study, *FDHs* were cloned from 12 different waterlogging tolerance oilseeds and *FDH* expression was detected by real time PCR in 12 lines. We want to know if there has any correlation between *FDH* and WTI.

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.

Extraction of total RNA: Total RNA was isolated with CTAB method from different tissues of the 12 materials (Jaakola *et al*., 2001) and the contaminated DNA in RNA samples was removed by digestion with RNase-free DNase I (TaKaRa).

RACE and 5' RACE for cloning FDH cDNA ends from *B. napus*: Five µg of equally proportioned (w/w) mixture of total RNA from various organs of Zhongshuang 9 was used as template to carry out Rapid Amplification of cDNA Ends (RACE) in terms of the user manual (Gene Racer kit, Invitrogen, USA). Based on multi-alignment of *FDH* sequences from *A. thaliana* and other plants, two forward and two reverse primers were designed corresponding to conserved sites of *FDH*: sense primers FDH-31 (5'- TGTACCA TGA CAGGCTCCAGAT -3') and FDH-32 (5'- GGACCA TCCATGGCGTTACAT -3') for 3' cDNA end(s) amplification of *B. napus FDH* gene(s), while antisense primers RFDH-51 (5'- GTCATG GTACAA CAAG TTGCA -3') and RFDH-52 (5'- TGCCTT CCAG ATCATACGCTCT -3') for 5' cDNA end(s). FFDH-31 and FFDH-32 were paired with kit primers 3' Primer and 3' Nested Primer respectively for primary and nested amplification of 3' cDNA ends. As for primary and nested amplification of the 5' cDNA end, kit primers 5' Primer and 5' Nested Primer were paired with RFDH-51 and RFDH-52, respectively. Polymerase Chain Reactions (PCRs) were carried out in kit-recommended 50-µL standard amplification system containing 1.5 units of *Taq* DNA polymerase (TaKaRa) and other ingredients. For primary amplification, 1 µL of total first strand cDNA was used as template. Then 0.1 µL of primary amplification product was used for nested amplification. The 4 reactions were carried out on PTC-200 (BIO-RAD, US) under following conditions: predenaturation at 94°C for 2 min, 30 cycles of amplification of 94°C for 1 min, 50°C for 1 min and 72°C for 1.5 min and followed by a final extension at 72°C for 10 min.

Amplification of full-length cDNA sequence: Based on the obtained 5' and 3' cDNA ends, a pair of primers was designed to amplify the cDNA of *BnFDH*: FBnFDH (5'- GGTTCTACAAAAATATCGACACGA -3') and RBnFDH (5'- GTCACGAAAT ATCAAC GTTACATAC-3'). In the cDNA amplification, 0.5 µL of total cDNA as template and 1.25 units of Pyrobest DNA Polymerase (TaKaRa) were used in a kit-recommended 50-µL PCR reaction. Cycling conditions were as follows: predenaturation at 94°C for 2 min, followed by 35 cycles of amplification (94°C for 1 min, 50°C for 1 min and 72°C for 3 min), succeeded by a final extension at 72°C for 10 min. After the reaction, 1.5 units of *Taq* DNA polymerase was added into each tube and the tubes incubated at 72°C for 20 min for dA-tailing of the PCR product.

PCR products detection, subcloning and sequence analysis: PCR products were analyzed by 1% agarose gel with GoldView/1×TAE electrophoresis and detected by UV light. DNA of target bands was recovered using a Gel Extraction Mini Kit (Watson Biotechnologies, Inc., China) and ligated to the pMD18-T vector. Competent cells of DH5α were prepared by a $CaCl_2$ method for transformation of recombinant vectors. Transformants were subjected to antibiotic selection and Isopropyl-beta-D-Thiogalactopyranoside (IPTG) /X-gal blue-white screening. White colonies were cultured and identified by PCR. Positive clones were sequenced at Beijing Sunbiotech 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 done 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).

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 as 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, radicle 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).

Evaluation of waterlogging tolerance of 12 materials: Real time PCR was performed to detect the expression of *BnFDH* 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 real time PCR reaction with primer pair QFFDH (5'- AAGAATCCTAACTTCCTTGGCTGC-3') /QRFDH (5'- GAAGGTCAGGGA TATGCTTCTT GA-3'). Annealed at 58°C, amplifications were taken for 35 cycles. Primers FACT (5'-TGGGTTTGC TG GTGACGAT-3') and RACT (5'-TGTGCCT AGGACG ACCAACA-3') were used as internal control. Ct value was recorded.

RESULTS

Sequence cloning of BnFDH: The results of 3' RACE showed a band about 400 bp and T-vector colonies showed plentiful variation of insert length. Representative colonies were sequenced. The accurate length of the 3' cDNA ends was 339, 369 and 384 bp, respectively (poly (A) not included) and the only difference of them was splicing site. NCBI Nucleotide-nucleotide BLAST (Blastn) (Altschul *et al*., 1997) proved sequences showed high homologies to *FDH* genes reported. The results of 5' RACE showed a bright band of about 700 bp and an accurate length of 727 bp, which were also in consensus with homology-based length prediction. Its homology to *FDH*s was proved by Blastn.

Gel detection of the cDNA amplification showed one band about 1400 bp and the accurate length was 1383 bp and the sequence showed identities to 5' and 3' cDNA ends in corresponding regions.

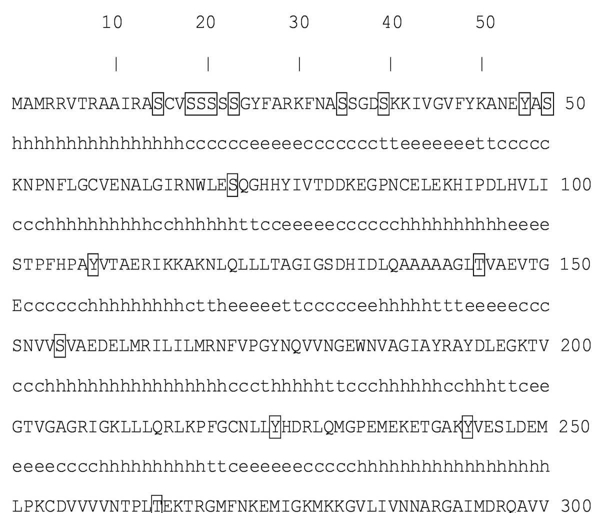

Fig. 1: Predicted secondary structures and phosphorylation sites of BnFDH-1
h: Helix; e: Extended strand; t: Beta turn; c: Random coil; The predicted significant phosphorylation sites were boxed

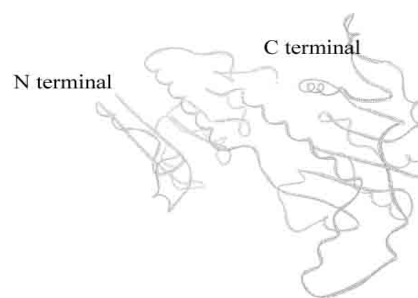

Fig. 2: Predicted tertiary structures

Molecular characterization of BnFDH nucleotide sequence: The mRNA of *BnFDH-1* was 1488 bp (poly A not included) and contained two UTRs (a 135 bp 5' UTR and a 97 bp 3' UTR) and an ORF of 1140 bp (with stop codon TAA).

BnFDH-1 encoded a polypeptide of 384 amino acids with a calculated molecular weight of 42.3 kD and an isoelectric point of 8.17. NCBI blastp indicated that BnFDH-1 showed wide similarities to FDHs from other plants, 73.2 and 73.2% identity to *A. thalina* and *Populus trichocarpa FDH* sequence, respectively. SignalP 3.0 (Bendtsen *et al*., 2004) predicted that BnFDH-1 had not signal peptide. Both WoLFPSORT and TargetP 1.1 predict BnFDH-1 probably located in Mitochondrion. TMpred prediction revealed 1 transmembrane helice (I_{133}-A_{157}) in BnFDH-1. NetPhos 2.0 search (Altschul *et al*., 1997) predicted 18 significant phosphorylation sites (10 for Ser, 4 for Thr and 4 for Tyr, respectively) (Fig. 1) suggesting that phosphorylation may be a posttranslational modification of BnFDH-1. NCBI Conserved Domain

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

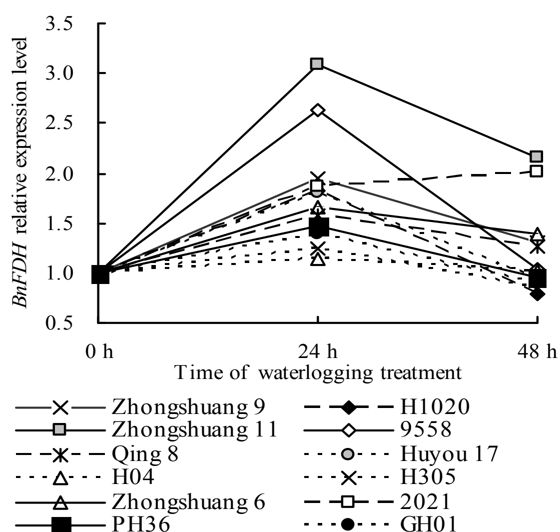

Fig. 3: *BnFDH* expression with time of waterlogging treatment

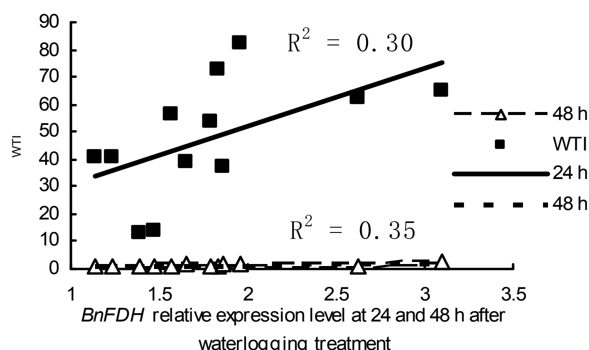

Fig. 4: Correlation between WTI and *BnFDH* expression level

search (Marchler-Bauer and Bryant, 2004) found it belongs to Rossmann-fold NAD (P) (+) -binding proteins. All proved BnFDH-1 was a representative FDH protein. SOPMA (Geourjon and Deleage, 1995) predicted that the secondary structure of BnFDH-1 was mainly composed of alpha helices (41.93%) and random coils (30.73%), while extended strands (18.75%) and beta turns (8.59%) contribute a little (Fig. 1). Alpha helices mainly distributed in the N terminal and the middle, which was similar with the predicted 3D structure (Fig. 1 and 2). *BnFDH-1* was cloned from other 11 materials also, but the results indicated all of them had same protein sequence.

Waterlogging Tolerance Index (WTI) of 12 materials: Waterlogging Tolerance Index (WTI) of 12 cultivates with different waterlogging tolerances was measured and the results were listed in Table 1. The results indicated WTI among 12 lines had significant difference, so the 12 lines could be used as materials for waterlogging research.

Transcription of BnFDH in root of 12 different waterlogging tolerances materials: The results suggested that transcription level of the *BnFDH* gene had significant difference in 12 cultivates with different waterlogging tolerances ability. *BnFDH* expression in Zhongshuagn 11 increased quickly after waterlogging treatment and slowly in some lines, for example, H305 and GH01 (Fig. 3). But all the *BnFDH* expression in 12 lines was induced by waterlogging and reached their peak at 24 h after treatment. The expression level decreased from 24 to 48 h.

BnFDH expression was uncorrelated with WTI: Correlation between WTI and *BnFDH* expression at 24 48 h after waterlogging treatment was analyzed and the results indicated the correlation coefficient was 0.31 and 0.04, respectively (Fig. 4). It indicated there was no significant correlation between them. The results proved that the transcription level of *BnFDH* gene was not the reason leading to different of waterlogging tolerance of *B. napus*.

DISCUSSION

The results of the response of plant to hypoxia stress in maize roots by two-dimensional electrophoresis shown 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, FDH, LDH and ADH (Chung and Ferl, 1999; Zeng *et al*., 1999). The difference genotypes in waterlogging tolerance could be distinguished according to the changed patterns of enzymatic activities because POD and CAT activity under waterlogging determine the status of oxidative stress (Zhang *et al*., 2007). A short anoxic stress (1-2 h) increased the production of superoxide production in soybean roots and longer time of anoxia (3-5 h) show less injury because of increased ability to cope with oxygen free radicals (Vantoai and Bolles, 1991). ADH deficient mutant can produce LDH and result in pH declining, so the mutant was very sensitive to waterlogging (Roberts *et al*., 1984a; 1984b; Sairam *et al*., 2008), but over-expression of *ADH* gene could not improve plant waterlogging tolerance. It may be some genes induce by waterlogging and response to hypoxia stress, but not decide the tolerance. In this

research, *BnFDH* also induced by waterlogging, but its expression was not correlated with WTI. It indicated *BnFDH* was not the key gene for waterlogging tolerance. Our research also indicated *BnADH* also responded to hypoxia stress and had no correlation with WTI (unpublished).

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REFERENCES

Agarwal, S. and A. Grover, 2006. Molecular biology, biotechnology and genomics of flooding-associated low O_2 stress response in plants. Crit. Rev. Plant Sci., 25(1): 1-21.

Altschul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller *et al*., 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res., 25(17): 3389-3402.

Bendtsen, J.D., H. Nielsen, G. von Heijne and S. Brunak, 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol., 340(4): 783-795.

Chung, H.J. and R.J. Ferl, 1999. Arabidopsis alcohol *dehydrogenase* expression in both shoots and roots is conditioned by root growth environment. Plant Physiol., 121(2): 429-436.

Cornelious, B., P. Chen, Y. Chen, N. de Leon, J.G. Shannon and D. Wang, 2005. Identification of QTLs underlying water-logging tolerance in soybean. Mol. Breed., 16(2): 103-112.

Dennis, E.S., R. Dolferus, M. Ellis, M. Rahman, Y. Wu, F.U. Hoeren *et al*., 2000. Molecular strategies for improving waterlogging tolerance in plants. J. Exp. Bot., 51(342): 89-97.

Dolferus, R., E.J. Klok, K. Ismond, C. Delessert, S. Wilson, A. Good *et al*., 2001. Molecular basis of the anaerobic response in plants. Iubmb. Life, 51(2): 79-82.

Drew, M.C., 1997. Oxygen deficiency and root metabolism: Injury and acclimation under hypoxia and anoxia. Ann. Rev. Plant Phys., 48: 223-250.

Geourjon, C. and G. Deleage, 1995. SOPMA: Significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments. Comput. Appl. Biosci., 11(6): 681-684.

Good, A.G. and D.G. Muench, 1993. Long-term anaerobic metabolism in root-tissue-metabolic products of pyruvate metabolism. Plant Physiol., 101(4): 1163-1168.

Jaakola, L., A.M. Pirttila, M. Halonen and A. Hohtola, 2001. Isolation of high quality RNA from bilberry (*Vaccinium myrtillus* L.) fruit. Mol. Biotechnol., 19(2): 201-203.

Kennedy, R.A., M.E. Rumpho and T.C. Fox, 1992. Anaerobic metabolism in plants. Plant Physiol., 100(1): 1-6.

Li, R., P.C. Bonham-Smith and J. King, 2001. Molecular characterization and regulation of *Formate dehydrogenase* in Arabidopsis thaliana. Can. J. Bot., 79(7): 796-804.

Marchler-Bauer, A. and S.H. Bryant, 2004. CD-search: Protein domain annotations on the fly. Nucleic Acids Res., 32(Web Server issue): W327-331.

Ricard, B., I. Couee, P. Raymond, P.H. Saglio, V. Saintges and A. Pradet, 1994. Plant-metabolism under hypoxia and anoxia. Plant Physiol. Bioch., 32(1): 1-10.

Roberts, J.K.M., J. Callis, O. Jardetzky, V. Walbot and M. Freeling, 1984a. Cytoplasmic acidosis as a determinant of flooding intolerance in plants. P. Natl. Acad. Sci-Biol., 81(19): 6029-6033.

Roberts, J.K.M., J. Callis, D. Wemmer, V. Walbot and O. Jardetzky, 1984b. Mechanism of cytoplasmic ph regulation in hypoxic maize root-tips and its role in survival under hypoxia. P. Natl. Acad. Sci-Biol., 81(11): 3379-3383.

Sachs, M.M., M. Freeling and R. Okimoto, 1980. The anaerobic proteins of maize. Cell, 20(3): 761-767.

Sairam, R.K., D. Kumutha, K. Ezhilmathi, P.S. Deshmukh and G.C. Srivastava, 2008. Physiology and biochemistry of waterlogging tolerance in plants. Biol. Plantarum., 52(3): 401-412.

Ueno, K. and H. Takahashi, 1997. Varietal variation and physiological basis for inhibition of wheat seed germination after excessive water treatment. Euphytica, 94(2): 169-173.

Vantoai, T.T. and C.S. Bolles, 1991. Postanoxic injury in soybean (glycine-max) seedlings. Plant Physiol., 97(2): 588-592.

Waters, I., S. Morrell, H. Greenway and T.D. Colmer, 1991. Effects of anoxia on wheat seedlings. 2. influence of o2 supply prior to anoxia on tolerance to anoxia, alcoholic fermentation and sugar levels. J. Exp. Bot., 42(244): 1437-1447.

Zeng, Y., Y. Wu, W.T. Avigne and K.E. Koch, 1999. Rapid repression of maize invertases by low oxygen. Invertase/sucrose synthase balance, sugar signaling potential and seedling survival. Plant Physiol., 121(2): 599-608.

Zhang, X., J. Cheng, H. Wang, J. Li and C. Zhou, 2007. Genetic difference of waterlogging tolerance in rapeseed (*Brassica napus* L.). Chin. J. Oil Crop Sci., 29(2): 204-208.