

Original Research Paper

Determination of Abscisic Acid and its Relationship to Drought Stress Based on Cowpea Varieties with Different Capability of Drought Resistance

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Abstract: Cowpea (*Vigna unguiculata* L.) is one of the most important grain legume supplying necessarily dietary proteins. Although its can tolerate drought environment, still the severe condition can limit the growth and development of this plant. Abscisic acid (ABA) has a crucial function in stress physiology, but little information is available for methods describing ABA determination in cowpea and its relationship to drought stress based on cowpea varieties with different capability of drought resistance. Here, an effectual approach based on high-performance liquid chromatography (HPLC) method was established to separate and determine the endogenous abscisic acid in cowpea and further the cowpea varieties with different capability of drought resistance were used to explore relationships between ABA content and drought stress. The results potentially provided useful reference for the cultivation of cowpea crop. Results-This method showed good liner regression ($R^2 = 0.9998$), coupled with high stability, precision and repeatability. The endogenous ABA decreased under drought treatment and the drought sensitive line had higher ABA content than drought resistant one, suggesting ABA performs a vital function in cowpea's response and resistance to drought stress. The studies potentially provided useful reference for the cultivation and breeding of cowpea crop.

Keywords: High-performance liquid chromatography, Cowpea, Abscisic acid, Drought

Introduction

Cowpea (*Vigna unguiculata* L.) is one of the most important drought-tolerant grain legume supplying necessarily dietary proteins, in particular for the vegetarian population of developing countries (Popelka *et al.*, 2004; Ramakrishnan *et al.*, 2005). Due to its super drought-tolerant ability, it is largely cultivated as an indispensable food or forage crop in the semi-arid tropic areas of Africa, Asia, Latin America, where the yield of most crop plants has been compromised by the abnormally high temperature and low level of water supplying (Paul *et al.*, 2011). Cowpea possesses several excellent nutritional qualities including high content of proteins, high calories, minerals and vitamins. It is an exclusively essential rural diet and animal feed, as well as a popular vegetable in the rest of the world. Besides, its biological

nitrogen fixation property grants it an even more vital niche in enhancing soil fertility and improving soil structure in the era of widely appealing for organic food and environmentally friendly agriculture (Bressani, 1985; Martins *et al.*, 2003), therefore, world demand for cowpea is increasing gradually. However, its production has, to some extent, been affected by drought stress, which has already become a hotspot topic in the field of agriculture (Bayuelo-Jimenez *et al.*, 2002; Chen *et al.*, 2007). Although cowpea can tolerate drought environment, still the severe condition can limit the growth and development of this plant (Munns, 1993). Abscisic acid (ABA) has a crucial function in stress physiology, but little information is available for methods describing ABA determination in cowpea and its relationship to drought stress based on cowpea varieties with different capability of drought resistance.

ABA, a type of sesquiterpenoid phytohormone, is commonly synthesized in all vascular plants, mosses and algae (Schwartz *et al.*, 2003; Rai *et al.*, 2011). Generally, ABA is maintained at relatively high level under the normal condition, but it will decrease significantly when plant is suffered from the environmental stresses (Xiong and Zhu, 2003; Sandhu *et al.*, 2011). ABA acts as a key signal molecule in the regulation and induction of the plant physiological responses to osmotic stress which is induced by salt, drought and cold tolerance (Rai *et al.*, 2011; Tuteja, 2007; Fujita *et al.*, 2011). In particular when plant encounters drought environment, ABA, with the content decreasing, stimulates stomatal closure and leaves wilt to mediate plant defense reaction (Bano and Yasmeen, 2010; Majeed *et al.*, 2011; Fukao *et al.*, 2011). These highlighted the importance of ABA in the plant's response to drought stress and great attention has been focused on the role of ABA in the stress response. Some analytical methods have been reported to determine this endogenous phytohormone, such as bioassays (Boerjan *et al.*, 1992), gas chromatography (GC) (Du *et al.*, 2000), enzyme linked immunosorbent assay (ELISA) (Gao *et al.*, 2006; Ferreira *et al.*, 2006), spectrometry, electrochemical technique (Carretero and Cruces-Blanco, 2004; Li *et al.*, 2005) and high-performance liquid chromatography (HPLC) (Dobrev *et al.*, 2005), in which HPLC has the highest sensitivity, the most specificity and the best repeatability in comparison with other methods mentioned, thus it is used extensively to determine ABA content in some plants such as soybean, *Arabidopsis thaliana* and *Glycyrrhiza uralensis* *etc.* (Ciha *et al.*, 1977; Ross *et al.*, 2004; Xu *et al.*, 2010). In the present study, an effectual approach based on HPLC method was established to separate and determine the endogenous abscisic acid in cowpea and further relationships were explored between ABA content and drought stress. The results potentially provided useful reference for the cultivation and breeding of cowpea crop.

Materials and Methods

Chemicals and Reagents

HPLC-grade acetonitrile and methanol was obtained from Fisher Chemical, USA. Phosphoric acid, ethyl acetate and hydrochloric acid were of analytical grade and were purchased from Guahua Chemical Ltd. (China). Water for HPLC analysis was purified using Milli-Q water system (Millipore Corporate, France). ABA was obtained from Sanland Chemical Co., Ltd (Los Angeles, USA). All of the chromatographic grade solutions were stored at 4°C in darkness and then the HPLC mobile phase was prepared fresh at a time, filtered through a 0.45 µm membrane filter.

Plant Material and Drought Stress Treatment

Cowpea materials used in current study were originated from Africa and contained two varieties: Dan lla (drought resistance variety) and TVu7778 (drought sensitive variety). Cowpea seeds were first surface sterilized in 1% NaClO for 10 min, then rinsed thrice in the sterile water. The sterilized seeds were set into earthen pots in a greenhouse at 30±2°C. Twenty-two days later, sixteen uniform seedlings from each variety were divided into two groups, each having eight seedlings. After flood irrigation, the control groups for each variety were irrigated lightly every three days with soil moisture of more than 14.5%; on the other hand, the experimental groups, was withheld irrigation for one month to maintain moisture around 5% (Fig. 1). The soil moisture was measured by soil temperature and humidity speed measuring device purchased from Handan Electronic Technology Limited Company (China).

Sample Preparation

About 1.0 g of fresh cowpea leaves were collected and put into liquid nitrogen, followed by grinding into powder and then the powder was extracted in 15 mL of methanol (80% v/v) for 12 h at 4°C. After air pump filtration, the filtrate was obtained; the filtrate residue was extracted for another 2 h with subsequent pumping filtration, two filtrates were combined together. Three steps were carried out, including acidifying with hydrochloric acid, extracting with ethyl acetate thrice and air drying by vacuum reduced pressure concentration, the final volume of extrate was made up to 10 mL with addition of methanol. At last, the samples were filtrated through the 0.45 µm membrane filter before being injected into HPCL system (Ma *et al.*, 2011).

Apparatus and Chromatographic Conditions

Separation and determination of ABA were performed with HPLC system (Waters 2695, miford, MA, USA), which consisted of a quaternary pump, an ultraviolet detector (ELSD, Waters 2487) and Waters Empower software. Chromatographic condition was slightly modified from the previous report (Xu *et al.*, 2010). Separation was carried out on a Waters Symmetry C18 column (4.6×250 mm, 5 µm) and this process was maintained at 25°C with a gradient elution at the flow rate of 0.6 mL min⁻¹. The mobile phase was composed of acetonitrile (solvent A) and phosphoric acid solution (0.1% v/v) (solvent B). The gradient elution program was: 20% A and 80% B (0 min), 25% A and 75% B (5 min), 30% A and 70% B (8 min), 35% A and 65% B (15 min), 45% A and 55% B (25 min). The target component was quantified by the peak areas at the maximum wavelength of 260 nm.

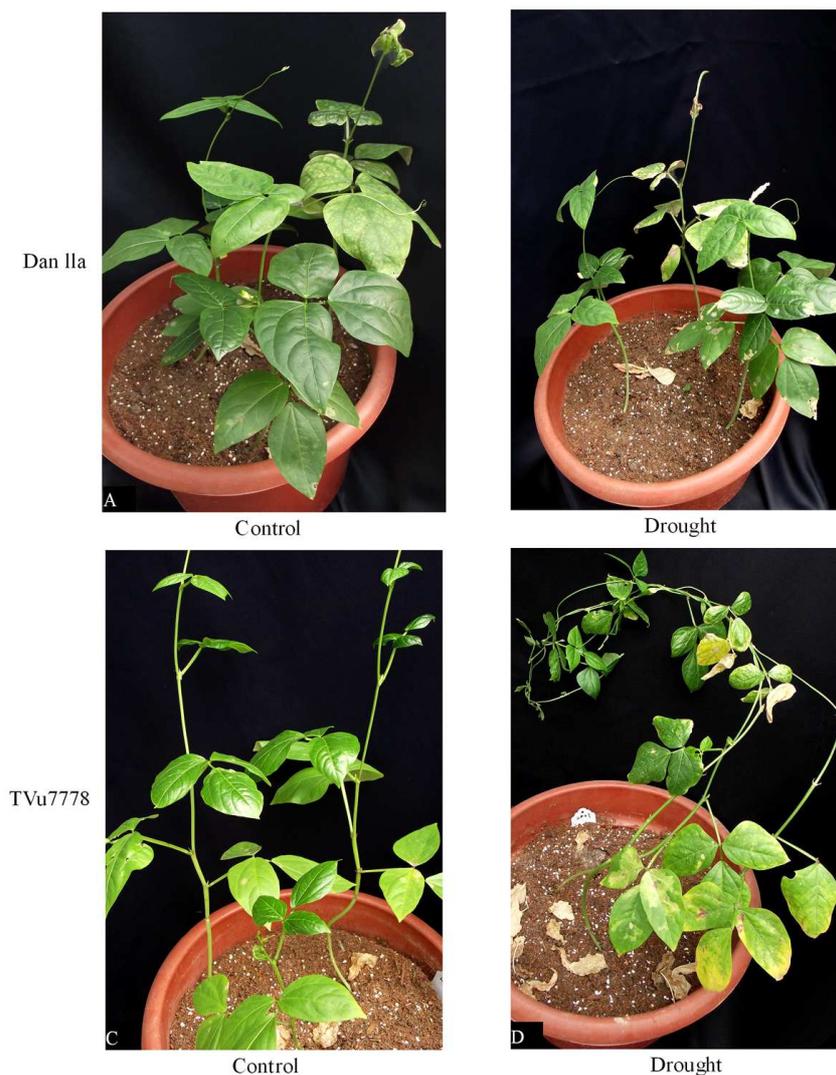


Fig. 1. Preparation of cowpea samples. A. the control group of Dan Ila (moisture 15.64%, n = 8); B. the treatment group of Dan Ila (moisture 6.39%, n = 8); C. the control group of TVu7778 (moisture 11.91%, n = 8); D. The treatment group of TVu7778 (moisture 6.19%, n = 8). Cowpea plants grown in soil were exposed to drought stress achieved by withholding irrigation for one month. The control plants were irrigated normally. A representative photograph is displayed

Preparation of Standard Solutions and Calibration Curve

Standard solutions of ABA were directly prepared in methanol. The assay linearity was determined for five concentrations of 33.6, 16.8, 6.73, 3.36 and 1.68 $\mu\text{g mL}^{-1}$, respectively. The calibration curve was constructed by the LC software and statistic analyses and the liner regression equation was obtained by plotting the peak areas vs. the nominal concentrations of ABA standard solutions. The linearity relationship was demonstrated by a correlation coefficient (R^2) higher than 0.999. LOD and LOQ were defined as the quantity of compound required for signal-to-noise ratios of 3:1 and 10:1, respectively.

Method Validation

The precision of this method was determined by repeated measurements of five times, the stability tests were carried out by analyzing ABA samples on 0, 2, 4, 6, 8 h and the repeatability was assessed by five independently prepared samples of ABA, with all indices calculated as R.S.D. In order to evaluate recovery of ABA in the current method, known amount of ABA standard solutions were injected at different levels of 33.6, 16.8, 6.72, 3.36, 1.68 $\mu\text{g mL}^{-1}$ based on the described procedure and calculated according to the following formula: Recovery rate (%) = (determined amount after spiking-original amount in sample)/spiked amount $\times 100\%$.

Results

Optimization of Chromatographic Conditions

The desired ABA compound from the samples was identified by comparing the retention time and UV spectra with that of the authentic standard. The remarkable agreement between the determined compound from the samples of cowpea and standard material suggested that the endogenous ABA was separated by gradient elution in about 21 min and determined accurately without interference from other components in the methanol extract of cowpea. As shown in Fig. 2, the typical chromatogram under the

proposed conditions was recorded about standard ABA (Fig. 2A) and methanol extract of cowpea (Fig. 2B).

Validation of Analytical Method

Some indices, including R^2 , LOD, LOQ, R.S.D, were used to evaluate precision, repeatability and stability of this method (Table 1). Both LOD and LOQ were examined to estimate the sensitivity of UV detector in the present study. The assay linearity was confirmed based on five gradient concentration of standard solutions and the regression equation coupled with calibration curve showed good fitness ($R^2 = 0.9998$) (Fig. 3).

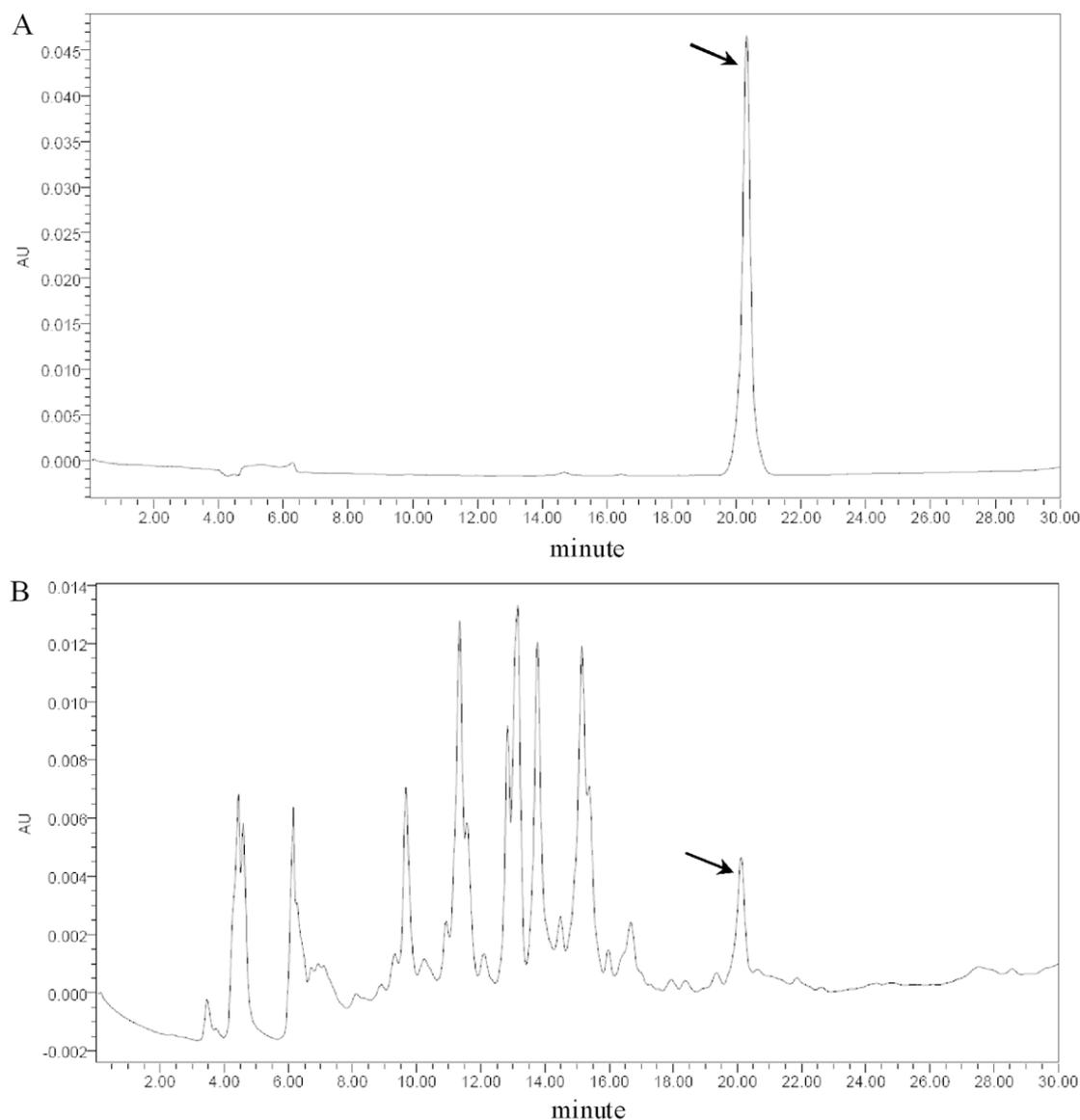


Fig. 2. Typical chromatogram of the standard solution (A) and methanol extract of cowpea (B). The arrows showed the ABA peak in both pictures (A and B)

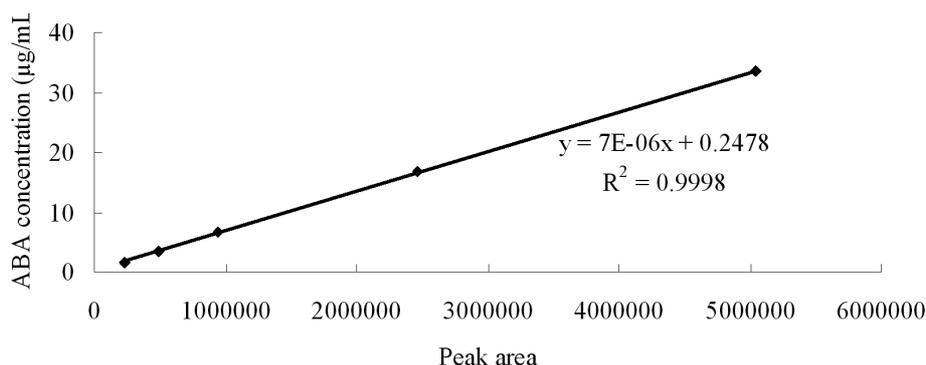


Fig. 3. Calibration curve of ABA standard solution

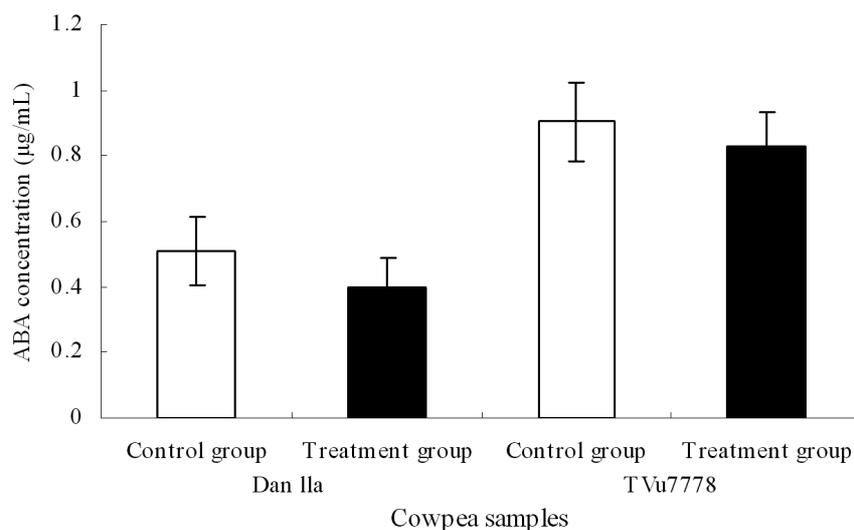


Fig. 4. Endogenous ABA contents of cowpea samples

Table 1. Validation data of the proposed HPLC method

Components	ABA
LOD (µg/mL) (S/N = 3)	0.770
LOQ (µg/mL) (S/N = 10)	2.568
Stability R.S.D (%)	2.054
Precision R.S.D (%)	1.992
Recovery rate (%)	91.56

Precision was determined by assaying standard solution at $6.72 \mu\text{g mL}^{-1}$ for five times and R.S.D was fixed on 2.9%, which demonstrated that this method is quite precise. Repeatability was tested using five independent samples (R.S.D 1.1%) and the result indicated that this method is reproducible and able to be used widely. The stability test was performed through analyzing sample solutions at 0, 2, 4, 6, 8 h, respectively and the R.S.D values of peak areas were more than 1%, manifesting that the experimental samples can keep stable for at least 8 h at 4°C . Accuracy test was carried out in terms of recovery rate, which was set out to be 99.7% with R.S.D of 1.7%.

Determination of ABA in Cowpea

A representative chromatogram of the methanol extracts was shown in Fig. 2B and the determination quantities of every sample were illustrated in Fig. 4, which showed the endogenous ABA decreased under drought treatment and the drought sensitive line TVu7778 had higher ABA content than drought resistant variety Dan Ila.

Discussion

ABA plays an important role in the signal transduction pathway of the environment stress response including drought, high salt, low temperature and so on (Liu *et al.*, 2007; Nagamune *et al.*, 2008). An HPLC method was successfully developed to analyze endogenous ABA in cowpea with a UV detector. The method was validated by some indices such as R^2 , LOD, LOQ, R.S.D, which represented precision, repeatability, stability and recovery rate, respectively.

Both LOD and LOQ were examined to estimate the sensitivity of UV detector in the present study and all of precision, repeatability and stability tests were evaluated using R.S.D value. Taken together, this developed method is acceptable, which was used to determine the endogenous ABA in all cowpea samples in this study. As indicated from these data, the HPLC method was efficacious and suitable for ABA determination and the results implying that ABA, as a signal of drought stress response, was synthesized more to resist the stress in the non-drought-tolerant variety, alternatively, ABA biosynthesis might be limited to some extent, which is enough to deal with environmental stresses in drought-resistant plants.

Conclusion

HPLC analysis is a rational and practical analytical strategy for authentic, stable and repeatable assay of endogenous ABA in cowpea. An HPLC method was successfully developed and applied to separate and determine ABA contents in cowpea. Based on cowpea varieties with different capability of drought resistance, it is elucidated that ABA performs a vital function in cowpea's response and resistance to drought stress. Moreover, this assay can be effectively applied in further researches to understand the physiological and genetic mechanisms of endogenous ABA during plant development and improve plant's drought tolerance by metabolic regulation of ABA biosynthesis.

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Author's Contributions

Wei Lei: Performed the study, wrote the manuscript and/or contributed to data analysis and interpretation.

Shian Huang: Performed the study and/or contributed to data analysis and interpretation.

Shaohu Tang: Performed the study and/or wrote the manuscript.

Xiaorong Shui: Wrote the manuscript and takes full responsibility for the work as a whole, including the study design, access to data and the decision to submit and publish the manuscript.

Can Chen: Performed the study and/or contributed to data analysis and interpretation.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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List of Symbols and Abbreviations

- Abscisic Acid, (ABA)
- Gas Chromatography (GC)
- Enzyme-Linked Immunosorbent Assay (ELISA)
- High-Performance Liquid Chromatography (HPLC)
- Limit Of Detection (LOD)
- Limit Of Quantification (LOQ)
- Relative Standard Deviation (RSD)