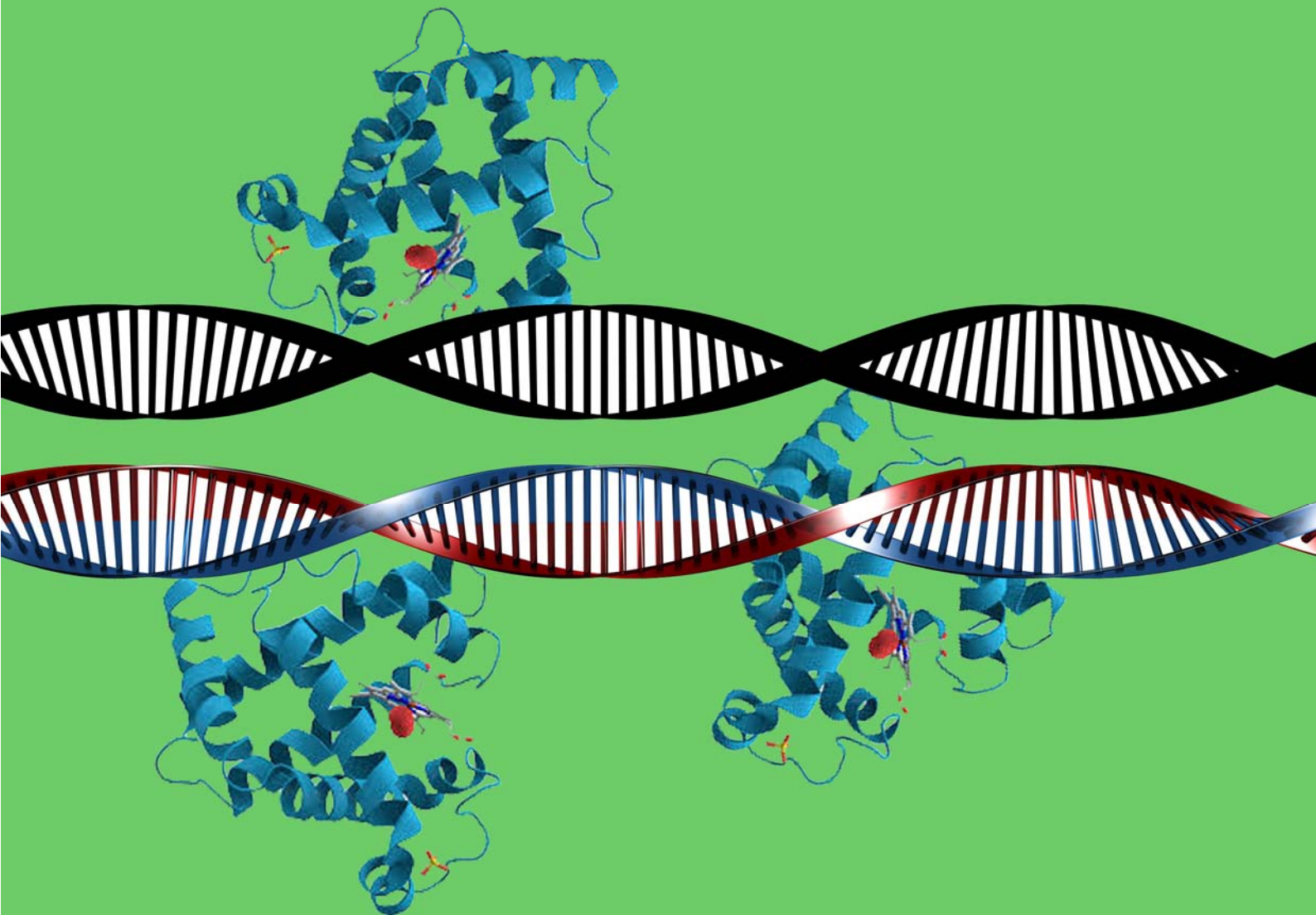


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Research Report

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Expression Analysis of *Arabidopsis thaliana* Genes *ARGAH1*, *ARGAH2* and the Response to Sodium Chloride Stress During Seed Germination

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Abstract *Arabidopsis thaliana* possesses two arginase-encoding genes (*ARGAH1*, *ARGAH2*), which catalyses the catabolism of arginine into L-ornithine and urea. In this research, we focus on the function of the two genes (*ARGAH1*, *ARGAH2*) encoding *Arabidopsis thaliana* arginase. The cDNA of cloned *ARGAH1* is 1347 bp, and 5' untranslated region (UTR) is 145 bp and the 3' UTR is 166 bp. The open reading frame of *ARGAH1* is 1029 bp, encoding 342 amino acids. *ARGAH2* is 1284 bp, and 5' untranslated region (UTR) is 34 bp and the 3' UTR is 215 bp. The open reading frame of *ARGAH2* is 1035 bp, encoding 344 amino acids. We studied the different expression models of arginase genes under NaCl stress by qRT-PCR and other biotechnology. Interestingly, we observed that the expression of *ARGAH1* and *ARGAH2* is both induced by NaCl stress. At the same time, the enzyme assay of arginase also showed an increase during seed germination. Therefore, we maintain that the arginase genes play a role against to NaCl stress especially during the germination.

Keywords *Arabidopsis thaliana*, ARGAHs, Salt Stress, Seed Germination

1 Introduction

The life circle of higher plants begins with the seed germination (Yan, 2014); the seed germination directly concerns the survival rate of seedlings and the condition of the follow-up growth (Guterman, 2002). Therefore, the germination of seed has a significant meaning to the growth and development. As we know, the seed of *Arabidopsis thaliana* is surrounded by the endosperm, which is made up by only a single layer cell and the episperm (Holdsworth *et al.*, 2008; Weitbrecht *et al.*, 2011). Then the seed germination is defined as two stages. The first stage is completed by the time that the radicle broken through endosperm. And the second stage is accomplished by the time radicle broken through episperm (Bentsink and Koornneef, 2008). During the seed germination period plants need to carry on a large amount of metabolism for accomplishing the growth and development. However, there are lots of environmental factors that restrict the seed germination and the salt stress is a kind of stresses.

Salt stress is a usual stress type, the half of the world irrigation soil and the twenty percent of cultivated land is suffered to the salt stress at different levels (Zhu, 2001). Salt stress causes ion poison, osmotic stress, and the accumulation of ROS, leading to the peroxidation of lipid and the inactivation of antioxidant enzyme (Mittler, 2002; Tanou *et al.*, 2009). It is reported that salt stress has a serious influence to seed germination. High salt density can decrease the germination rate of seed, delaying the germination time and reducing the rate that seedling grown up (Almansouri *et al.*, 2001). Consequently, researching the influence of salt stress in the seed germination has a significant meaning in order to find out the suitable method to decrease even remove the effect of salt to seed germination.

A lot of nitrogen elements largely in seed is stored in the form of storage proteins, providing an adequate nitrogen source for the subsequent seed germination and seedling growth in the early development period.

Studies show that the main form of storage proteins is arginine in the plant seed (Chat Thai and Misra, 1998; King and Gifford, 1997). During the seed germination period, a large number of storage proteins degraded, and we can detect a significant increase in arginine content (Herman and Larkins, 1999). For example, during the seed germination of Loblolly Pine (*Pinus taeda L.*), the storage proteins existing in megagametophyte degraded, and then the amino acid content of seedlings increased largely. Among the amino acid, the part of arginine is the most, almost accounted for half of the nitrogen storage in the storage proteins in megagametophyte (King and Gifford, 1997). Consequently, we can know that arginine play an important role in seed germination progress.

Arginase (E. C. 3. 5. 3. 1) is an enzyme contained manganese metal, and it has three subunits, each of which requires two Mn atoms, and arginase specificity catalyzes L-arginine into L-ornithine and urea (da Silva *et al.*, 2008; Di Costanzo *et al.*, 2005; Reczkowski and Ash, 1992). Currently, the arginase of many plants has been studied. In soybean, there is a sharp increase expression during germination period, which reached the maximum 3 or 5 days later after germination (Goldraij and Polacco, 1999); In rice, the only arginase gene plays a key role in the development of panicles and grain yield, especially when the external source of nitrogen deficiency (Ma *et al.*, 2013); There are two arginase genes in tomato -*LeARG1*, *LeARG2*, both of which are abundantly expressed in reproductive tissues such as the bud, not open mature flowers, already open matured flowers and unripe fruits (Chen *et al.*, 2004; Alabadí *et al.*, 1996); In *Arabidopsis thaliana*, there are two arginase genes which has been cloned, namely *ARGAH1*, *ARGAH2* (Flores *et al.*, 2008; Krumpelman *et al.*, 1995). *ARGAH1* can be transcribed and expressed (Flores *et al.*, 2008). And the expressing of *ARGAH2* is induced by *Botrytis cinerea* and methyl jasmonate (Brauc *et al.*, 2012; Brownfield *et al.*, 2008); during germination and post-germination in loblolly pine, the arginase activity is also increased largely, accompanying with the increase of arginase gene expressing (King and

Gifford, 1997; Todd *et al.*, 2001). In addition, arginase activity has a rapid increase during the germination in many plants, such as pumpkin (Splittstoesser, 1969), broad bean (Kollöffel and van Dijke, 1975), soybean (Kang and Cho, 1990), *Arabidopsis thaliana* (Zonia *et al.*, 1995).

In addition, it is reported that arginase has a response to various biotic stresses and abiotic stress through the regulation of arginine metabolism and polyamine pathway. For example, the mutant of *Arabidopsis thaliana* arginase accumulated less ROS, which indicated that inhibiting the expression of *ARGAHs* can improve the tolerance to dehydration, high salt stress and chilling stress (Shi and Chan, 2013). Furthermore, Brown and his team have found out that *Botrytis cinere* induced the expression of *ARGAH2* and the increase expression of *ARGAH2* increased the tolerance to *Botrytis cinere* in *Arabidopsis thaliana* (Brauc *et al.*, 2012; Brownfield *et al.*, 2008). Similar to this, previous studies have shown that the deletion mutant of *ARGAHs* genes led to an increase accumulation of nitric oxide, and the overexpression of *ARGAH2* enhanced the development of callus tissue when the plant is infected by Clubroot. In tomato, the expression of *LeARG2* is induced by wounding. However, *LeARG1* is not (Chen *et al.*, 2004). In conclusion, we can summarize that arginase genes are largely expressed during seed germination, and arginase plays a role in the response to many adversity stresses in plants.

In this study, we take Columbia-type *Arabidopsis* as experimental material. By the means of real-time fluorescence quantification PCR (QRT-PCR) and Semi-quantitative RT-PCR (SqRT-PCR), we analysis the expression characteristics of *ARGAHs* genes during seed germination, especially under NaCl treatment, and aim to know about the function of arginase genes responding to salt stress during seed germination.

2 Results and Discussion

2.1 Homologous comparison analysis of *ARGAH1* and *ARGAH2* genes

There are two genes who encode arginase: *ARGAH1*, *ARGAH2*. Biological chemistry analysis shows that

these two genes both had arginase activity. We successfully cloned these genes: *ARGAH1*, *ARGAH2*; meantime, we got matched the sequence data on NCBI. The full length of the cloned cDNA sequence of *ARGAH1* is 1347 bp; 5' non-coding region lasts 145 bp, and the 3' non-coding region length is 166 bp; the ORF length is 1029 bp, encoding 342 amino acids, and the molecular mass prediction is 37344 Da. The predicted isoelectric point is 6.11. On the other hand, the full length cDNA of *ARGAH2* is 1284 bp; its 5' non-coding region lasts 34 bp, and 3' non-coding region is 215 bp. The ORF of *ARGAH2* is 1035 bp. *ARGAH2* encodes 344 amino acids, and the molecular mass prediction is 37980 Da. The predicted isoelectric point is 5.90. The similarity of *ARGAH1* and *ARGAH2* amino acids is 99%; and 86% with *Brassica napus*, 81 % with Arginase 1 in tomato.

To further investigate the relationship between these two genes in *Arabidopsis thaliana*, and the cognate relation with other species, we select the arginase genes from other species to do the comparison. The conclusion will be drawn that the *Arabidopsis thaliana* arginase1 has the closest relationship with *Arabidopsis lyrata* and *Brassica napus*; however, the *Arabidopsis thaliana* arginase2 has the closest relationship with wheat and *Brachypodium distachyon*. Two arginases in tomato have a close relationship with each other (Figure 1).

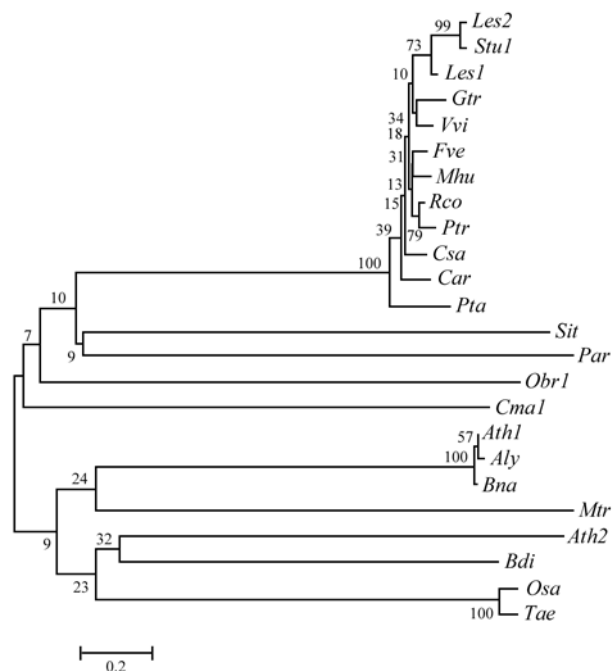


Figure 1 The phylogenetic tree of arginase in selected higher plants. Species abbreviations are as follows *Lycopersicon esculentum* (Les), *Solanum tuberosum* (Stu), *Gentiana triflora* (Gtr), *Vitis vinifera* (Vvi), *Fragaria vesca subsp.vesca* (Fve), *Malus hupehensis* (Mhu), *Ricinus communis* (Rco), *Populus trichocarpa* (Ptr), *Cucumis sativus* (Csa), *Cicer arietinum* (Car), *Pinus taeda* (Pta), *Setaria italica* (Sit), *Prunus armeniaca* (Par), *Oryza brachyantha* (Obr), *Glycine max* (Gma), *Arabidopsis thaliana* (Ath), *Arabidopsis lyrata* (Aly), *Brassica napus* (Bna), *Medicago truncatula* (Mtr), *Brachypodium distachyon* (Bdi), *Oryza sativa* (Osa), *Triticum aestivum* (Tae)

2.2 The expression analysis of *ARGAHs* genes during different development periods

To clarify the expression characters of arginase gene in different growth periods in *Arabidopsis thaliana*, this research selects dry seed, normal raised seedling after 36 hours and 2-week seedling as experimental material. Using QRT-PCR and SqRT-PCR methods to research the expression of the arginase genes in different periods of growth. *ARGAH1* and *ARGAH2* genes expressed a lot in dry seed, and the least in 36-hour seeding. This result shows that the expression of arginase genes is abundant in germination, which is consistent with the findings about these two genes expressed a lot in tomato reproductive tissue (Chen *et al.*, 2004).

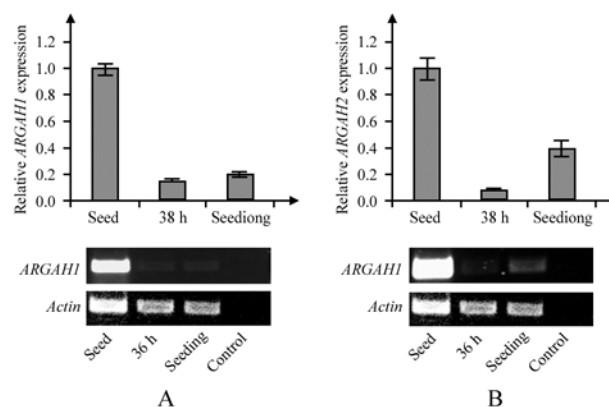


Figure 2 Expression analysis of *ARGAH1* gene and *ARGAH2* gene during different developmental stages. A represented the expression change of *ARGAH1* gene by QRT-PCR and SqRT-PCR; B represented the expression change of *ARGAH2* gene by QRT-PCR and SqRT-PCR

2.3 Expression Analysis of arginase genes under NaCl treatment during seed germination in *Arabidopsis thaliana*

The results of the analysis of the arginase genes expression show that the arginase genes expressed abundantly during seed germination. To further investigate the change of the arginase genes expression under the NaCl treatment, this research conducted SqRT-PCR and QRT-PCR methods to do the experiments to study it.

2.4 Expression Analysis of arginase genes under the NaCl treatment in different concentrations

To choose the suitable NaCl density, we read numerous academic papers, finally choose 0, 50, 100, 150, 200 mM as 5 concentration gradients to be treatment conditions.

The result shows that the expression levels of *ARGAH1* and *ARGAH2* genes had risen with the increasing of NaCl concentration. Under 200 mM NaCl treatment, the expression levels of *ARGAH1* and *ARGAH2* reached the highest.

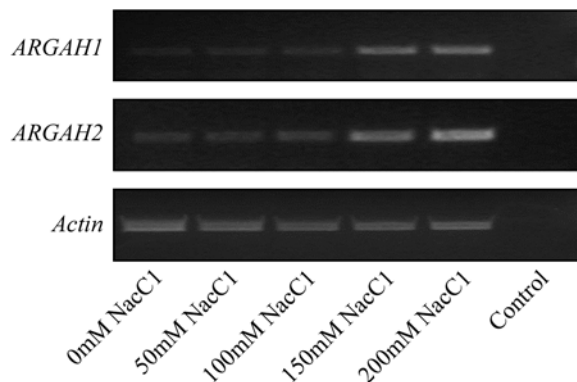


Figure 3 Expression analysis of *ARGAHs* genes under NaCl treatment by SqRT-PCR

2.5 Expression analysis of arginase genes during different germination stages under NaCl treatment

We can see that the expression levels of *ARGAH1* and *ARGAH2* were increased with increasing NaCl concentration. In order to detect the expression levels of *ARGAH1* and *ARGAH2* under NaCl treatment, we chose 100 mM as the experimental concentration. The seed sterilized was sown in 1/2 MS medium and 1/2 MS medium adding 100 mM NaCl. The seed was

vernalized at 4 °C for two days and then transferred to the culture chamber to culture. RNA was extracted from the germinating seed after different culture time (0, 12, 24, 36, 48) h, and then was reverse transcribed to cDNA, the expression levels of *ARGAH1* and *ARGAH2* was detected by Real-time quantitative PCR. Experimental results are shown in Figure 4, both of *ARGAH1* and *ARGAH2* were largely expressed in the seed; the expression level of *ARGAH1* gene is induced by 100 mM NaCl treatment compared with the control group with no NaCl added. The expression level of *ARGAH1* is a little higher after 0 h culture. The speculated reason is that the material was taken out from low temperature (4°C), and it is reported that low temperature induce the expression of *ARGAHs*. In this study, the expression model of *ARGAH2* is similar to *ARGAH1*, except for the time after culture 36 h and 48 h at which time expression of *ARGAH2* gene under NaCl treatment was a bit lower than control. It is not clear that the reason causing this situation, so further research is needed to find it out.

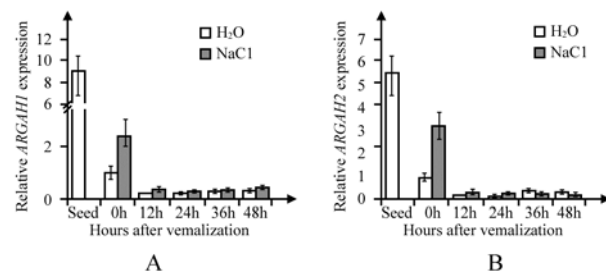


Figure 4 Real-time quantitative expression analysis of *ARGAHs* gene under NaCl treatment during seed germination. A represented the expression change of *ARGAH1* gene treated with 100mM NaCl during seed germination; B represented the expression change of *ARGAH2* gene treated with 100mM NaCl during seed germination

2.6 The enzyme activity assays of arginase

In this study, we analyzed the arginase activity under 100 mM NaCl treatment during seed germination. We extracted the whole protein from fresh materials and detected the arginase activity. We can see that the arginase activity in seed was the highest, and the arginase activity is higher under NaCl treatment compared with control. There is an increase in 36 h, while the possible reason needs further analysis to find out.

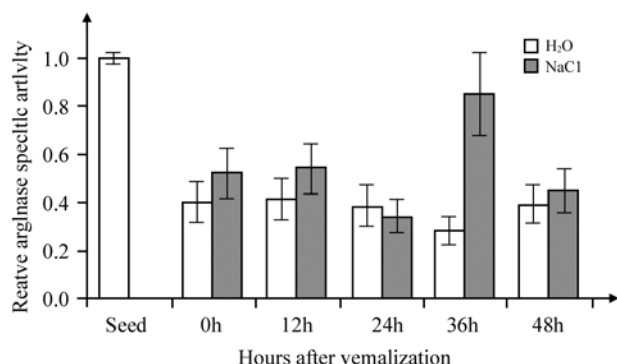


Figure 5 The analysis of arginase activity during seed germination under 100 mM NaCl treatment in *Arabidopsis thaliana*

3 Materials and Methods

3.1 Materials and Treatments

Arabidopsis thaliana ecotype Columbia seed was preserved in the Alkali Soil Natural Environmental Science Center (ASNEC), Northeast Forestry University, and Harbin, China.

The seed was sterilized with 75 % ethanol and sodium hypochlorite, and then was sown in glass containers filled with 1/2 MS medium in the growth chamber after stratification at 4°C for two days in darkness. For experiments about analysis of expression level during different developmental stages, RNA was extracted from seed just after sterilization, 36-hour-old seeding, 3-week-old seeding. For experiments about analysis of expression model under NaCl treatment, seed was sown in glass container filled with 1/2 MS medium, adding different concentrations of NaCl (0,50,100,150,200) mM. For experiments about the analysis of arginase genes under NaCl treatment during seed germination, RNA was extracted from the seed treated by 100 mM NaCl after culture different time (0,12,24,36,48) h.

3.2 Arginase phylogeny

Members of the arginase in plants were obtained by BLAST searches in NCBI. The cDNA of *ARGAH1* and *ARGAH2* was obtained from TIGR databases. A total of 28 sequences were used for construction of the phylogenetic tree (Figure 1).

3.3 Semi-quantitative reverse-transcription PCR and real-time quantitative PCR

Total RNA was isolated from 0.2 g seed using a modified method as described previously by Martin (Martin *et al.*, 2005), and treated by RNase-free DNase (Takara) to remove genomic DNA. First-strand cDNA was synthesized using Prime Script RT reagent kit (Takara) from 1 µg total RNA, and 1 µl RNA was added to 30 µl PCR mixture for SqRT-PCR. For real-time quantitative PCR, a 20 µl mixture was used with SYBR-green fluorescence (TransGen Biotech) and the comparative $\Delta\Delta CT$ method was used as previously described (Yang *et al.*, 2011). All the primers were referred to Brauc (Brauc *et al.*, 2012).

3.4 Enzyme assays

The method used to measure arginase activity is as described by King (King and Gifford, 1997). Specific operation will not be described.

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