PHYSIOLOGICAL ANALYSES OF AN OVER-EXPRESSING CYTOKININ METABOLIC GENE *GmIPT10* UNDER NORMAL AND DROUGHT CONDITIONS

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SUMMARY

Cytokinin (CK) is an important phytohormone, which not only plays significant role in plant development but also involves in mediating plant stress tolerance. Previous studies showed that the drought tolerance can be improved by stress-inducible overexpression of adenine isopentenyl transferase (IPT), which is a critical enzyme in CKs biosynthesis. To study the role of soybean GmIPT10 in regulating plant tolerance, we successfully generated *GmIPT10*-overexpressing transgenic soybean plants and screened a line carrying homozygous, single copy of transgene. Analyzing several physiological traits of this line demonstrated that it possessed stress tolerance characteristics, including increased primary root and shoot lengths, better production of shoot biomass, higher number of trifoliate leaves, and higher survival rate than the non-transgenic plants under drought condition. The net house experiments also showed that the *GmIPT10*-overexpressing transgenic soybean had a greater relative water content compared to the control genotype under applied drought condition. Therefore, this report indicated that plant drought tolerance might be enhanced via regulating expression of *GmIPT10*.

Keywords: *Cytokinin, drought tolerance, GmIPT10, soybean, transgenic plant*

INTRODUCTION

Soybean (*Glycine max*) is an important crop used commonly in producing vegetable oil, protein and nutrition feed. However, drought stress has been concerned as one of the most critical factors determining the final yield of soybean (Tran and Mochida, 2010). Previous studies have reported drought can reduce soybean production up to 40% (Thao and Tran, 2012). Making stable genetically modified soybean cultivars by genetic engineering has been considered as a productive and rapid method to improve drought-tolerant traits (Guttikonda *et al*., 2014). By utilizing *in silico* analysis-based approach, a large number of cytokinin (CK) – related genes involved in drought adaptation have been systematically characterized and functionally studied (Hwang, Sheen, 2001; Inoue *et al*., 2001). Regarding aspect of plant physiology, CK is well known as a regulator in morphological (Muller *et al*., 2008; Werner *et al.,* 2010) and physiological development (Aloni *et al*., 2006) as well as in plant adaptation to environmental stresses,

such as tolerant response to drought stress (Muller *et al*., 2007; Kuppu *et al*., 2013). Protective responses to drought in plants could be, therefore, modified by genetic engineering through manipulation of endogenous CK levels (Le *et al*., 2012).

In the past 20 years, a great deal of effort on research has been conducted to draw a detailed picture of CK metabolism. In plants, CK metabolic homeostatic is consistently regulated by adenosine phosphate-isopentenyl transferases (IPTs) and CK oxidases/dehydrogenases (CKXs). There are two groups of IPTs affecting adenine aromatic moiety found in *Arabidopsis thaliana*, including seven genes for ATP/ADP IPTs (*IPT1, IPT3, IPT4, IPT5, IPT6, IPT7,* and *IPT8*) and two genes for transfer RNA IPTs (*IPT2* and *IPT9*). Further research of various *ipt* mutants revealed that transfer RNA IPTs are responsible for biosynthesis of *cis*-zeatin- type CKs while ATP/ADP IPTs are involved in synthesis of isopentenyl adenine- and *trans*-zeatin- type CKs (Ha *et al*., 2012; O'Brien, Benkova, 2013). Moreover, relative interaction of CK with other phytohormones including abscisic acid and auxin, which are

concerned as key phytohormones, has a critical role in plant development and adaptation (Růžička *et al.*, 2009; Bishopp *et al.*, 2011; Thu *et al.*, 2017).

Although detailed understanding of molecular mechanism and pathways of CKs signaling is still limited, the great number of scientific evidence showed that CKs level adjustment and CK-encoding gene modulation would be a potentially powerful tool to enhance plant drought resistance. Normally, reducing CK concentration approaches by over-expressing a specific *CKX* gene in root will induce root development and biomass accumulation without shoot retardation (Ha *et al.*, 2012). However, there were reports on enhancement of *IPT* expression that could reduce root growth but still significantly contributes to drought resilience through reducing leaf senescence, free radical oxidation, and improving photosynthetic intensity (Rivero *et al.*, 2010; Merewitz *et al.*, 2011). Recent evidence demonstrated that over-expressing *Agrobacterium tumefaciens IPT* in cassava could increase drought tolerance and delay senescence in the transgenic plants (Zhang *et al.*, 2010). Likewise, over-expressing the bacterial gene in peanut demonstrated significantly improved performance in photosynthetic rate, stomatal conductance and transpiration compared to the wild-type plants under water deficit (Qin *et al.*, 2011). In another report, rice (*Oryza sativa*) over-expressing the *ITP* gene also exhibited enhanced grain yield quality and improved drought tolerance (Peleg *et al.*, 2011).

IPT genes in soybean (*GmIPTs*) have been isolated and under functional characterization. In 2012, Le *et al.,* analyzed their expression under normal and water stress conditions. According to their findings, among the studied *GmIPTs*, *GmIPT08* transcripts were found to consistently increase in the leaves and shoots of young soybean seedling under dehydration conditions. The data also showed high transcriptional expression activity of another gene, *GmIPT10*, in roots and root hairs under drought stress condition. Taken together, these findings suggest that *GmIPT8* and *GmIPT10* are likely to involve in drought responses in soybean and thus could be employed to improve drought tolerance by genetic engineering approach.

To study the function of GmIPT10, different transgenic lines with *RD29A*-inducible promoter were generated at the University of Missouri (USA) using the *Agrobacterium*-mediated transformation method. In this study, we endeavored to analyze a number of main physiological characteristics involved in plant response to water deficit of a *GmIPT10*-over-expressing line and compared its performance with the non-transgenic soybean counterparts.

MATERIALS AND METHODS

Growing conditions

The plants were grown in net house condition with temperature range of 27-34°C, humidity of 60-70%, natural photoperiod.

Selection of homologous transgenic soybean carrying *GmIPT10*

The seeds of transgenic (carrying *RD29A::GmIPT10* and selective marker *bar* gene), positive control (carrying *bar* gene) generated by University of Missouri (USA) and wild-type (WT) (cultivar W82) soybean were germinated in trays and then transferred to net house with daily watering. To select the homozygous and single copied transgenic soybean line(s), the plants at V4 stage (22 days after germination) were sprayed with Basta (glufosinate ammonium) (80 mg/l, 3-ml dose per plant). Upon this treatment, the transgenics remained healthy and green while the non-transgenic plants displayed yellow, paled and/or wilted leaves. The screening for the line carrying homologous, singled copied transgene was performed based on Mendelian laws of inheritance and segregation.

Shoot growth and root growth assay

The method described in Thu *et al.* (2014) was adopted. In brief, the WT and homozygous transgenic plants carrying *GmIPT10* were planted in plastic tubes (80-cm height, 10-cm diameter) filled with Tribat soil (Saigon Xanh Bio-Technology Ltd Company, Vietnam). The 14-day-old plants were subjected to drought condition by withholding water for the next 16 days. Another set of plants for both genotypes remained watered for being used as controls. After the stress application period, the drought-treated and non-drought treated plants were removed gently out of the containers for recording lengths and fresh weights (FWs) of shoot and tap root of each individual plant. Next, these tissues were dried in oven at 65°C for 2 days before their dry weights (DWs) were measured. To evaluate the relative water content (RWC), additional step was performed between fresh weight and dry weight

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measurement, upon which the aerial part of each plant was soaked in water overnight then weighed to get the turgid weight (TW) (Ha *et al*., 2013). The RWC was determined using equation: RWC = (FW – DW) / (TW – DW) × 100.

Plant drought tolerance evaluation

Following the protocol described in Thu *et al*. (2014) with modification, 20-day drought treatment was applied to 14-day-old plants (grown in plastic tubes with 50 cm in height and 30 cm in diameter) by stopping watering, followed by water resumption. Control plants of both genotypes which were adequately watered were included. Soil moisture content (SMC) in each pot was monitored by using moisture meter (Total Meter, Taiwan), during the stress treatment, number of non-withered plants was recorded every 2 days.

Statistical analyses

The data were analyzed by Student's t-test (one tail, unpaired, equal variance) to identify the statistical significance with *p*-value < 0.05.

RESULTS AND DISCUSSION

Successful selection of homologous transgenic soybean carrying *GmIPT10*

In this experiment, *bar* gene was used as an effective selectable marker for identification of the transgenic plants. This gene encodes phosphinothricin N-acetyltransferase (PAT) enzyme, which confers resistance to Basta herbicide containing glufosinate ammonium (De Block *et al*., 1987; Song *et al*., 2013). Due to the large number of transgenic plants that were used for screening soybean events, Basta application was chosen as a quick, cheap but accurate method. After five days since Basta application, the whole leaves of the positive control plants (Fig. 1c) and transgenic plants possessing *bar* gene remained healthy and green (Fig. 1d). On the other hand, non-transgenic soybean and negative control were vulnerable and their leaves mostly turned yellow (Fig. 1a, b). Based on the Basta results and screening for several generations following Mendelian laws, we have identified one stable homozygous and single-copied line carrying *GmIPT10* (line 175-27). Another notice was that development retardation was not observed in this line. Therefore, the line was used for subsequent experiments (Fig. 1d).

Transgenic plants had better root and shoot-related traits under normal condition

Shoot and root growth at vegetative stage (30 days of age) of WT and *GmIPT10*-over-expressing plants were examined under full irrigation condition. To ensure the plants were well watered, the soil moisture content (SMC) was regularly monitored. Following this, the SMC value was maintained within the range of 60~70% (Fig. 3a) throughout the experimental period, which was in agreement with other studies such as Thu *et al*., (2014). According to our record, although the transgenic and WT plants displayed similar average tap root length under normal condition (Fig. 3c), the transgenic plants had significantly higher trifoliate leaf number (Fig. 3g, *p*-value < 0.0001) and had higher average shoot length of 10.1 cm (Fig. 3d, *p*-value < 0.05) than those of the WT, suggesting that the former might have a stronger photosynthesis performance and grain yield in normal condition (Qin *et al.,* 2011).

It was also found out that there was a clear difference in biomass accumulation between the two examined genotypes. The transgenic plants had 40.8% higher mean of root and 28.22% higher mean of shoot dry matters (*p*-values < 0.005) compared to the corresponding parameters of the WT (Fig. 3e, f). In soybean, there is the strong linear relationship between mean dry matter and mean seed yield production (Mayers *et al*., 1991).

Taken all of these together, the transgenic plants displayed improved root and shoot traits compared to the WT counterpart in terms of number of trifoliate leaves, shoot length, and root and shoot dry matters. These obtained data indicate that the transgenic line is likely to have capacity in producing greater seed yield under field condition.

### Transgenic plants had better root- and shoot-related traits under drought condition

To evaluate the drought tolerance, we ceased to water both WT and *GmIPT10*-over-expressing transgenic plants after 14 days growing them under normal condition, when both genotypes showed similar size. At the end of the drought period, the SMC in the containers of treated plants dropped substantially to around 30% (Fig. 3b). At this stage, the drought-treated transgenic plants were visibly much larger and stayed greener than the WT plants (Fig. 2). This clear observation was also found in transgenic cassava carrying *Agrobacterium IPT* upon

drought treatment, which could be explained by delay of leaf senescence and relatively higher chlorophyll content compared with the WT (Zhang *et al.*, 2010).
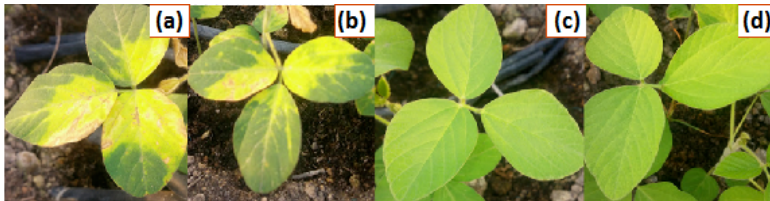
Figure 1. Identification of the transgenic soybean plants based on Basta resistance phenotype. Each plant was sprayed with 3-ml Basta solution at concentration of 80 mg/l. (a) Negative control; (b) Sensitive plant; (c) Positive control; (d) Transgenic plant.
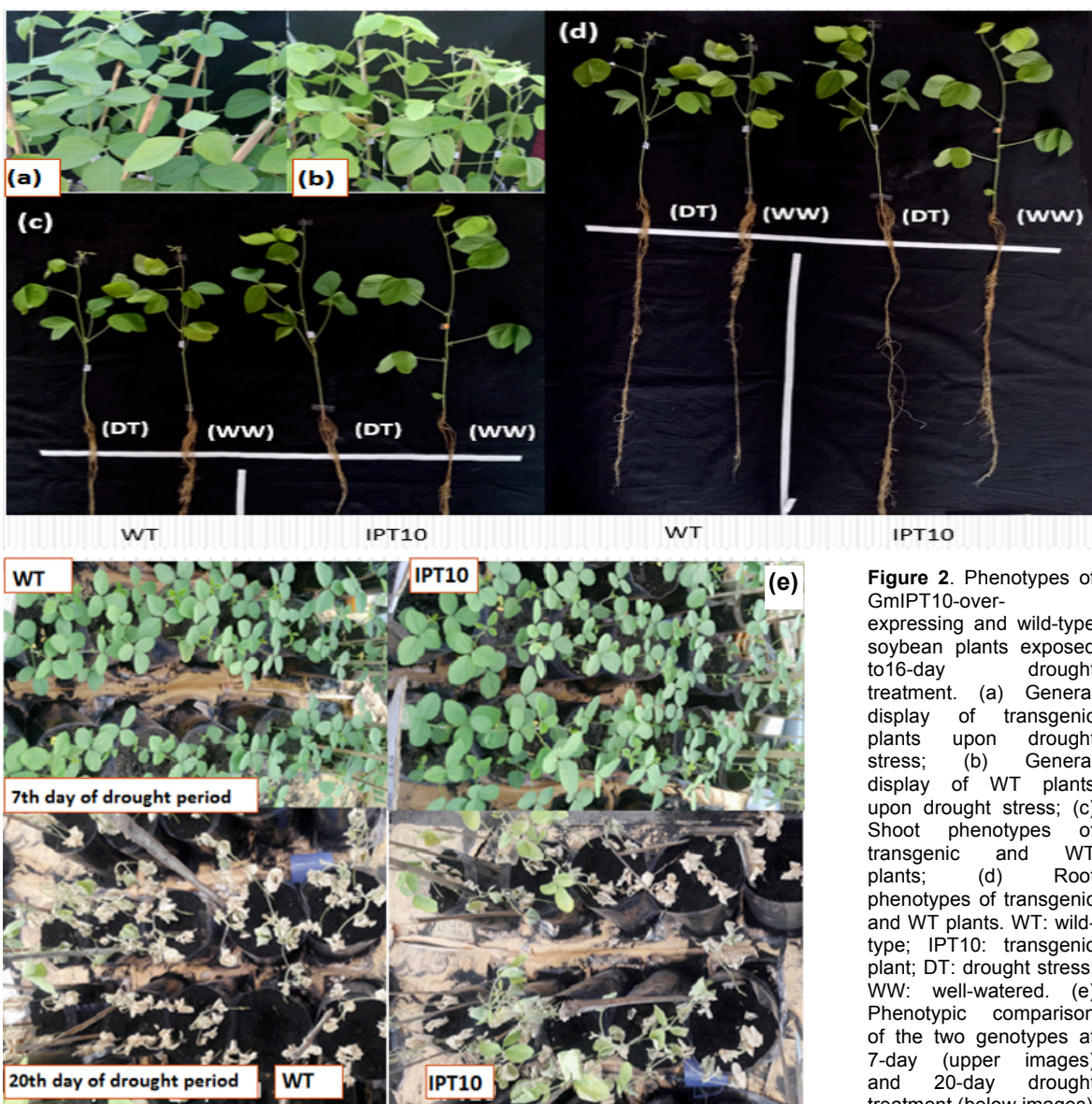
Figure 2. Phenotypes of GmIPT10-over-expressing and wild-type soybean plants exposed to16-day drought treatment. (a) General display of transgenic plants upon drought stress; (b) General display of WT plants upon drought stress; (c) Shoot phenotypes of transgenic and WT plants; (d) Root phenotypes of transgenic and WT plants. WT: wild-type; IPT10: transgenic plant; DT: drought stress; WW: well-watered. (e) Phenotypic comparison of the two genotypes at 7-day (upper images) and 20-day drought treatment (below images)
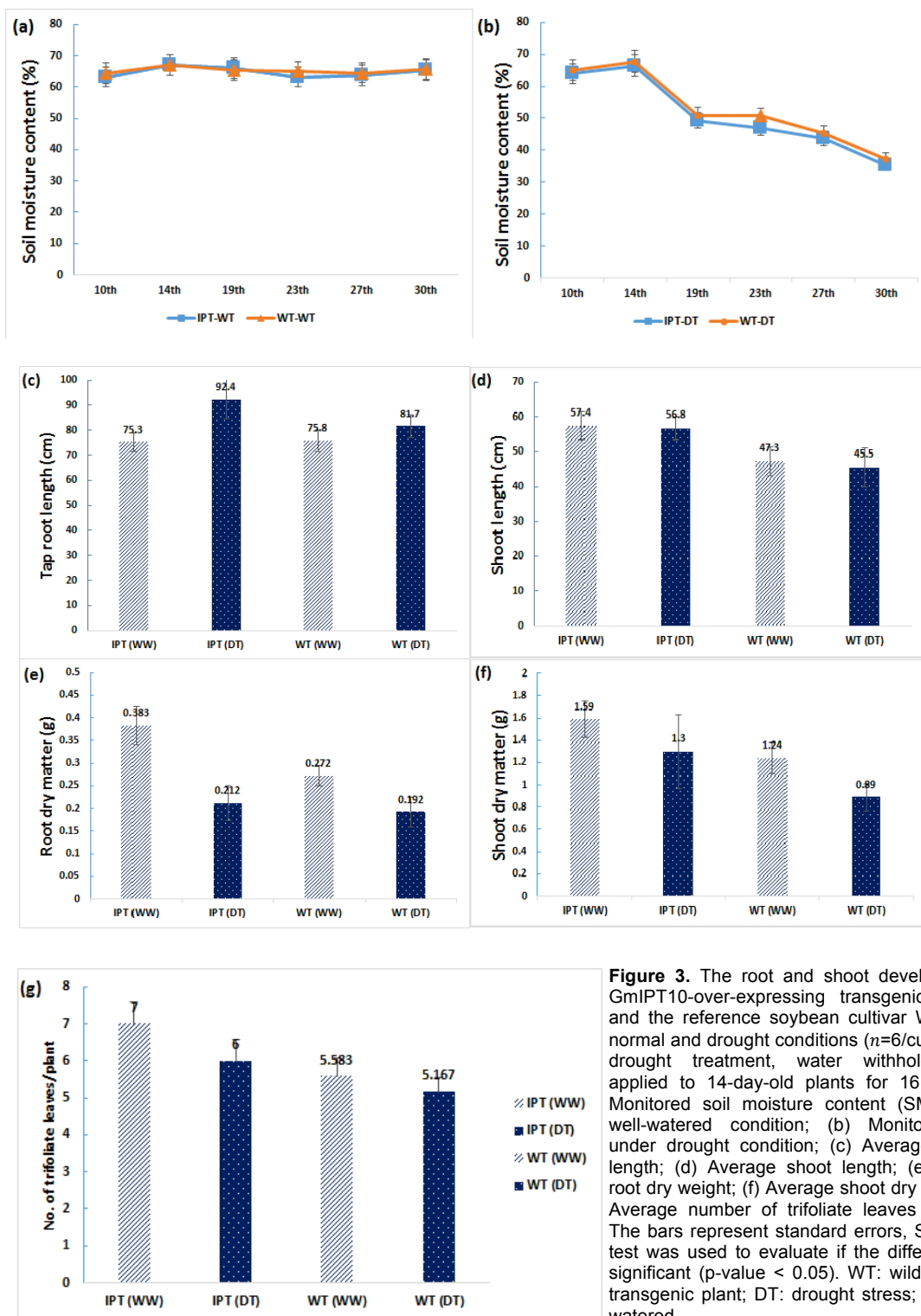
Figure 3. The root and shoot development of GmIPT10-over-expressing transgenic soybean and the reference soybean cultivar W82 under normal and drought conditions (*n*=6/cultivar). For drought treatment, water withholding was applied to 14-day-old plants for 16 days. (a) Monitored soil moisture content (SMC) under well-watered condition; (b) Monitored SMC under drought condition; (c) Average tap root length; (d) Average shoot length; (e) Average root dry weight; (f) Average shoot dry weight; (g) Average number of trifoliate leaves per plant. The bars represent standard errors, Student's *t*-test was used to evaluate if the difference was significant (p-value < 0.05). WT: wild-type; IPT: transgenic plant; DT: drought stress; WW: well-watered.

Under drought treatment condition, analyzing root trait revealed that the average tap root length of the *GmIPT10*-over-expressing line (92.4 cm) was significantly greater than that of the WT (81.7 cm)

(*p*-value < 0.05) (Fig. 3c). With similar trend, the drought-treated transgenic plants had considerably longer shoot than that of the WT counterparts (56.8 cm and 45.5 cm, respectively, *p*-value < 0.05) (Fig. 3d). These results provide a good comparison between the transgenic and WT plants, as the former also displayed increased shoot dry weight (*p*-value < 0.01) and trifoliate leaf number (*p*-value < 0.0001) compared to those of WT soybean (Fig. 3f, g). Higher biomass accumulation in soybean transgenic plant under water deficit could be induced by higher photosynthetic rates, higher stomatal conductance and higher transpiration and improved water use efficiency since these improved biochemical parameters were found in peanut over-expressing *IPT* gene (Qin *et al.*, 2011).

When evaluate the effect of drought to each genotype, the water shortage led to a significant reduction in shoot dry weight of the WT (*p*-value < 0.03, Fig. 3f). Meanwhile, tap root of the transgenic plants under water deficit tended to be statistically much longer (92.4 cm) than the mean root length of the transgenic plants grown under well watering condition (75.3 cm) (*p*-value < 0.001, Fig. 3c), although the drought stress still caused a substantial reduction in root dry matter (*p*-value < 0.001, Fig. 3e). The observation in transgenic plant with strong development of primary root and reduction of lateral root was a good accordance with the criteria specified for genotype with improved phenotypic traits and better drought tolerance under drought stress (Thu *et al.*, 2014). Promotion of primary root growth is to increase the probability of accessing water at deeper soil layer when water becomes limited while lateral root (LR) development is concerned as an adaptive adjustment to nutrient deficiency (Linkohr *et al.*, 2002; Zhan *et al.*, 2015). Exogenous CK was known as inhibitor of LR development (Li *et al.*, 2006; Laplaze *et al.*, 2007). It was reported that CKs inhibit LR development via regulating *abscisic acid insensitive4* (*ABI4*), which encodes an ABA-regulated AP2 domain transcription factor in *Arabidopsis*, causing a reducing of polar auxin transport to promote LR formation (Shkolnik-Inbar, Bar-Zvi, 2010). Therefore, it is generally postulated that extending primary root length under water deficit seen in the studied *GmIPT10*-carrying transgenic plants might be due to CKs adjustment.

Taken together, our results indicated that the *GmIPT10*-over-expressing soybean line displayed improved drought tolerant traits, which are consistent with the results reported previously in studies by Oneto *et al.*, (2016) in maize, Kuppu *et al.*, (2013) in cotton and Qin *et al.*, (2011) in peanut.

Transgenic plants had lower penalty in RWC reduction upon drought stress exposure

Relative water content was considered as one of the main parameters to evaluate the drought tolerance capacity in plants (Yan *et al.*, 2016). The RWC reflects the plant ability to store water and minimizing cellular water loss due to drought effects will bring advantage for the plant to survive as well as to maintain its growth and development. The analyzed results indicated that under the same growing condition of drought application, the aerial parts of the transgenic and non-transgenic plants shared similar RWC values although the value of the former was slightly higher (Fig. 4b). However, the WT plants had a noteworthy penalty in RWC upon drought exposure (decreased by 6.37%, *p*-value < 0.001) in comparison with its counterpart. For the transgenic plants, there were no significant difference in RWC values between the plants grown under normal and drought conditions. The results suggest that the transgenic line may have advantages in tolerance to drought stress.

Transgenic plants had higher survival rate upon drought exposure

Soybean plants were first grown in a net house with normal irrigation for 2 weeks, and then non-irrigation was applied in the next 20 days in order to evaluate the drought effect on the plant survival. According to our record, there were no phenotypic differences between the two genotypes, or between the drought treated and non-drought-treated plants within the early stage of drought stress exposure. However, after 18 days of water deficit, wilting symptoms were clearly seen. At this time-point, interestingly, the transgenic plants possessed higher survival percentage compared to the survival rate of the drought-treated WT (97% versus 80%, respectively, Fig. 5a). At the end of the drought treatment, 80% of the transgenic plants were still alive while the survival rate of the WT decreased to 50% (Fig. 5). Statistical comparison analyses showed that the transgenic line displayed significantly improved water-deficit tolerance in green house conditions (*p*-value < 0.002).
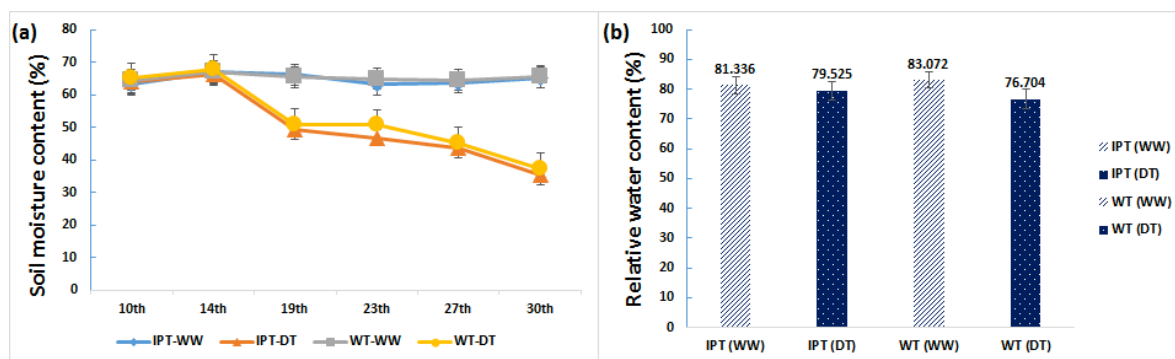
Figure 4. Examination of relative water content (RWC) of aerial part of *GmIPT10*-over-expressing and wild-type soybean plants. Sixteen-day drought period was applied, started when plants were 14 days old. (a) The SMC was monitored during the experiment (*n*=10); (b) RWC values (*n*=10/cultivar).
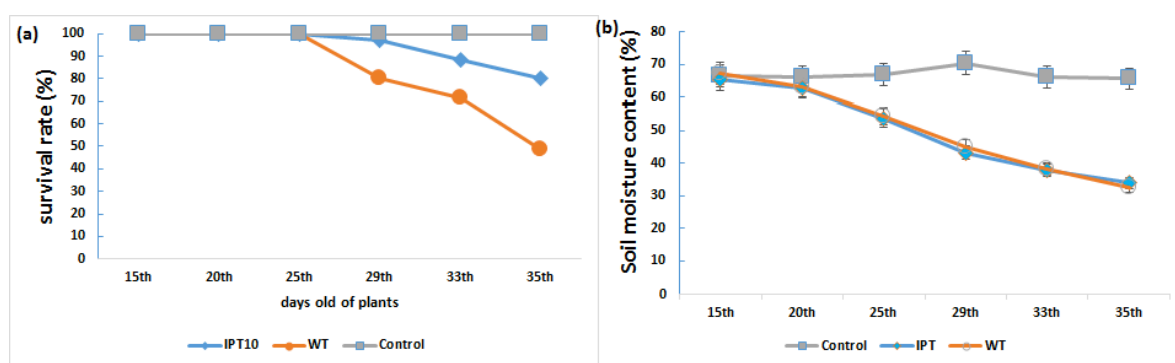
Figure 5. Evaluation of drought tolerance capacity of the *GmIPT10*-overexpressing and wild-type plants (*n*=35 per genotype). The non-irrigation was applied for 20 days after growing the plants for 14 days under normal condition; (a) Survival rates of the two genotypes upon drought treatment; (b) Monitored soil moisture content during the experiment (n=10); The bars indicate standard errors.

CONCLUSION

This study was the first example demonstrating that over-expression of *GmIPT10* in soybean plants could improve root and shoot – related traits which would bring advantages for plants to cope with drought stress, including increased primary root and shoot lengths, better production of shoot biomass, higher number of trifoliate leaves, and higher survival rate than the non-transgenic plants under the stress condition. The obtained results indicate this transgenic line might have better drought tolerance capacity, and thus worthwhile to perform in-depth studies to precisely evaluate its tolerance ability to drought and its potential of economic application, as well as to get understanding of modulating mechanisms mediated by *GmIPT10*.

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PHÂN TÍCH MỘT SỐ TÍNH TRẠNG SINH LÝ CỦA CÂY CHUYỂN GEN TĂNG CƯỜNG BIỂU HIỆN *GmIPT10* Ở ĐIỀU KIỆN TRỒNG BÌNH THƯỜNG VÀ CÓ XỬ LÝ STRESS HẠN

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TÓM TẮT

Cytokinin (CKs) là một loại hormone thực vật có vai trò quan trọng không chỉ trong quá trình phát triển mà còn trong quá trình chống chịu stress ở cây trồng. Các nghiên cứu trước cho thấy việc nâng cao khả năng chống hạn của cây bằng cách dùng kích thích stress để tăng cường biểu hiện của gen mã hóa enzyme adenine isopentenyl transferase (IPT) liên quan đến tổng hợp CK, là một trong những giải pháp khả thi. Để nghiên cứu vai trò của GmIPT10 ở đậu tương trong giúp cây chống hạn, chúng tôi đã tạo được cây chuyển gen có biểu hiện vượt mức *GmIPT10* và sàng lọc thành công dòng đậu tương mang một bản sao của gen chuyển *GmIPT10* ở dạng đồng hợp. Đánh giá các tính trạng sinh lý cho thấy so với cây không chuyển gen đối chứng, dòng cây chuyển gen mang nhiều tính trạng liên quan đến khả năng chống chịu hạn bao gồm tăng cường chiều dài rễ chính và thân, tăng khối lượng sinh khối, có số lá kép ba lá nhiều hơn và có tỉ lệ sống sót cao hơn khi được thử nghiệm stress hạn. Kết quả thực nghiệm trong nhà lưới cũng cho thấy dòng chuyển gen *GmIPT10* có chỉ số hàm lượng nước tương đối trong mô chồi cao hơn dưới điều kiện stress. Những kết quả này cho thấy việc nâng cao khả năng chịu hạn của cây có thể thực hiện được thông qua điều phối sự biểu hiện của *GmIPT10*.

Từ khóa: *Cây chuyển gen, chống chịu hạn, Cytokinin, đậu tương, GmIPT10*