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Characteristics of Expansins in Soybean (*Glycine max*) Internodes and Responses to Shade Stress

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ABSTRACT

In order to obtain better knowledge about the physiological mechanism of endurance shade in different soybean cultivars, two soybean cultivars soybean with different levels of shade endurance was plant under two different light condition and the characteristics of expansins in soybean internodes and their responses to shade stress were studied. Expansin proteins were extracted from the cell walls of soybean internodes by the methods of $CaCl_2$ and Hepes. The activities of expansins were measured with an extensometer. The expansins extracted with $CaCl_2$ had higher activity than Hepes. The results demonstrated for the first time the presence of expansins in soybean internodes, it could induce extension of cell walls in soybean and in cucumber hypocotyls. The expansins activities of endogenous and reconstituted cell wall extension depended on acidic pH. The changes in activity of expansins in different soybean cultivars in response to shade stress suggest that expansins may play a significant role in the endurance of relay cropping soybean to shade stress.

Key words: Soybean, shade stress, expansins, relay cropping, cell wall

INTRODUCTION

It has been recognized that biodiversity is key to securing global food supply (Thrupp, 2000). Relay or inter-cropping is a simple, effective approach to boosting crop yields and increasing land equivalent ratios (LERs) and widely adopted in the global challenge of securing the food supply (Li *et al*., 2009; Emuh, 2007). Corn-soybean relay cropping is one of the successful modes which used in South China to increase soybean production (Wang and Yang, 2007).

During the practice of relay cropping, soybean at vegetative growth stage grows under the canopy of surrounding corn. The absorption of solar radiation by surrounding vegetation result in a shade stress, which are vital cues for soybean to start elongation of their internodes. The excessive elongation of internodes causes lodging frequently at reproductive growth stage and decreases soybean yield (Wu *et al*., 2007). The endurance to shade stress for relay cropping soybean is positive correlation with it`s internode length (Chen *et al*., 2003; Liang and Liang, 1997). Therefore, a better understanding of how tolerant and sensitive varieties differ in their responses to shade stress, the physiological mechanism of soybean internode elongation in response to the stress are required.

There is clearly evidence that stem elongation mostly involves cellular expansion in plants. Increased cell wall extensibility is made possible by certain proteins that act on the molecular framework of the cell wall and thus allow the walls to stretch out, a process termed wall loosening.

The most well-characterized group of cell wall-loosening proteins is the expansions (Cosgrove, 2005; McQueen-Mason and Cosgrove, 1994; Song *et al*., 2007). However, for the internode of the soybean, the existences of expansins with the correlation between the activity of this protein and the elongation of internodes does not always hold. In order to obtain better knowledge about the physiological mechanism of endurance shade in different soybean cultivars, the first aim of the present work was to demonstrate the existence and characteristics of expansins in the internode of soybean. In addition, we used two soybean cultivars soybean with different levels of shade endurance to reveal the correlation between internode elongation characteristics and expansin proteins activity.

MATERIALS AND METHODS

Plant material and shade treatments: Two emi-determinate cultivars which have similar maturity (late), plant height and seed yield in sole croping system (monoculture), were chosen for the experiment. Both cultivars were supplied by the National Soybean Comprehensive Test Station, Nanchong city, Sichuan province, China. One was NANDOU12, which is extended in Southwest China in the corn-soybean relay cropping system because it is highly tolerant to shade stress. The other one, NAN022-2 is planted in sole croping system in the same zone and standard shade sensitive type.

The study was conducted from 2009 to 2010 in the crop planting laboratory of Sichuan Agriculture University, China. The seeds were sown in 500 mL plastic pots in a mixture of 1:1 (v/v) with perhite and vermiculite. Prior to sowing, each pot received approximately 150 mL of nutrient solution containing 7.5 M$(NH_4)_2SO_4$, 15 mM KH_2PO_4, 15 mM KNO_3, 3.3 μM $MnSO_4$, 1.8 μM $ZnSO_4$, 0.32 μM $CuSO_4$, 43 μM H_3BO_3, 0.53 μM Na_2MoO_4 and 86 μM Fe-EDTA. They were planted for 12 days at VC stage(unrolled unifoliolate leaves) in a climate-controlled plant growth chamber (15 h photoperiod, 150 μmol/ m^2/ sec photosynthetically active radiation, 9 h of dark), after which half of them were transferred to green shade growth chamber.

Green shading mimicking shade condition under the canopy in a relay cropping system was achieved using one layer of Lee 122 Fern Green, which reduced the PAR to 52 μmol/ m^2/ sec, the R/FR to 0.21 and the blue light photon fluence rate to 2 μmol/ m^2/ sec. (Measured by FieldSpec HandHeld, ASD).Wherever mentioned, controls refers to data from plants grown in light conditions with an unaltered spectral composition and PAR of 150 μmol/ m^2/ sec. All light treatments were started at approximately 8 AM each time the experiments were performed.

Measurement of plant growth: In order to measure internode elongation, the topmost internode lengths were measured using a caliper every day at the same time to calculate daily growth increments. Care was taken to choose seedlings that had similar starting lengths(about 1 cm). For each light treatment, a total of at least 15 to 20 seedlings were measured.

Acid-induced extension of endogenous expansins in soybean internodes: Acid-inducing Extension(AIE) was measured using a custom-built constant-load extensometer, modified from the design of GAO Qiang (Gao *et al*., 2007), with a pulling weight of 22.0510 g. This acid-induced extensibility of endogenous and reconstructive cell walls reflects the expansins content in the cell wall (Cosgrove, 1996). The topmost internodes from soybean were harvested for measurements and immediately frozen in liquid nitrogen. These internodes were then thawed, abraded and pressed and 10 mm segments were clamped in the extensometer plastice cuvette. The internodes were first

bathed in 1 mL of a 50 mM Hepes (pH 6.8) buffer for 30 min, after which the buffer was replaced with 50 mM acetate sodium buffer (pH 4.5). AIE was measured as the difference in the slopes of lines fitted through 120 min intervals before and after the observed bending point obtained upon the change in pH of the buffer.

Extension rate of reconstitution cell wall: The reconstituted AIE of extracted expansin proteins to cucumber hypocotyls was measured to further determine expansins activity according to the method of McQueen-Mason *et al.* (1992) with some modification. Frozen cucumber hypocotyls were thawed, abraded and boiled in water (20 sec) to eliminate edogenous expansins activity and then pressed and clamped on the extensometer. The hypocotyls were first bathed in 1 mL of a 50 mM Hepes (pH 6.8) buffer for 30 min, after which the buffer was replaced with expansin proteins extraction solution. The extension value was readed every 10 min.

Isolation of cell walls: The first internodes from plants of NANDOU12 cultivars growing under normal light conditions were harvested on the 16th d after sowing. Harvested materials were immediately frozen in liquid nitrogen and stored at -80°C until they were used. Isolation of cell walls were prepared as described (Feiz *et al.*, 2006). The materials were washed with distilled water and transferred into 500 mL of 5 mM acetate buffer, pH 4.6, 0.4 M sucrose and protease inhibitor cocktail (Sigma) 1 mL per 30 g of material fresh weight. The mixture was ground in a blender at full speed for 15 min. After adding PVPP (1 g per 10 g fresh weight of internode), the mixture was incubated in cold room for 30 min while stirring. Cell walls were separated from soluble cytoplasmic fluid by centrifugation of the homogenate for 15 min at 1000x g and 4°C. The pellet was further purified by two successive centrifugations in 500 mL of 5 mM acetate buffer, pH 4.6, respectively 0.6 M and 1 M sucrose. The residue was washed with 3 L of 5 mM acetate buffer, pH 4.6, on a nylon net (25 μm pore size). The resulting cell wall fraction was ground in liquid nitrogen in a mortar with a pestle prior to lyophilization. Starting with 15 g fresh weight of internodes, this process resulted in 4.1 g dry powder.

Expansins extraction by two methods: Typically, 0.7 g of lyophilized cell walls was used for one experiment. The main difference between the two methods was the extraction buffer. One was the $CaCl_2$ (5 mM acetate buffer, pH 4.6, 0.2 M $CaCl_2$ and 10 μL protease inhibitor cocktail).Another was Hepes solution (1 M NaCl, 25 mM Hepes, pH 7.0, 3 mM metabisulfite, 2 mM EDTA and 5 mM DTT) (McQueen-Mason *et al.*, 1992). Proteins were extracted by two extractions each time with 6 mL above buffer respectively for 2 h on ice and squeezed through nylon net.

The wall proteins were slowly precipitated with ammonium sulfate 0.39 g mL^{-1}. Precipitated proteins were pelleted by centrifugation (3500x g, 10 min, 4°C), and then resuspended in 50 mM acetate sodium buffer (pH 4.5) or 50 mM Hepes buffer (pH 6.8). Aliquots of the solutions were dialyzed. Protein concentration was determined according to Bradford (1976) and adjusted to 2 mg mL^{-1} for measurement of reconstituted to bioled cucumber hypocotyle.

RESULTS AND DISCUSSION

Acid-induced extension of endogenous expansins in soybean internodes: Expansins are consider as the primary enzymes involved in cell wall extension during plant growth and development and can mediate the acid-induced extension of the native cell wall. Figure 1 showed that native cell walls of soybean inter nodes exhibited long-term irreversible extension in the acidic

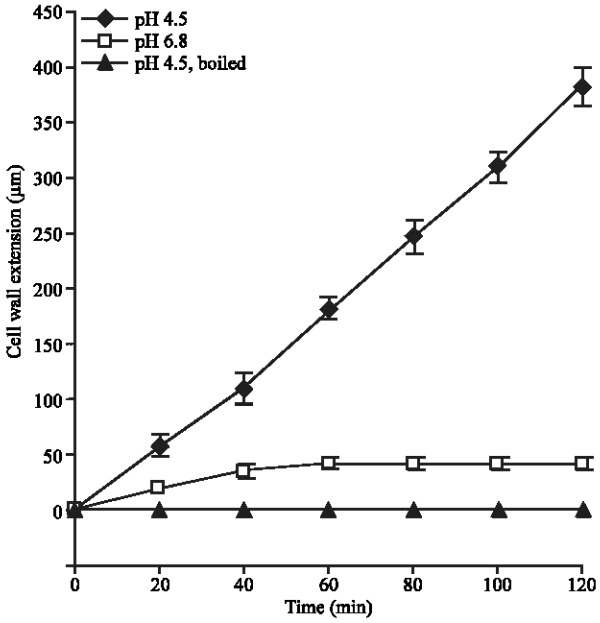

Fig. 1: Extension of endogenous expansins in soybean internode. The topmost internodes of soybean treated with green shade for 4 days was use to measured the extension of native cell walls and validate the existence of expansins in soybean internodes. After the live internodes had been suspended under tension in Hepes buffer (50 mM L^{-1}, pH 6.8) for 30 min, the solution was then replaced by sodium acetate buffer (50 mM L^{-1}, pH 4.5) (black diamonds). Live internodes suspended in Hepes buffer (squarses) and bioled internodes suspended in sodium acetate buffer (black triangles) were used as controls. The extension values were recorded every 20 min after the buffers were changed and data points represent means of 4 internodes (Mean±SE, n = 4). Experiments were carried out twice with similar results

buffer (pH 4.5), but did not in neutral buffer (pH 6.8). Boiled cell walls did not elongate in the same buffer. These results imply that in soybean internodes, there are expansins and the endogenous proteins have the acid-induce activity to the cell walls extension of soybean internode. They were in agreement with those in previous reports on cucumber (McQueen-Mason *et al.*, 1992) and demonstrated firstly the presence of expansins in soybean internodes.

Activity of expansins extracted by different methods: Expansins are one kind of weakly bound cell wall proteins which can be extracted from purified cell wall with low ionic strength salt solutions, such as NaCl, LiCl or $CaCl_2$. Of these salts, the most efficient for extraction of higher plant cell wall proteins is $CaCl_2$ (Feiz *et al.*, 2006). But the classical method of extracting expansin proteins was using Hepes solution (1 M NaCl, 25 mM Hepes, pH 7.0) as extracting buffer (McQueen-Mason *et al.*, 1992). To compare the efficiency of extraction methods in working on soybean internods, the cell wall was prepared as described (Feiz *et al.*, 2006). The expansins in cell wall were extracted with $CaCl_2$ and with Hepes (Fig. 2). The data suggested that the expansins extracted with $CaCl_2$ had higher activity than Hepes. So we propose that $CaCl_2$ method can be used to measure the expansins activity in soybean internodes.

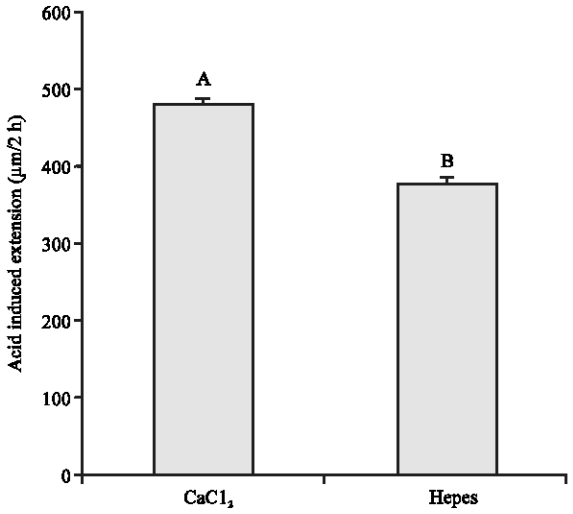

Fig. 2: Analysis of expansins activity at cell wall in soybean internodes with different protein extraction methods. The cell wall of soybean internodes which were grown under normal light condition for 4 days were isolated and extracted by $CaCl_2$ and Hepes buffer. The extracts were resuspended in 10 mL acetate sodium buffer (50 mM, pH 4.5) and the expansins activities to boiled cucumber hypocotyls were measured for 2 h. Data are expressed as Means±SE of three independent extracting experiments. Different letters above each bar indicate statistically significant differences ($p<0.01$, Tukey's b test)

Cross reconstitution activity of extracted expansins with cucumber hypocotyls: The Cucumber Hypocotyls boiled to eliminate edogenous expansins activity were used to test the expansins extracted from other material. The cell wall extension was termed cross reconstitution activity of expansins. Figure 3 showed that the expansin proteins extracted from soybean internodes could induce extension of cell walls in cucumber hypocotyls. The protein exhibited inducing activity in acidic buffer, which was similar with the activity of endogenous expansins in native soybean internodes (Fig. 1). The reversibility of cell wall extension induced by expansins was the other property of expansins, which was also observed in extracted expansins to cucumber hypocotyls. The cell wall extension was observed in acidic protein solution but not in neutral protein solution and the phenomenon can be repeated by alternately switching incubation buffer, implying that change in pH of bathing solution could only affect the conformation of expansins but not the affinity of it to cell walls (Gao *et al.*, 2007).

Internode elongation of different soybean cultivars responded to shade stress: Soybean of both cultivars were grown under shade stress conditions, which mimics canopy shade of wheat or corn in the relay cropping system, with combined reductions in blue, R/FR and total light intensity. The increase in the length of the topmost internode was measured every day for 8 days. We found both cultivars responded within 1 day with rapid internodal elongation and growth rates that were significantly higher than controls (Fig. 4). Also, the growth rates stayed significantly higher than those in controls until the last day of measurement. These results imply that whether the soybean is tolerant to shade or not, the internodes will elongate under canopy shade. It must be noted that, although the tolerant shade cultivar (NANDOU12) did respond with higher growth

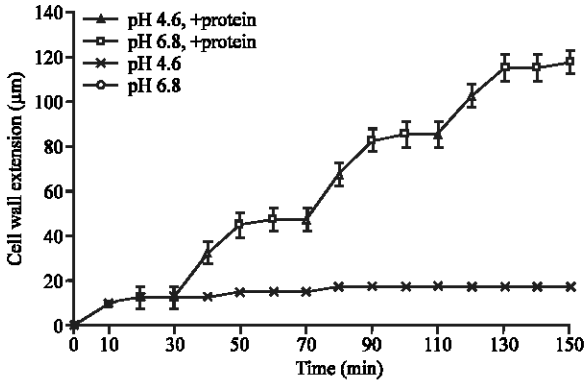

Fig. 3: The activity of extracted expansins from soybean internodes to boiled cucumber hypocotyls. The expansins in soybean topmost internodes which were treated with normal light for 4 days were extracted by $CaCl_2$ solution (5 mM acetate buffer, pH 4.6, 0.2 M $CaCl_2$ and 10 μL protease inhibitor cocktail) and resuspended in 50 mM acetate sodium buffer (pH 4.5) and 50 mM Hepes buffer (pH 6.8). Protein concentration was adjusted to 2 mg mL^{-1}. After the bioled cucumber hypocotyls had been suspended under tension in Hepes buffer (50 mM L^{-1}, pH 6.8, without proteins, marked by circle) for 30 min, the solution was then replaced by sodium acetate protein solution (triangles) and Hepes protein solution (squares).The different protein solutions were used alternately every 20 min. The same hypocotyls suspended in 50 mM acetate sodium buffer (pH 4.5, without proteins, marked by forks) were used as controls. The extension values were recorded every 10 min from beginning and data points represent means of 4 internodes (Mean±SE, n = 4). Experiments were carried out twice with similar results

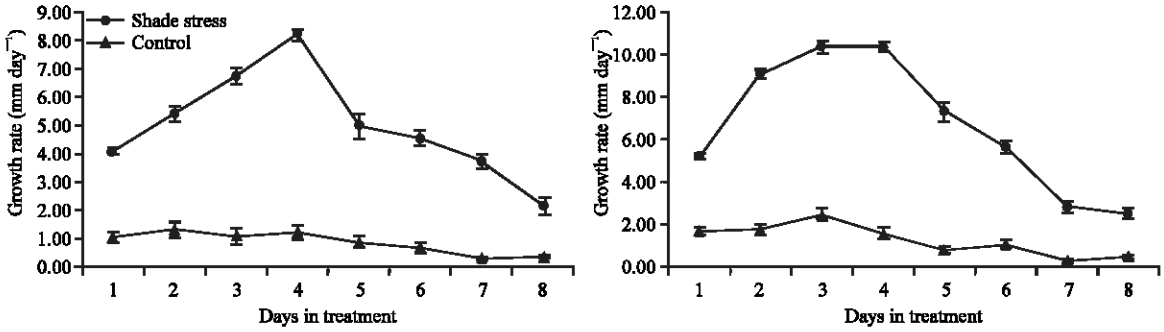

Fig. 4: The effects of low PAR on the growth rates of soybean internode. The elongation rate was measured every day for 8 days. The cultivars of NANDOU12 and NAN022-2 are under green shade (low PAR, red circles). Controls (black triangle) were grown under normal light conditions (spectral composition unaltered). Growth rates were calculated from length measurements of the first internode obtained using a caliper. Data points represent means of 10 Seedlings (Mean±SE, n = 10). Experiments were carried out twice with similar results. Growth rates show statistically significant differences relative to controls, at all time points, in both cultivars. (Student's t-test, $p<0.05$). Under normal light, the growth rates of NAN022-2 were similar relative to NANDOU12 at any time point. But under green shade, NAN022-2 grown fasterly than NANDOU12 (Student's t-test, $p<0.05$)

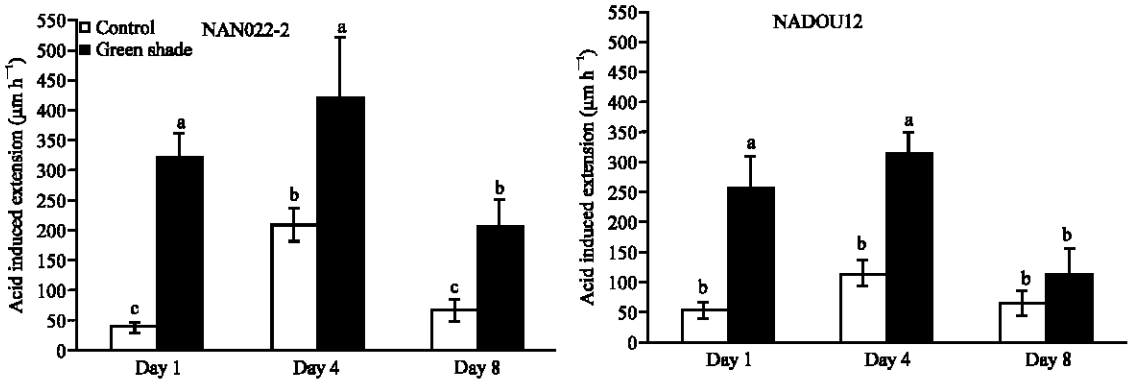

Fig. 5: The effects of green shade on the AIE of internodes from soybean. AIE of the topmost internodes of ramets from cultivars NANDOU12 and NAN022-2 plants grown under normal light (control, white bars) and green shade (black bars) growth conditions for 1, 4 and 8 day. AIE was measured using a constant-load extensometer with a pulling weight of 22.0510 g and was calculated as the difference in the slopes of lines fitted through 30-min intervals before and after the bending point observed due to a change in pH from 6.8 to 4.5. Data points represent Mean±SE (n = 6-8). Each biological replicate consisted of the topmost internode from different plants from different pots. Different letters above each bar indicate statistically significant differences ($p<0.05$, Tukey's b-test)

rates relative to controls when subjected to shade stress, the growth rates were much lower than NAN022-2 under similar conditions. So the seedling of NAN022-2 are taller than that of NANDOU12 under the shade. The growth rates of NAN022-2 reached the max at the third day, earlier than NANDOU12 and lasted to the fourth day. It suggested that NAN022-2 were more sensitive to green shade than NANDOU12.

Acid-induced extension of internodes of both NANDOU12 and NAN022-2 cultivars correlated with growth under shade stress: There is evidence that the regulation of cell wall extensibility via the control of the expression and/or activity of expansins in response to shade signals (Sasidharan *et al.*, 2008). But for soybean, there is no studies on this. Acid-induced extension (AIE) is one of the reflections of expansins activity. AIE activities of both cultivars under shade stress were measured at the days 1, 4 and 8. In both cultivars. AIE activity of in the internodes of soybean seedlings were responsive to shade and a significantly higher in green shade, relative to controls at days 1 and 4 (Fig. 5). For the un-resistant NAN022-2 cultivar, AIE activity kept higher level relative to controls over the 8 days growth period. These trends for AIE activity (assessed at days 1, 4 and 8) were significantly related to relative growth rate for the first internode elongation, measured at days 1-8. This correlation of AIE values with growth suggests a role for expansins in shade-induced internode elongation in soybean cultivars. Furthermore, under shade conditions, AIE activity in NAN022-2 cultivar was high relative to that in NANDOU12 cultivar of approximately 27, 33 and 90% at days 1, 4 and 8, respectively. This shows that there may be a correlation between expansins activity in soybean internode and the resistance shade.

Reconstitution activity of expansin proteins extracted from NANDOU12 and NAN022-2 cultivars grown under green shade: Expansin proteins activity can also be reflected by measuring the reconstitution extension activity with cucumber hypocotyls in pH 4.5 buffer. Since

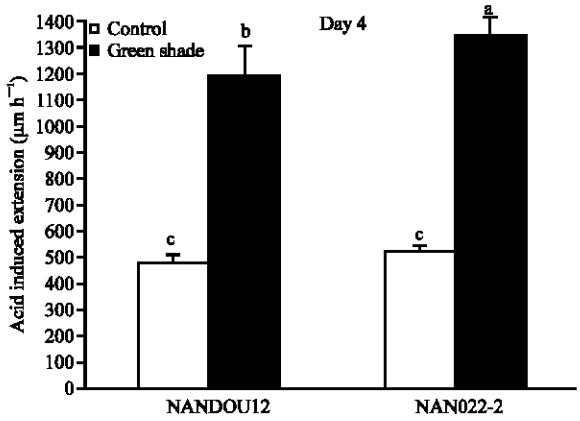

Fig. 6: The reconstitution activity of expansins extracted from NANDOU12 and NAN022-2 cultivars grown under green shade at days 4.Expansin proteins of the first internodes from cultivars NANDOU12 and NAN022-2 plants, grown under normal light (control, white bars) and green shade (black bars) growth conditions at days 4, was extracted by $CaCl_2$. The reconstitution activity with cucumber hypocotyls was measured using a constant-load extensometer with a pulling weight of 22.0510 g and was calculated as the difference in the slopes of lines fitted through 30-min intervals before and after the bending point observed due to a change in pH from 6.8 to 4.5. Data points represent Mean±SE (n = 6-8). Each biological replicate consisted of the topmost internode from different plants from different pots. Different letters above each bar indicate statistically significant differences ($p<0.05$, Tukey's b-test)

the internode growth rates of both soybean cultivars were at their maximum levels under green shade at the days 4th day and the diversities came to be significant between two varieties and light conditions (Fig. 4), the expansin proteins were extracted from both cultivars under green shade at the days 4th day and it`s reconstitution AIE activities were measured for 2 h (Fig. 6). By contrasted, expansin proteins activity from two cultivars under green shade condition increased significantly with approximately 150 and 160%, respectively. For the control, the difference of expansin proteins activity between the cultivars was not detected. However, under green shade condition, the expansin proteins activity of NAN022-2, which is not unresistant to shade, was significantly higher than that of NAND12.The result suggests further that expansin proteins play an important role during soybean suffered shade stress.

As a further focus of the physiological mechanism of soybean responding to shade stress, gene expression of these proteins should be studied. We hope to use the expansins activity as one of the key indicators for selecting soybean cultivars or species which are endurance shade stress and suitable for being planted in relay cropping system.

ACKNOWLEDGMENTS

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