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Research Article

Alcohol dehydrogenase (ADH) activity in soybean (*Glycine max* [L.] Merr.) under flooding stress

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Abstract:
Sowing time of soybean (*Glycine max* [L.] Merr.) often coincides with the early onset of rainy season. Germinating seeds encounter a transient to prolonged period of water-logging that causes anoxia (absence of oxygen) and hypoxia (insufficient oxygen) resulting in poor germination. This reduces crop stability and yield. One of the factors responsible for flood tolerance is activity of alcohol dehydrogenase (ADH) during flood. The effect of ADH activity during flooding and difference in flood tolerance level were investigated using two soybean cultivars, Peking and Tamahomare, and their F_9 recombinant inbred lines (RILs). Tamahomare showed higher ADH activity than Peking. There was a great variation in ADH activity among the RILs. QTL analysis detected five QTLs for ADH activity (*qAas*1-5) on five linkage groups, LG_A2, D1a, F, K and L. The QTL *qAas*4 was close to a QTL for shoot damage and conductivity of germinating seeds after flooding treatment.

Keywords: Soybean, Alcohol dehydrogenase (ADH), Flood tolerance, Quantitative Trait Loci (QTL), Linkage group

Introduction

Soybean is the world's foremost provider of protein and oil (Black *et al.*, 2006). This bushy, green leguminous plant is grown almost everywhere in the world. The sowing time of soybean coincides with the rainy season in most parts of the world. Of numerous stresses during its life cycle, stress due to water-logging during germination is detrimental to germinating seeds, seedling growth, crop establishment and finally the yield (Hou and Thseng, 1992). Plants respond to stresses by altering the expression of various genes (Chaves *et al.*, 2009). Alcohol dehydrogenase (ADH) activity increases under different stress conditions, including anoxia (Crawford, 1977). ADH null mutants were found sensitive to anoxia during flooding (Russell *et al.*, 1990). Previous studies have shown that ADH activity increases during the anaerobic stress in many plant species including maize (Drew *et al.*, 1979). Since ADH null mutants were more sensitive to anoxia, ADH was considered essential for anoxia survival, presumably because it recycles NAD+ for continued glycolysis in the absence of oxygen. In absence of aerobic respiration, ADH regenerates NAD+ by reducing acetaldehyde to ethanol; the reaction, which permits continued glycolysis without cytoplasmic acidosis, in contrast to the alternative pathway of lactic acid formation (Roberts *et al.*, 1989). Exposure to oxygen deficits is widespread in biological systems beyond the induction of alcoholic or lactic acid fermentation as the biochemical response to hypoxia/anoxia. Depending upon the species, anaerobic metabolism encompasses much more than simple glycolytic metabolism (Kennedy *et al.*, 1992). However, the effect of ADH on flooding tolerance of germinating soybean seeds is not known. Thus, it was necessary to know the role and mechanism of ADH during the seed germination under the flooding stress. In this study we investigated ADH activity of germinating seeds of recombinant inbred lines and elucidated the effect of ADH on flooding tolerance. We detected five putative QTLs for ADH activities.

Material and methods

Plant material

Two soybean cultivars, Peking, Tamahomare and 96 F_9 Peking Tamahomare recombinant inbred lines (PT-RILS) were used in this experiment. Peking is a highly flood tolerant Chinese cultivar and Tamahomare is a flood susceptible Japanese cultivar. Peking has thick black seed coats and Tamahomare has thin, yellow colored seed coats (Fig. 1). The recombinant inbred lines were maintained through single seed descent of the second filial generation

(F_2) seeds from the cross between Peking and Tamahomare.

Assay of alcohol dehydrogenase

The assay of ADH activity was carried out according to Russell *et al*., (1990) with some modifications. Three seeds per line were soaked in water for three hours, to create anaerobic condition. The water was drained out, extraction buffer was added and the seeds were crushed and homogenized using Polytron. The extraction buffer consisted of 50 mM Tris-Cl [pH 6.8]. 15% v/v glycerol, 5% v/v β Metacaptoethanol, and 1 mM PMSF (phenylmethylsulfonyl fluoride). The samples were spun at 12,800 g for 5 minutes; supernatant was saved as crude protein. The enzyme reaction mixture contained 150 µL of 1 M Tris-Cl (pH 8.0), 20 µL of 10 mg/mL NAD+, 10 µL of sample extract, and 800 µL of water. It was mixed three times by inversion, placed in the spectrophotometer, and adjusted to 0.000 at A_{340}. The assay was started by adding 20 µL of 50% ethanol. After mixing, the A_{340} value was recorded every 15 seconds for first 1 minute and every 30 seconds thereafter up to 3 minutes. Data were checked for linearity over time. The ΔA/min was also shown to be directly proportional to the amount of extract added. The data was converted to units of ADH activity (U). One unit of ADH activity (U) is defined as the amount of ADH which catalyzes the reaction to produce 1 µM NADH per min at 25 ^{0}C. Samples were normalized on the basis of A_{280} units, a measure of crude extract concentration. The standard ADH activity was determined by measuring the change in NADH absorbance at 340 nm (E34 NADH = 6220 m^{-1} min^{-1}) in a spectrophotometer at 25 ^{0}C. In presence of ADH and NAD+, ethanol is oxidized to acetaldehyde and NADH, which can be monitored spectrophotometrically at 340 nm.

Phenotyping

The germination rate after a day of flooding treatment, healthy growth rate, damage of roots and shoots after flooding stress were recorded, and used as indices to measure the flooding tolerance in the germinating seeds. On the basis of phenotypic indices, the levels of flooding tolerance of Peking and Tamahomare and PT RILs were evaluated.

QTL analysis

For genotyping, total genomic DNA of plants was extracted from young leaves by the Cetyl trimethylammonium bromide (CTAB) method (Murray and Thompson, 1980). PCR analysis was carried out in a 10 µL reaction volume. Reaction mixture consisted of 1 µL of 30 ng DNA, 1 µL 10xPCR buffer, 2 mM dNTPs, 0.6 µL nTaq Polymerase, 0.003 µL of 100 PM solution of forward and reverse SSR primers and 6.34 µL distilled water. PCR was carried out in 33 cycles, 60 s denaturation at 93 ^{0}C, 30 seconds of annealing at 47 to 57 ^{0}C based on the annealing temperature of the primers and 1 minute of extension at 68 ^{0}C. To detect polymorphism, the PCR products were electrophoresed in 12% polyacrylamide gel at 500 V, 500 A for 2 hours. Polymorphic bands in RILs were visually noted against the position of the bands of the parents. A total of 288 SSR markers distributed over the 20 linkage groups (LGs) of each were used for the QTL analysis. Linkage maps were constructed from the genotype data with MAPMAKER/EXP 3.0 (Lander *et al.,* 1987). The genotypic distance was estimated by using Kosambi map function (Kosambi, 1944). Putative QTLs were detected by using the composite interval mapping (CIM) function of Windows QTL Cartographer Version 2.5 (Wang *et al.*, 2007). The CIM threshold was based on the result of 1000 permutation tests at the 5 % level of significance (Churchil and Doerge, 1994) and walking speed was set to 1 cM. The additive and dominance effects and the phenotypic variance explained by each QTL were analyzed. Coefficient of determination (R2), also known as the contribution, was estimated at maximum LOD score value for each peak. The LOD score peaks above the threshold value set to 2, were used as the most likely position on the linkage map. The peaks were named according to a convention based on R2 and LOD score.

Results and discussion

The ADH activity of seeds germinating under water for different time periods was studied. The ADH activity was found to be low in flood tolerant cultivar Peking and high in flood sensitive Tamahomare. Thus, the ADH activity and flooding tolerance showed a unique inverse relationship. Flooding stress is manifested in two forms, physical injury and anaerobic stress. The physical injury occurs due to the rapid absorption of water, breakdown of seed structure, out flow of seed contents all of which add to hamper normal germination. We found the rate of absorption of water by Peking was slower than that of Tamahomare and the physical strength of Peking was more than that of Tamahomare, which could be due to thick seed coat of Peking. When the experiment was repeated without seed coat the trend of ADH activities was similar (Fig. 2). Therefore, we concluded that the difference was not just only due to the permeability of seed coat.

Although Brzezinski *et al*. (1986) reported that there was no indication of ADH under anaerobic condition in soybean cultivar 'Prize', later researchers detected

the ADH activity in soybean (Newman and Van Toai, 1992). No further research reports on 'Prize' were found. Flooding seed for 1 to 3 days after the start of imbibitions or for 1 to 24 hours was more detrimental to germination at 15 ^{0}C than at 25 ^{0}C. One hour of flooding caused physical and imbibitional damage to the seed that is further aggravated by chilling temperature (Wuebker *et al*., 2001). In seeds tolerant to soaking, ethanol production was limited during anoxia. In seeds intolerant to soaking, anoxia causes an acceleration of glycolysis induction of ADH activities and production of a large quantity of ethanol as exclusive end product of glycolysis (Crawford, 1997).

ADH activities in PT RILs

The ADH activities in PT RILs showed a wide variation. The activity was as low as 50 U in PT 37 to as high as 320 U in PT 13. The parents, Peking and Tamahomare had 160 U and 280 U of ADH activities, respectively (Fig. 3).

Only the difference in seed coat pigmentation and thickness could not explain the difference in flood tolerance level of PT-RILs. So, to identify genetic factors of ADH, QTL analysis was done. The QTL analysis using genotyping data was performed using win Cart QTL. The analysis detected the presence of 5 QTL in 5 linkage groups (Table 1 and Fig. 4).

Based on the contributing factor (R2) and on Log of Odds (LOD) set to by permutation, the peaks were named *qAas*1- *qAas*5, as the abbreviation of QTL(q) for ADH (A) activity (a) in seeds(s); the digits represent their number on the basis of R2. The five QTLs were located in the LG_A2, D1a, F, K and L. The name of the QTL, the closest marker, LOD score, contribution and additive effects are shown in the table1. One of the QTLs, i.e. *qAas*-4 (Fig. 4) was found closer to two of the flood tolerance related traits i.e. QTL for electrical conductivity (EC) and QTL for shoot damage (DS) in the seedlings from the flood treated seeds, the function and expression of genes of other loci are yet to be clarified.

ADH is a group of dehydrogenase enzymes that occur in many organisms and facilitate the inter-conversion between alcohols and aldehydes or ketones. Most of the alcohol dehydrogenase characterized at gene level in plants belong to 3 groups of dimeric Zn containing enzymes, NAD+ dependent 'Classical" ADH active on ethanol is one of them (Chase, 1999). Soybean ADH molecules exist as functional dimmer. The dimeric ADH structure has at least 2 stable hetero dimers (Gorman and Kiang, 1978). Three distinct classes of soybean ADH cDNAs were revealed by sequence analysis. RNA blot hybridization showed differential expression for these genes. One gene expressed in all seedling organs was inducible by anaerobiosis, second was expressed only in anaerobic organ and the third was expressed predominantly in anaerobic roots (Newman and Van Toi, 1992). Therefore, ADH is considered to play significant role in flood tolerance.

ADH activities were studied in other crops too. Comparison of the behavior of wild type (Adh1+) and mutant Adh1- m9 barley plants in flooded conditions showed a specific requirement for a functional ADH1 polypeptide in the immersed seed but not at later stages of development (Harberd and Edwards, 1982). ADH was one of the candidate gene for the control of stage shift from diving cells to endoreduplication, 7 and 21 days after flowering (just before and after the initiation of seed fill) (Beilinson *et al*., 2005). Short term cold stress induces ADH 1 gene expression in rice and maize (Christie *et al*., 1991). Regulatory elements different from those involved in cold and osmotic responses were required for induction of ADH by anoxia and hypoxia. This implies that transcription complexes for ADH gene under cold, osmotic and low oxygen stress may share common factors but probably also embody some discrete factors (Conley *et al.*, 1999). Tolerance to anoxia differs with season. Anoxia during warmer condition is more detrimental than in cooler condition as during warm condition the rate of metabolism is high and the demand for oxygen becomes acute (Crawford, 2003). ADH activities were lost when seeds were stored at -10 ^{0}C (Duke *et al.*, 1977).

While ADH activities help cell survival during low oxygen stress in water logged roots, hypoxic regions around vascular core, it also has additional metabolic roles including aerobic fermentation, acetaldehyde detoxification, carbon reutilization and even the production of aromatic compounds in the nectary (Garabagi *et al.,* 2005). The study ranging from the response of enzymes to anoxia to gene induction and regulation at molecular level only help to understand a segment of anaerobic metabolism. The serious drawback being the study of flood tolerance is mostly done in crops with extremely limited tolerance to anoxia, like pea, maize, soybean, etc. (Kennedy *et al*., 1992).

ADH and also pyruvate decarboxylase are abundant in pollens, help in the production of acetaldehyde and ethanol, thus resulting in fermentation in fully oxygenated cells (Tadege *et al*., 1999). ADH was also active throughout callus tissue development in

cactaceae (Torquato *et al*., 1995). The protein was found in roots, seeds, leaves, pollen, fruits of both monocot and dicot species, besides ADH expression was induced by anaerobiosis and 2- 4 dichlorophenoxyacetic acid (Wolyn and Jelenkovic, 1990). Thus ADH activity is universal in plant and its activities during flooding stress are important for the survival. Although a few reports were published on the QTL analysis of flooding tolerance on mature soybean plants (Cornelious *et al.*, 2005, Reyna *et al.*, 2003 and VanToai *et al.*, 2001) this is the first report on the QTL for the ADH activities during germination stage.

The crop potential for quantity and quality of yield is hampered by soil water-logging starting from seed germination. It can be prevented effectively with the knowledge of ADH activity level of the line or cultivar and by taking preventive measures like avoidance of soil waterlogging and selection of relatively tolerant crops. For the stable production of soybean it is a prime need to have soybean cultivars with flooding tolerance ability during germination. In case of soybean, cultivars with low ADH activity should be chosen to sow when and if the sowing time coincides with rainy season.

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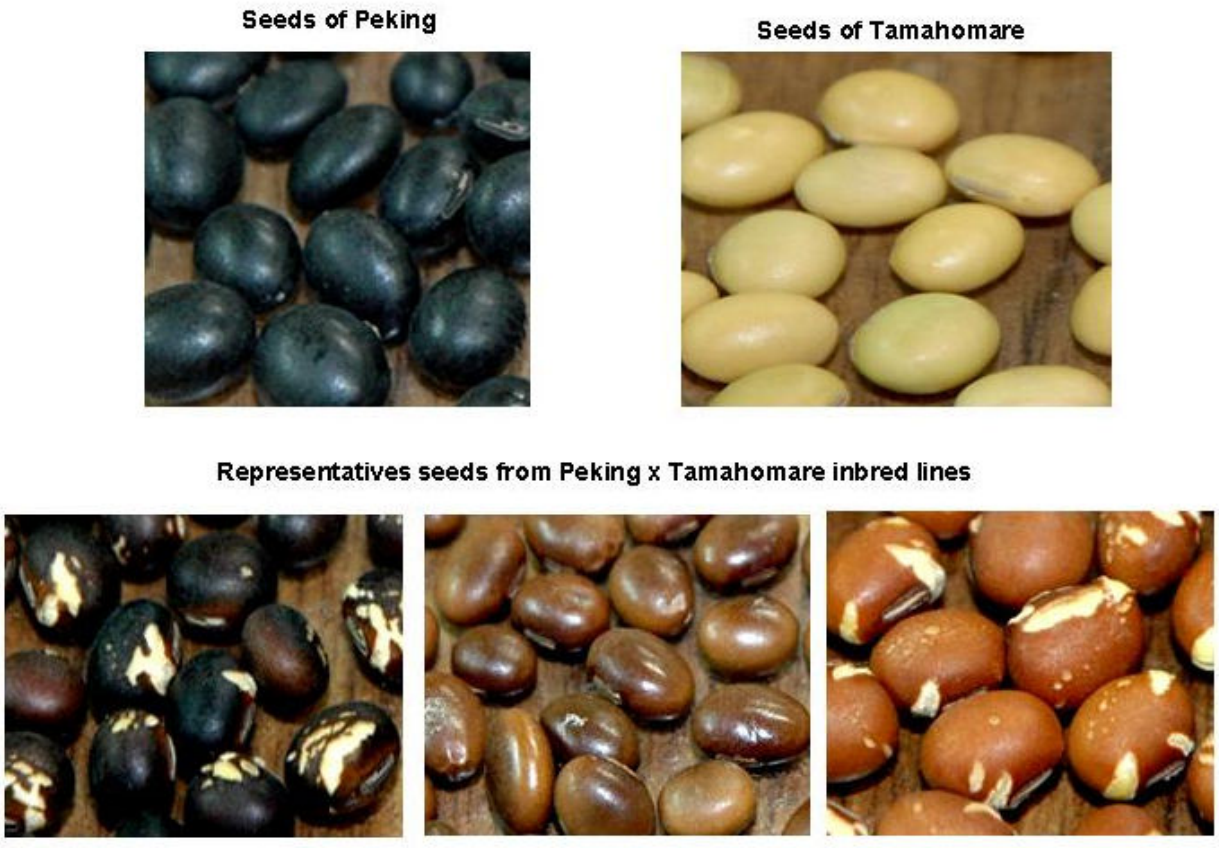

Fig. 1 Seed coat colors of parental lines and their recombinant inbred lines.

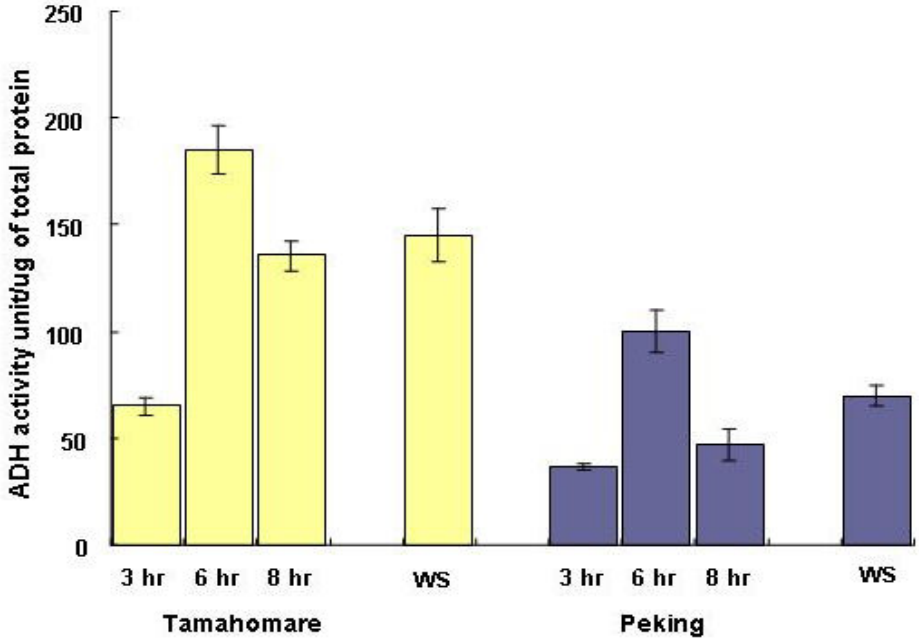

Fig. 2 The ADH activities in Tamahomare and in Peking after 3, 6 and 8 hours of flooding stress with seed coat and average of activities without seed coat (WS).

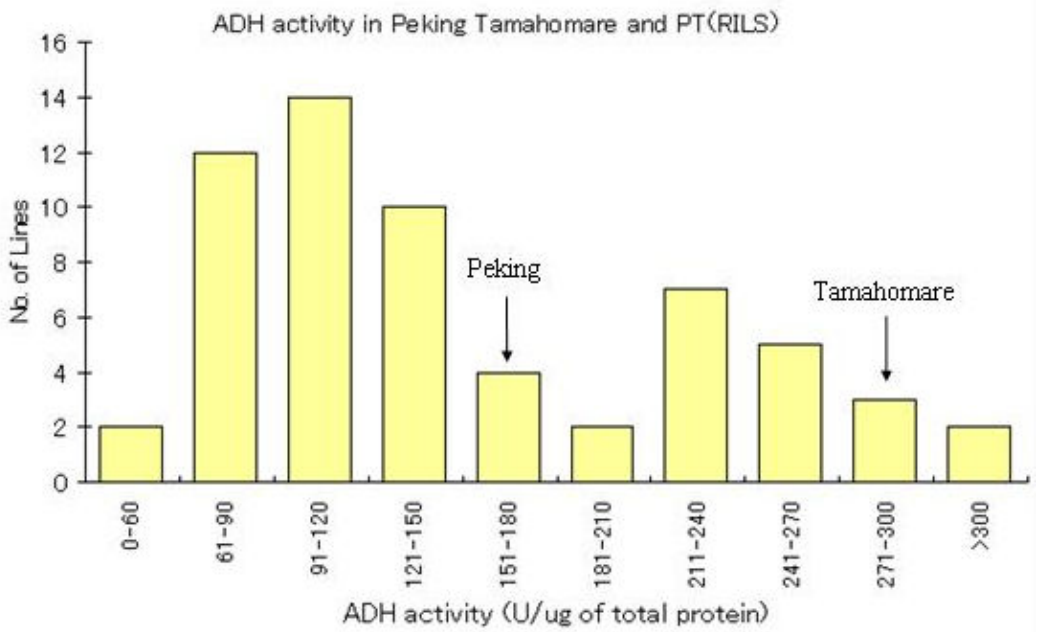

Fig. 3 ADH activities in the PT RILs. The bar height show the number of lines falling into the groups shown in the X axis.

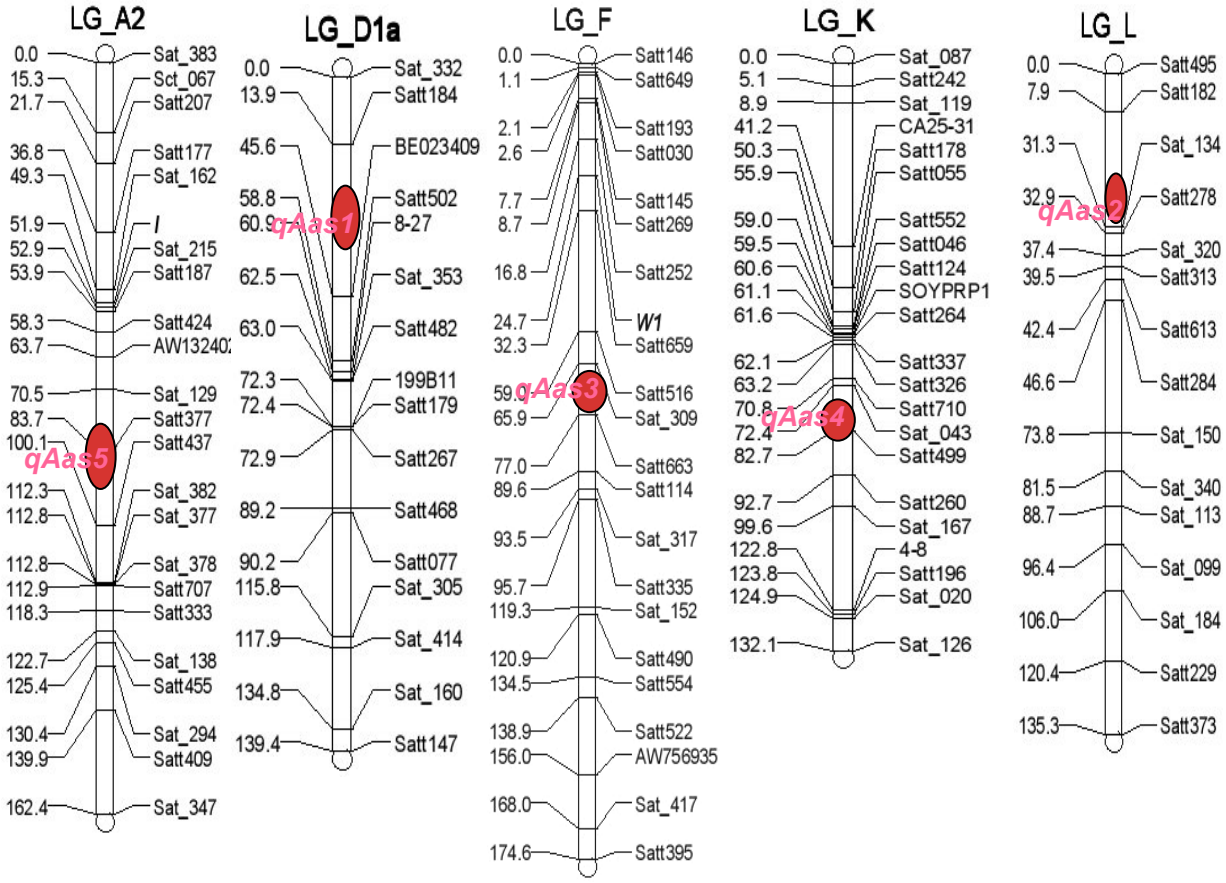

Fig. 4 The position of QTLs in the linkage group. Only the linkage groups in which QTL were detected are shown.

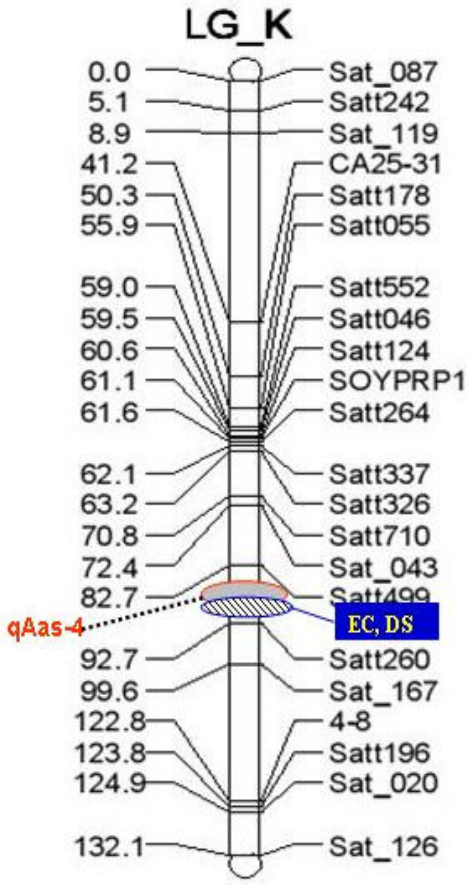

Fig. 5 QTL *qAas*-4 on the linkage group K was found closely located to two QTLs related to flood tolerance traits (EC for electron conductivity and DS for damage of shoots from flood treated seeds).

Table 1. The statistical description of QTLs for ADH activities.

QTL	Linkage Group	Closest Marker	Position	LOD Score	Additive Effect	R2
qAas1	D1a	Satt184	28.1	2.31	48.00	0.349
qAas2	L	Sat_134	19.9	2.51	-41.09	0.274
qAas3	F	Sat_309	69.9	3.40	-29.60	0.138
qAas4	K	Satt499	87.5	3.32	25.49	0.104
qAas5	A2	Satt377	82.5	3.03	-25.23	0.103