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Running title: *AaNAC1* enhances the content of artemisinin

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Overexpression of a novel NAC domain-containing transcription factor (*AaNAC1*) enhances the content of artemisinin and increases tolerance to drought and *Botrytis cinerea* in *Artemisia annua*

Running title: *AaNAC1* enhances the content of artemisinin

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Abbreviations: ADS, amorpha-4,11-diene synthase; ALDH1, aldehyde dehydrogenase 1; CYP71AV1, amorphadiene 12-hydroxylase; DBR2, artemisinic aldehyde Δ11(13) reductase; DMADP, dimethylallyl diphosphate; DHAA, dihydroartemisinic acid; FDS, fanesyl diphosphate synthase; NAC, NAM/ATAF/CUC; SA, Salicylic Acid; YFP, yellow fluorescent protein.

Abstract

The NAC (NAM, ATAF, and CUC) superfamily is one of the largest plant-specific transcription factor families. NAC transcription factors always play important roles in response to various abiotic stresses. A NAC transcription factor *AaNAC1* containing a complete ORF of 864 bp was cloned from *Artemisia annua*. The expression of *AaNAC1* could be induced by dehydration, cold, salicylic acid (SA) and methyl jasmonate (MJ), suggesting that it might be a key regulator of stress signaling pathways in *A. annua*. AaNAC1 was shown to be localized to the nuclei by transforming tobacco leaf epidermal cells. When *AaNAC1* was overexpressed in *A. annua*, the content of artemisinin and dihydroartemisinic acid was increased by 79 and 150%, respectively. The expression levels of artemisinin biosynthetic pathway genes, *i.e.* amorpha-4,11-diene synthase (*ADS)*, artemisinic aldehyde Δ11(13) reductase (*DBR2)* and aldehyde dehydrogenase 1 (*ALDH1),* were increased. Dual luciferase (Dual-LUC) assays showed that AaNAC1 could activate the transcription of *ADS* in vivo. The transgenic *A. annua* exhibited increased tolerance to drought and resistance to *Botrytis cinerea*. Overexpression of *AaNAC1* in Arabidopsis, the transgenic Arabidopsis were markedly more tolerant to drought. The transgenic Arabidopsis showed increased resistance to *Botrytis cinerea*. These results indicate that *AaNAC1* can be potentially used in transgenic breeding for improving the content of artemisinin and drought tolerance in *A. annua*.

Keywords: NAC transcription factors *A. annua* artemisinin Drought resistance *Botrytis cinerea*

Introduction

Environmental stresses such as drought, salinity and extreme temperature, impose osmotic stress on plants and significantly affect both biomass and grain yields of crops worldwide (Mao et al. 2012). The NAC (NAM, ATAF, and CUC) superfamily is one of the largest plant-specific transcription factor families. The N-terminus of NAC proteins is conserved and has a DNA-binding domain, which is constituted by 150 amino acids and contains five conserved regions. The N-terminus also usually has a nuclear localization signal. The C-terminus of NAC proteins has a transcriptional activation domain. The NAC transcription factors can promote root development (Fu et al. 2013; Hao et al. 2011; Xie et al. 2000) and enhance disease resistance (Kim et al. 2012; Shan et al. 2015; Sun et al. 2012). Many reports indicate that the NAC transcription factors play important roles in response to drought, salt, cold, and chilling (Fang et al. 2015; Yang et al. 2015; You et al. 2015). More recent studies show that overexpression of NAC transcription factors can enhance drought stress tolerance in rice and wheat (Gao et al. 2010; Jeong et al. 2013; Saad et al. 2013; Tang et al. 2012). NAC transcription factors interact specifically with several stress-responsive *cis*-acting elements with the core-binding motif 'ACACGCATGT' (Franco-Zorrilla et al. 2014; Hu et al. 2006; Tran et al. 2004).

The necrotrophic fungus *Botrytis cinerea* can bring about significant economic losses in agriculture (Lu et al. 2013a). NAC transcription factors can modulate the resistance to *B. cinerea* in Arabidopsis (Bu et al. 2008; Wu et al. 2009). Besides balancing the relative abundance of different stresses, NAC transcription factors can modulate the biosynthesis of terpenoids. The NAC transcription factors *NAC2*, *NAC3*, and *NAC4* of *Actinidia arguta* can bind to the terpene synthase 1 (*TPS1)* gene promoter and regulate monoterpene production (Nieuwenhuizen et al. 2015). *SlNAC4* in tomato can increase the ethylene synthesis and promote fruit ripening (Zhu et al. 2014).

Artemisia annua L (sweet wormwood; Chinese wormwood) is a member of the Asteraceae family of plants (Brown 2010), which is a genus of small herbs and shrubs and can produce a many different natural compounds, including artemisinin and its precursors. Artemisinin has been isolated from mature leaves or buds of *A. annua*. Artemisinin has been identified as the anti-malarial principal of the plant and it was discovered by Youyou Tu (Miller and Su 2011). In addition to malaria, artemisinin can cure other diseases including different types of cancer, other parasitic diseases like schistosomiasis, and some viral diseases such as hepatitis B (Cao et al. 2015; Nguyen et al. 2011).

In the process of artemisinin biosynthesis, the first and key enzyme is amorpha-4,11-diene synthase (ADS) (Bouwmeester et al. 1999; Mercke et al. 2000), which converts farnesyl diphosphate (FPP) to the sesquiterpenoid amorpha-4,11-diene. In the following step, the amorpha-4,11-diene produced is converted to artemisinic alcohol by a cytochrome P450 dependent hydroxylase (CYP71AV1) (Teoh et al. 2006). CYP71AV1 can also oxidize the artemisinic alcohol to artemisinic aldehyde. In two steps, catalyzed by the artemisinic aldehyde Δ11(13) reductase (DBR2) (Zhang et al. 2008) and aldehyde dehydrogenase 1 (ALDH1) (Teoh et al. 2009) dihydroartemisinic acid is produced which is the final intermediate of artemisinin biosynthesis. Artemisinin is an effective drug for curing malarial, but the content of artemisinin in the plant is low (0.4-1%) and currently the increasing worldwide demand cannot be met.

Drought and salt are the major abiotic stresses that cause decreased yields of crops. It has been estimated that drought and salt cause an average yield loss of more than 50%. The major artemisinin producers are China and Vietnam while the production of artemisinin in Africa has been increased in recent years. However, drought is a main factor that affects artemisinin yields in Africa. For this reason, enhancing the tolerance of *A. annua* to drought can bring about immediate benefits for farmers of the

developing world, as well as improve the existing artemisinin supply chain by reducing production costs and stabilizing supplies (Graham et al. 2010).

There are 151 non-redundant NAC genes in rice and 117 in Arabidopsis (Nuruzzaman et al. 2010), while there are 55 NAC genes in *A. annua* glandular trichome transcriptome. We chose the NAC transcription factors according to the rank of top ten RPKM (Reads Per Kilobases per Million reads) value and *AaNAC1* was induced by SA, MJ. Because SA and MJ can improve the artemisinin content and increase the tolerance of various abiotic stresses, so we overexpressed *AaNAC1* in *A. annua* and tested its functions. The content of artemisinin was increased when *AaNAC1 was* overexpressed in *A. annua*. In addition, *AaNAC1* could increase tolerance to drought and *B. cinerea* in transgenic plants of *A. annua.* When *AaNAC1* was overexpressed in Arabidopsis, the transgenic plants showed increased tolerance to drought. These findings are the first to demonstrate that NAC transcription factors can modulate artemisinin biosynthesis when *AaNAC1* was overexpressed in the *A. annua*. Therefore, *AaNAC1* may be applied in genetic engineering strategies for improving the content of artemisinin and drought stress tolerance in *A. annua*.

Results

The identification and sequence analysis of drought stress-responsive NAC proteins

A full length nucleotide sequence of the *AaNAC1* containing a complete ORF of 864 bp was obtained from the *A. annua* RNA-Seq data. The GenBank accession number of *AaNAC1* is KX082975. Blastx from the NCBI showed that *AaNAC1* was highly homologous to *ATAF1* (identity 78%, ABZ89746.1) and *CmNAC1* (identity 81%, ADQ20114.1). The AaNAC1 encodes a protein of 287 aa with a calculated

isoelectric point of 5.11 and molecular weight of 71.56 kDa. Scansite analysis indicates that *AaNAC1* has an N-terminal region (1-129 amino acid residues) and a C-terminal region (130-287 amino acid residues). The N-terminal region is highly conserved (Fig. S1) and can bind to DNA and some proteins. The C-terminal region plays different roles under different environmental conditions and the main function is thought to be involved in regulating gene expression (Olsen et al. 2005).

Phylogenetic analysis of AaNAC1 proteins

The amino acid sequences of AaNAC1 and NAC protein sequences from other species were used to construct a phylogenetic tree to investigate divergence of AaNAC1 protein with other NAC proteins during evolution. AaNAC1 clustered in the same clade as MnATAF1 and CmNAC1 (Fig. S2). *MnATAF1* was induced and strengthened by wounding and *CmNAC1* was proved to enhance salt stress in transgenic tobacco (Li et al. 2012; Liu et al. 2011). Both of them are related to regulation of plant defense and stress responses. Most NAC genes are induced by wounding while other NAC genes are induced by ABA, drought (DR), cold, salt or pathogens (Puranik et al. 2012; Olsen et al. 2005).

The stress responsive characteristics of *AaNAC1*

Because many NAC transcription factors have some roles in resistance of abiotic stresses, so we treated *A. annua* with various abiotic stresses, such as NaCl, cold and dehydration to check the expression level of *AaNAC1.* When wild type seedlings of *A. annua* were treated by NaCl, the expression level of *AaNAC1* was increased 3-fold after 30 min (Fig.1A). However, at later time points (1, 3, 6, 12 and 24h), the expression level of *AaNAC1* was not changed. Therefore, *AaNAC1* may not play an important role in salt-resistance. Under the treatment of cold (Fig.1B), the mRNA of *AaNAC1* was

increased 5-fold. *AaNAC1* can also be induced by dehydration (Fig.1C). However, under the treatment of SA, MJ and SA+MJ, the mRNA of *AaNAC1* was increased about 30-, 40- and 60-fold, respectively (Fig.1D). Consequently, *AaNAC1* appears to be a stress-related gene and may enhance the drought tolerance and disease-resistance.

The stress environments may increase the gene expression levels of artemisinin biosynthetic pathway in *A. annua*. Previous studies indicated that under the treatment of cold, the mRNA levels of artemisinin biosynthetic pathway genes *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* are increased to 1- to 4-fold, while the treatment of UV light and heat shock are increased few of the expression levels of *ADS* and *CYP71AV1* (Yin et al. 2008; Liu et al. 2016). Water deficit can increase the artemisinin accumulation also (Marchese et al. 2010). Under the treatment of drought, the expression levels of *ADS*, *CYP71AV1*, *DBR2* and *ALDH1* are increased to 3- to 7-fold (Fig. S3). The stress environments increase both the artemisinin biosynthetic pathway genes and *AaNAC1*, therefore, there are some relationships between *AaNAC1* and artemisinin biosynthesis. So we checked the role of *AaNAC1* in *A. annua*.

Subcelluar localization of AaNAC1

Transcription factors are typically localized to cell nuclei where they can bind DNA and perform transcriptional activation role. To confirm the subcellular localization of the AaNAC1 protein, a yellow fluorescent protein (YFP) was fused to the C-terminus of AaNAC1 and the AaNAC1-YFP construct was expressed under the control of double 35S CaMV promoter. After agroinfiltration into *Nicotiana benthamiana*, the AaNAC1-YFP fusion protein was detected in the nucleus of epidermal cells by Confocal Laser Scanning Microscope. Microscopy showed that the AaNAC1 protein was localized to nuclei (Fig. 2).

Overexpression of *AaNAC1* improves the content of artemisinin in *A. annua*

When the ORF of *AaNAC1* was overexpressed under the control of a double 35S promoter in *A. annua*, the mRNA level of *AaNAC1* changed a lot. According to the different expression levels of *AaNAC1*, we selected the transgenic lines NAC1-30, NAC1-35 and NAC1-37 for further study. The expression level of *AaNAC1* were increased 2- to 12-fold in transgenic lines NAC1-30, NAC1-35 and NAC1-37 (Fig.3). Many studies indicate that transcription factors are likely to increase the expression of genes of the artemisinin biosynthetic pathway. Overexpression of *AabZIP1* and *AaERF1*/*AaERF2* increased the transcription levels of *ADS* and *CYP71AV1* (Lu et al. 2013a; Yu et al. 2011; Zhang et al. 2015), and overexpression of *AaORA* increased the transcription levels of *ADS*, *CYP71AV1* and *DBR2* (Lu et al. 2013b). Therefore, the transcription levels of genes of the artemisinin biosynthetic pathway were detected. In this study, the mRNA level of *ADS* was increased 1.5- to 4.5-fold, *DBR2* was increased 1- to 5-fold and *ALDH1* was increased 1- to 3-fold, while *CYP71AV1* was not significantly increased (Figs.4A-D). The expression of *HMGR* acting upstream of the artemisinin biosynthetic pathway was unaffected by overexpression of *AaNAC1* (Fig.S4). The content of artemisinin (ART) was enhanced by 14, 46 and 79% in the transgenic plants NAC1-30, NAC1-35 and NAC1-37, respectively (Fig.5A). At the same time, the content of dihydroartemisinic acid (DHAA) was enhanced by 30, 59 and 150% in the transgenic plants NAC1-30, NAC1-35 and NAC1-37, respectively (Fig.5B).

AaNAC1 activates the transcription of *ADS in vivo*

Since overexpression of *AaNAC1* significantly increased the expression level of *ADS*, the dual-LUC method was used to detect whether AaNAC1 protein activates the transcription of *ADS in vivo* or not. The vector *ADSpro:LUC* was used as the reporter and 35s:*AaNAC1* was used as effector. The effector and reporter constructs were introduced into *A. tumefaciens* strain EHA105 and coinfiltrated into tobacco leaves for dual-LUC assays. As expected, *AaNAC1* improved the transcription of *ADS* 2.3-fold (Fig.6). However, AaNAC1 protein activated the transcription of *ADS* in tobacco while binding of

AaNAC1 directly to the *ADS* promoter was not found by yeast one-hybrid (Y1H)(data not shown). Consequently, AaNAC1 may indirectly activate the transcription of *ADS*.

Overexpression of *AaNAC1* improves drought tolerance in *A. annua* and Arabidopsis

The expression patterns character of *AaNAC1* is mainly expressed in roots and mature leaves (Fig.S5A). Since *AaNAC1* can be induced by dehydration (Fig.1C), we investigate whether *AaNAC1* is able to improve drought tolerance in *A. annua* under drought conditions. Figure 7 shows that the transgenic *A. annua* appear to be more tolerant to drought than the wild type (Fig.7). Most of the leaves of the wild type were withered while the transgenic plants were normal.

To characterize the function of *AaNAC1* in Arabidopsis, T3 generations of three homozygous transgenic lines were tested under drought stress. The transcription level of *AaNAC1* was detected in Arabidopsis by qRT-PCR (Fig.S5B). After 14 days of water deficit treatment, the wild type Colombia Arabidopsis (Col) plants turned purple for 2 days and then began to wither, whereas all the seedlings of the *AaNAC1* transgenic lines remained green. On the 17th day, most of the Col plants were dead and the transgenic lines displayed a purple colour (Fig.8A). Re-watering for 3 days, the survival rate of Col plants was less than 10 % but for the transgenic lines it was nearly 90 % (Fig.8B). These results suggest that overexpression of *AaNAC1* can significantly improve drought tolerance in Arabidopsis. Since roots have a close relationship with drought tolerance, the root length of *AaNAC1* was measured. Compared to control plants, the 35S::*AaNAC1* overexpressing lines exhibited increased root lengths (Figs. 8C, 8D). The water loss rate was also determined. All the transgenic Arabidopsis plants showed lower water loss rates than the controls at each time point (Fig. S6). In addition, the expression levels of stress-related genes were increased in transgenic Arabidopsis plants (Fig. S7).

AaNAC1*-overexpression in *A. annua* and Arabidopsis results in the increase of disease resistance to *B. cinerea

Many studies indicate clearly that many transcription factors can act as a positive regulator of *B. cinerea* resistance, such as *AaORA,* which positively regulates the resistance to *B. cinerea* in *A. annua* (Lu et al. 2013b). *PLANT DEFENSIN1.2 (PDF1.2)* is the marker gene for several fungal infections in Arabidopsis, including *B. cinerea*. The expression level of *PDF1.2* was increased in transgenic Arabidopsis plants overexpressing AaNAC1 (Fig.S7). Therefore, we assumed that *AaNAC1* may have some roles in pathogen resistance. To investigate whether overexpressed *AaNAC1* in *A. annua* affects the resistance to infection by *B. cinerea*, the transgenic plants were treated with *B. cinerea* for one week. In fact, the transgenic plants appeared to be more resistant to infection by *B. cinerea* (Fig.9). About 80% of the leaves of control plants showed infection symptoms, while only 10% of the leaves of the transgenic plants were infected by *B. cinerea*. When *AaNAC1* was overexpressed in Arabidopsis, the transgene played a significant role in resisting the infection by *B. cinerea* (Fig.10).

Discussion

AaNAC1 is one of the NAC (NAM, ATAF1, 2 and CUC2) proteins, which is a major transcription factor family renowned for its roles in response to biotic and abiotic stresses (Puranik et al. 2012; Zhong et al. 2012). In this study, we cloned a new *NAC* gene (*AaNAC1*) from *A. annua* and found that it was mainly expressed in roots and mature leaves (Fig.S5A). In addition, this gene was induced by SA and MJ, dehydration and cold, which indicate that it may play important roles in abiotic or biotic stresses

resistance. Therefore we investigated the function of *AaNAC1* by overexpressing it in *A. annua* and Arabidopsis. In addition, we studied the molecular mechanism of *AaNAC1* resulting in improved artemisinin content. Our results indicated that *AaNAC1* is an important positive regulator that can be used for engineering artemisinin biosynthesis in *A. annua.*

The NAC transcription factors are induced by many abiotic stresses (Chen et al. 2014; Zhu et al. 2014). *AaNAC1* was strongly induced by dehydration and the expression level was increased 9-fold (Fig.1C). Previous studies indicated that NAC transcription factor could play important roles in enhancing tolerance to drought and disease resistance (Du et al. 2014; Fang et al. 2015; Vlot et al. 2009). Hence, we hypothesized that *AaNAC1* may improve the tolerance to drought and disease resistance. Firstly, we checked whether overexpression of *AaNAC1* in Arabidopsis could increase the expression of *RAB18*, *RD29A*, and *COR47* genes. These genes encode low-molecular-weight hydrophilic proteins, which reduce the rates of water loss under stress conditions (Mao et al. 2012). The expressions of these genes were increased 9- to 14-fold in transgenic Arabidopsis. Secondly, root length is relevant to drought resistance (Ekanayake et al. 1985). Longer root system should facilitate water absorption from deeper soils. Thus, long root systems can strengthen drought tolerance and increase biomass under water-deficit conditions (Mao et al. 2012). The length of root is the marker of tolerance to drought. The root length of the transgenic plants was significantly longer than the mock plants and the survival rate was higher than wild type plants when exposed to the drought environment. Based on these data, the transgenic Arabidopsis seedlings were treated under drought and *B. cinerea,* respectively. The results indicated that the transgenic Arabidopsis seedlings showed enhanced tolerance to drought and disease resistance (Figs.8 and 10). Therefore, *AaNAC1* is an important gene which plays great roles in

tolerance of freezing, drought and pathogen resistance in Arabidopsis. At the same time, *AaNAC1* was induced by SA, MJ, NaC1, cold and dehydration in *A. annua*, it also suggest that *AaNAC1* plays important roles in response to various abiotic stresses in *A. annua*.

SA and MJ play important roles in regulating plant defenses that are triggered after pathogen or insect attack (Van der Does et al. 2013). MJ can increase the expression level of transcription factors *AaERF1/ AaERF2* and *AaORA* (Lu et al. 2013a; Lu et al. 2013b), and leads to increase the mRNA level of the defense marker genes *PDF1.2* and *BASIC CHITINASE (ChiB)* (Jensen et al. 2008). Therefore, MJ can increase the resistance to *B. cinerea* in plant. SA inhibits the expression level of *PDF1.2* and shows decreased resistance to *B. cinerea* (Ndamukong et al. 2007). Plant hormone SA induces *AaNAC1* about 30-fold, while MJ induces *AaNAC1* reach to 40-fold (Fig.1D). SA may synergize with MJ to increase the expression level of *AaNAC1.* To further investigate this phenomenon, SA+MJ used to treat *A. annua* at same time. Under the treatment of SA+MJ, the expression level of *AaNAC1* can reach to 60-fold (Fig.1D). Therefore, SA+MJ combination plays an important role in enhancing mRNA level of *AaNAC1.* Both the plant hormone MJ and SA can improve the artemisinin content, while SA has some roles in plant drought tolerance (Kang et al. 2012; Maes et al. 2011). Hence, we postulated that *AaNAC1* can increase the artemisinin content and plays some roles in regulating defenses. The fact that overexpression of *AaNAC1* improves the content of artemisinin (Fig.5) and increase the tolerance of drought (Figs.7, 8). Many studies identified an antagonistic interaction between the MJ and SA pathways, but few synergistic interactions had been reported as well (Mur et al. 2006). Since the synergistic role of MJ and SA, we conclude that *AaNAC1* is an important gene for improving artemisinin content and plant defense.

Then *AaNAC1* was overexpressed in *A. annua*. The transgenic plants were shown to exhibit significant

increase in tolerance to drought and disease resistance (Figs.7 and 9). However, only a few abiotic stress genes have been cloned from *A. annua*, such as *AaPYL9* and *AaORA* (Lu et al. 2013b; Zhang et al. 2013). The molecular mechanism of *AaNAC1* improving tolerance to drought and disease resistance is unknown. NAC-type transcription factors can bind the regulatory *cis*-element "TTGCGTG" in the promoter of the target genes (Franco-Zorrilla et al. 2014). In the *AaNAC1* transgenic *A. annua*, the expression level of *ADS* was increased about 5-fold. The expression levels of *ALDH1* and *DBR2* were increased only 2-3 folds. Hence, the transcription factor can mainly activate the expression level of *ADS* resulting in increased artemisinin content. Nevertheless, the protein AaNAC1 could not bind the promoter of *ADS* directly as shown by Y1H experiments (data not shown). Consequently, AaNAC1 may indirectly activate the promoter of *ADS*. On the other hand, the dual-LUC assays indicated that the transcript level of *ADS* was activated by AaNAC1 only 2-fold, the mRNA level of *ADS* may be activated by others transcription factor at the same time. Previous studies indicated that *AaWRKY*1, *AaERF*1 and *AaORA* can increase the expression of *ADS* (Franco-Zorrilla et al. 2014). Therefore *AaNAC1* may interact with these transcription factors and co-activate the promoter of *ADS*. In addition, the increased expression level of *ADS* is about 4-fold in the transgenic plant NAC1-30 (the expression level of *AaNAC1* is 4-fold contrasted with CK), while the expression level of *ADS* was only 2-fold in NAC1-35 (the expression level of *AaNAC1* is 7-fold contrasted with CK). For some reason, the observed overexpression effects were not always correlated with the differences in *AaNAC1* expression levels. Nevertheless, our results clearly show that *AaNAC1* overexpression leads to an increase in these outputs in several independent transgenic lines.

In conclusion, *AaNAC1* is a stress responsive NAC-type transcription factor in *A. annua*. Overexpression of it can up-regulate the expression levels of several artemisinin-related genes,

resulting in enhancement of artemisinin content in *A. annua*. *AaNAC1* can also play the potential roles in positively regulating the resistance to drought stress and *B. cinerea* in *A. annua*. These results demonstrate that *AaNAC1* can be used for engineering artemisinin biosynthesis in *A. annua*.

Materials and methods

Sequence and phylogenetic analysis of *AaNAC1* gene

The sequence of *AaNAC1* was obtained from RNA-Seq data in our lab. To analyze the relationship between *AaNAC1* and *NAC* genes from other plant species, the BLASTX algorithm with default parameters from http://blast.ncbi.nlm.nih.gov/ was used. The ORF of *AaNAC1* was identified by Vector NTI version 15 with the ORF analysis tools.

The AaNAC1 protein was deduced from the sequence of *AaNAC1* by primer premier5 and the isoelectric point was calculated by expasy (http://cn.expasy.org/tools/pi_tool.html). Scansite program (http://scansite.mit.edu) was used to predict several conserved sites. For homology analysis of protein sequences the Vector NTI version 15 with the Protein Align was used. MEGA5 was performed to generate Neighbor-Joining tree.

Plant material

Seeds of *Arabidopsis thaliana Col-0* were surface sterilized with 10% sodium hypochlorite solution and 0.01% Triton X-100 for 5 mins and then washed three times with sterilized water . Sterilized seeds were planted on MS solid culture medium containing 20% sucrose (w/v), 6% agar (w/v) and 0.043% MS (w/v) basal salts at pH 5.8. The seeds on the MS solid culture medium were cultured for 3 days at 4°C in the

refrigerator, and then transferred to the greenhouse with 16/8-h light/dark photoperiod at 24°C for 5-7 day. The seedlings were grown in soil mixture (vermiculite: perlite: peat moss =7:0.5:2).

Seeds of *N. benthamiana* were planted on the same soil mixture as Arabidopsis. Five weeks later, the tobacco plants could be used for infiltration experiments.

The seeds of *A. annua* were obtained from the School of Life Sciences, Southwest University, Chongqing, P. R. China, and grown in the greenhouse with 16/8-h light/dark photoperiod at 26°C.

Hormonal and stress treatments

For stress treatments, 4-week-old plants of *A. annua* were sprayed with 1 mM SA, 100 μM MJ, 1 mM SA+100 μM MJ and 150 mM NaCl solution. Water was used as mock. Samples were analyzed 0, 0.5, 1, 3, 6, 12 and 24 h after treatment. For cold treatments, seedlings were chilled at 4°C for 0, 1, 3, 6, 12, and 24h. Dehydration was induced by putting plants on dry filter papers in air for 0, 0.5, 1, 1.5, 2, 2.5, 3, and 3.5 h. After treatments, the leaves of *A. annua* were collected for RNA extraction.

Water-deficit stress

Transgenic Arabidopsis and Col-0 seedlings were planted in pots filled with soil mixture and well-watered in the greenhouse with 16/8-h light/dark photoperiod at 24°C for 9 days. For a drought treatment, water was withheld for a period of 14 days. After 14 days of drought treatment, pots were rewatered for 3 days to supply plants with normal culture conditions. The number of surviving transgenic and Col-0 plants was determined.

Two-month old transgenic *A. annua* seedlings were used for drought treatment in green house. The water-deficit stress treatment of *A. annua* was the same as for Arabidopsis.

Root length assays

To measure root length of wild type and transgenic seedlings, seeds of Arabidopsis were germinated on MS plates for 7 days then transplanted to the new MS plates and vertical culture under the normal conditions. Root length of Arabidopsis was measured after 7 days culture on the new MS plate.

Water loss rate determination

Transgenic and wild type Arabidopsis seedlings were planted in pots filled with soil mixture and then well watered for 3 weeks. Then the above-ground parts of all the seedlings were immediately weighed (fresh weight, FW) and placed on the filter papers with a relative air humidity of 45–55%, at 25°C. At time points of 0.5, 1.5, 2.5, 3.5 and 5 days the seedlings were weighed (WW). Water loss rate (WLR) can be calculated as follows: WLR (%) =FW- [(FW–WW) / FW]×100.

Overexpression of *AaNAC1* in *A. annua*

The *AaNAC1* was cloned into the PHB vector and driven by the double cauliflower mosaic virus 35S promoter (35S). The vector obtained was introduced into *A. annua* by *Agrobacterium*-mediated EHA105 transformation (Lv et al. 2016; Zhang et al. 2009). The primers used in this investigate are listed in Table S1.

RNA isolation and reverse transcription (RT)

Total RNA extraction was performed by using RNA prep pure Plant Kit (Tiangen Biotech., Beijing, China) according to the manufacturer's instructions. TaKaRa cDNA synthesis kit (TaKaRa, Dalian, China) was used to synthesize the first-strand of cDNA.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed with SYBR Green (Tiangen Biotech., Beijing, China) according to the manufacturer's instructions. Three replicate cDNA samples were tested for each experiment. The comparative cycle-threshold (CT) method was used to determine relative mRNA

levels of samples. The *β-ACTIN* gene (internal reference) of *A. annua* and the *UBIQUITIN* gene (internal reference) of Arabidopsis were used to quantify relative transcript levels. The $2^{-\Delta\Delta CT}$ method was used to detect the relative express level of *AaNAC1* (Mao et al. 2012).

Subcellular localization of AaNAC1

The full-length ORF of *AaNAC1* without a terminator codon (TAA) was fused upstream of the *YFP* gene and put under the control of the constitutive cauliflower mosaic virus 35S promoter in the PHB-YFP expression vector to construct a AaNAC1-YFP fusion protein. After sequencing, the positive plasmids were transformed into *Agrobacterium tumefaciens* strain EHA105. *Agrobacterium* cells were harvested by centrifugation in late exponential phase and resuspended in MS medium (containing 10 mM MES and 150 μM acetosyringone) to an OD600 nm of 0.6. The mixture was incubated for 3 hours at room temperature. The bacteria were injected into young leaves of 5 weeks old *N. benthamiana* plants. Leaves of *N. benthamiana* were checked by Confocal Laser Scanning Microscope after 2 days.

Dual-LUC assay

For dual-LUC assay, the promoter of *ADS* and the vector pGreenII 0800-LUC were digested with restriction enzymes *Kpn*I and *Bam*HI at the same time and then fused with T4 DNA ligase to generate a new transformation vector *ADSpro-LUC*. The sequenced plasmid was introduced into *Agrobacterium tumefaciens* strain EHA105 by a conventional freezing and thawing method (Zhang et al. 2009). Incubated *Agrobacterium* cells were harvested by centrifugation and resuspended in MS medium (containing 10 mM MES and 150 μM acetosyringone) to an OD600 nm of 0.6. After 3 hours, the mixted bacteria (reporter strain *ADSpro::LUC* mixed with effector strain *35Spro::AaNAC1-PHB* = 1:1) were injected into young leaves of 5 weeks old *N. benthamiana* plants (the effector strain *35Spro*:PHB as

control). Two days later, the injected area of *N. benthamiana* leaves were collected and used for dual-LUC detection by dual-LUC reaction reagents (Promega, Durham, NC, USA).

Pathogen infections

B. cinerea was used to assay the pathogen resistance of transgenic plants. *B. cinerea* was grown on potato dextrose agar medium for 2-week and washed by sterile water. The pathogenic microbes were resuspended in sterile water at a concentration of 2×10^5 spores mL^{-1} and sprayed on the transgenic plants (Lu et al. 2013a). After 7 days control and transgenic plants were assayed for resistance to *B. cinerea*.

Quantification of artemisinin using HPLC-ELSD (HPLC-evaporative light scattering detection)

The leaves of upper region of 6 month-old *A. annua* (Nair et al. 2013) were collected and dried at 50°C for 24 h, then ground into powder. The powder (0.1 g) of each sample was suspended in ethanol (1 ml) for ultrasonication 30 min. After ultrasonication, the samples were centrifuged 12000 rpm for 10 min. After 10 min, the supernatant was filtered through a 0.25 mm pore size filter. A Waters Alliance 2695 HPLC system was used to analyze the samples (Zhang et al. 2009).

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Disclosures

The authors have no conflicts of interest to declare.

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Figure legends

Fig.1. Regulation of the expression of *AaNAC1* by NaCl (A), cold (B), dehydration (C) and SA, MJ and SA+MJ (D). Four-week-old plants of *A. annua* were sprayed with 1 mM SA, 100 μM MJ, 1 mM SA+100 μM MJ and 150 mM NaCl respectively, and then harvested after 0, 0.5, 1, 3, 6, 12 and 24 h. For cold treatment, seedlings were chilled in 4°C for 0, 1, 3, 6, 12 and 24 h. For dehydration treatment, seedlings were placed on a dry filter paper in air for 0, 0.5, 1, 1.5, 2, 2.5, 3 and 3.5 h. Expression of *AaNAC1* was detected by quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Error bars are SE (n= 3).

Fig.2. Subcellular localization assay of AaNAC1. Subcellular localization of AaNAC1 in tobacco epidermal cells. A constructed vector *35S::AaNAC1-YFP* was induced into *Agrobacterium* and introduced into tobacco *(Nicotiana benthamiana)* epidermal cells by infiltration. The subcellular localization of the AaNAC1-YFP fusion protein is mainly nuclear. The YFP protein is observed in cytosol and nucleus.

Fig.3. The expression level of *AaNAC1* in the transgenic plants was determined by qRT-PCR analysis. Error bars are SE (n= 3).

Fig.4. The expression levels of *ADS, CYP71AV1, DBR2* and *ALDH1* in the transgenic plants were determined by qRT-PCR analysis. Error bars are SE (n= 3).

Fig.5. The contents of artemisinin (ART) and dihydroartemisinic acid (DHAA) were determined in transgenic plants. Three different upper parts of each *A. annua* plants were collected. Each sample was measured once by HPLC for the ART and DHAA. Data represent means ± SE from three replicates. Statistical significance is determined by Student's t-test (**, $P < 0.01$; *, $P < 0.05$). Asterisks indicate the difference between CK and transgenic *A. annua* plants.

Fig.6. Transient dual-LUC was determined in tobacco leaves. (A) Vectors used in the transient dual-LUC assays. The vector of effector contains the sequence of *AaNAC1* and is driven by the CaMV35S promoter. *ADSpro::LUC* is used as reporter constructs. The empty vector does not carry the *AaNAC1* as negative control. (B) *AaNAC1* can activate the promoter of *ADS* by transient dual-LUC analysis in *N. benthamiana* leaves. The values of LUC/REN (the ratio of LUC activities to REN activities) represents the level of *AaNAC1* activation of the ADS promoter. Error bars represent SE (n= 3).

Fig.7. Drought tolerance of transgenic *A. annua*. (A) The phenotypes of seedlings before water deficit treatment. (B) The phenotypes of seedlings after water deficit treatment. Most of the transgenic plants are still alive while the control plants withered after 14 days of water deficit treatment. The picture in the white box is the magnificent picture of B.

Fig.8. Drought tolerance and root length of transgenic Arabidopsis. (A) The phenotype of drought treatment between Col and transgenic plants. Three transgenic typical lines are picked out and used in the drought tolerance assays. Most of the transgenic plants are still alive while the Col 0 plants are dead after 14 days of water deficit treatment. The upper 4 pot plants are grown in the normal conditions as control and lower parts plants are grown in the drought conditions for drought tolerance assays.

(B) The survival rate of transgenic plants is significantly higher than control plants under the treatment of drought (**, $P < 0.01$; *, $P < 0.05$). Three lines of transgenic plants NAC1-1, NAC1-2 and NAC1-4 were chose and used for drought treatment respectively. Sixteen plants of each line were used. (C) The transgenic plants have longer roots when compared the col. (D) The root lengths of the transgenic lines are significantly longer than col (**, $P < 0.01$; *, $P < 0.05$). The root lengths of 30 plants of NAC1-1, NAC1-2 and NAC1-4 were measured.

Fig.9. Resistance of transgenic *A. annua* against *B. cinerea*. (A) The phenotypes of control and three independent transgenic *A. annua* lines overexpressing *AaNAC1*, without inoculation with *B. cinerea*. (B) The phenotypes of control and three independent transgenic *A. annua* lines overexpressing *AaNAC1*, seven days after inoculation with *B. cinerea*. Most of the leaves of the control are infected by *B. cinerea* while most of the transgenic plants exhibit resistance to *B. cinerea.*

Fig.10. Resistance of transgenic Arabidopsis against *B. cinerea*. The phenotypes of control and three independent transgenic Arabidopsis lines overexpressing *AaNAC1*, four days after inoculation with *B. cinerea*. Most of the leaves of the control are infected by *B. cinerea while* most of the transgenic plants exhibit resistance to *B. cinerea.*

Figure 1

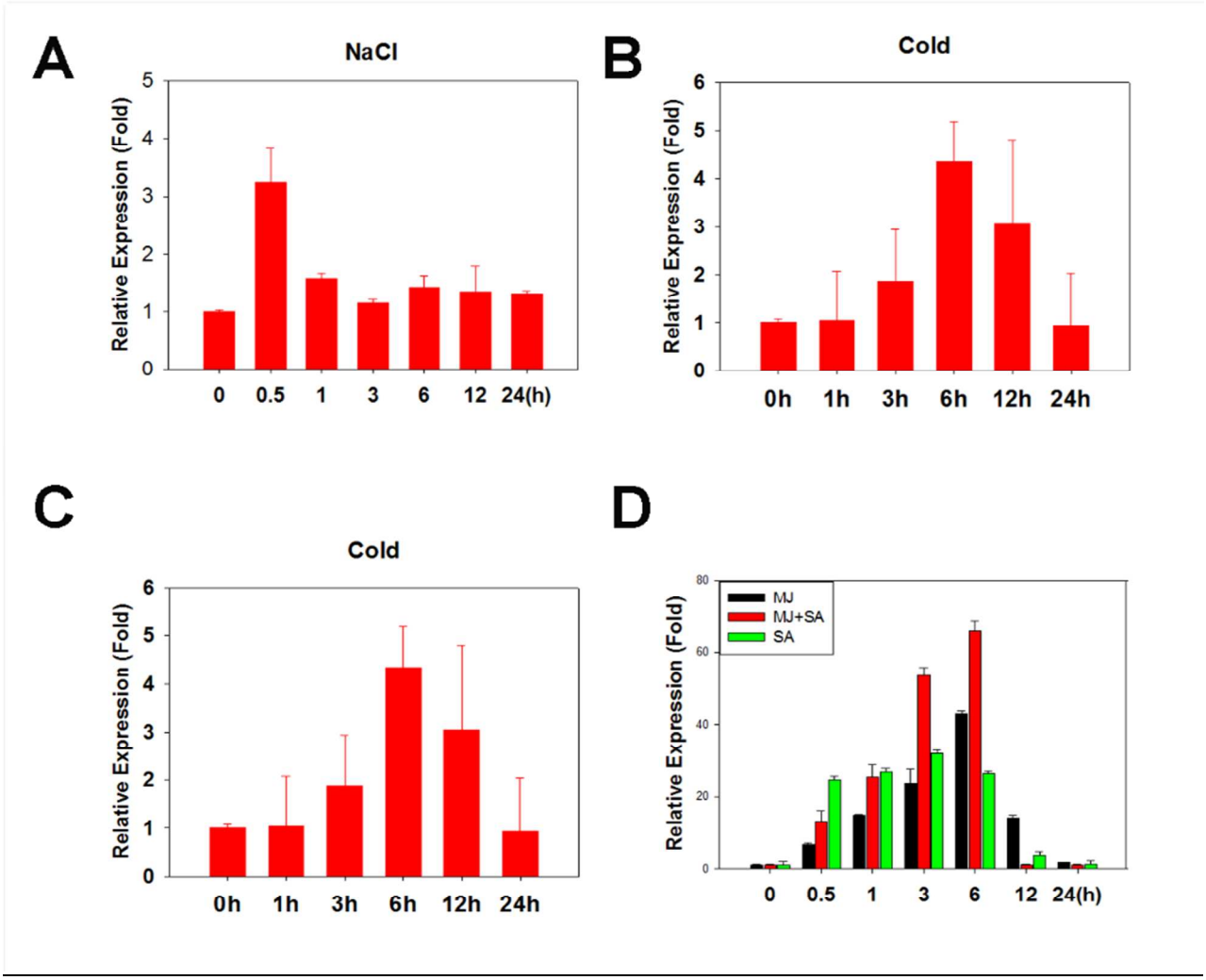

Figure 2

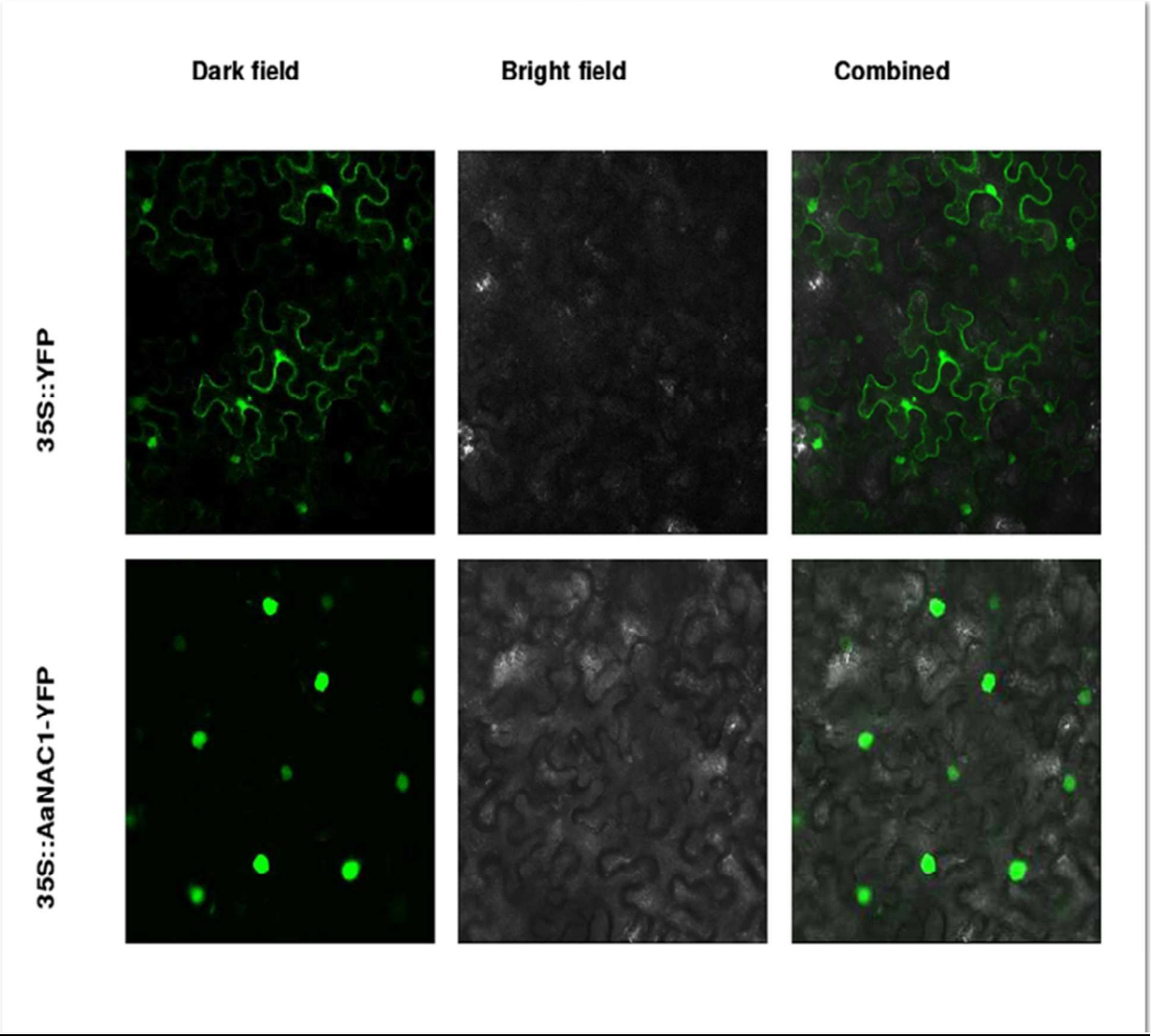

Figure 3

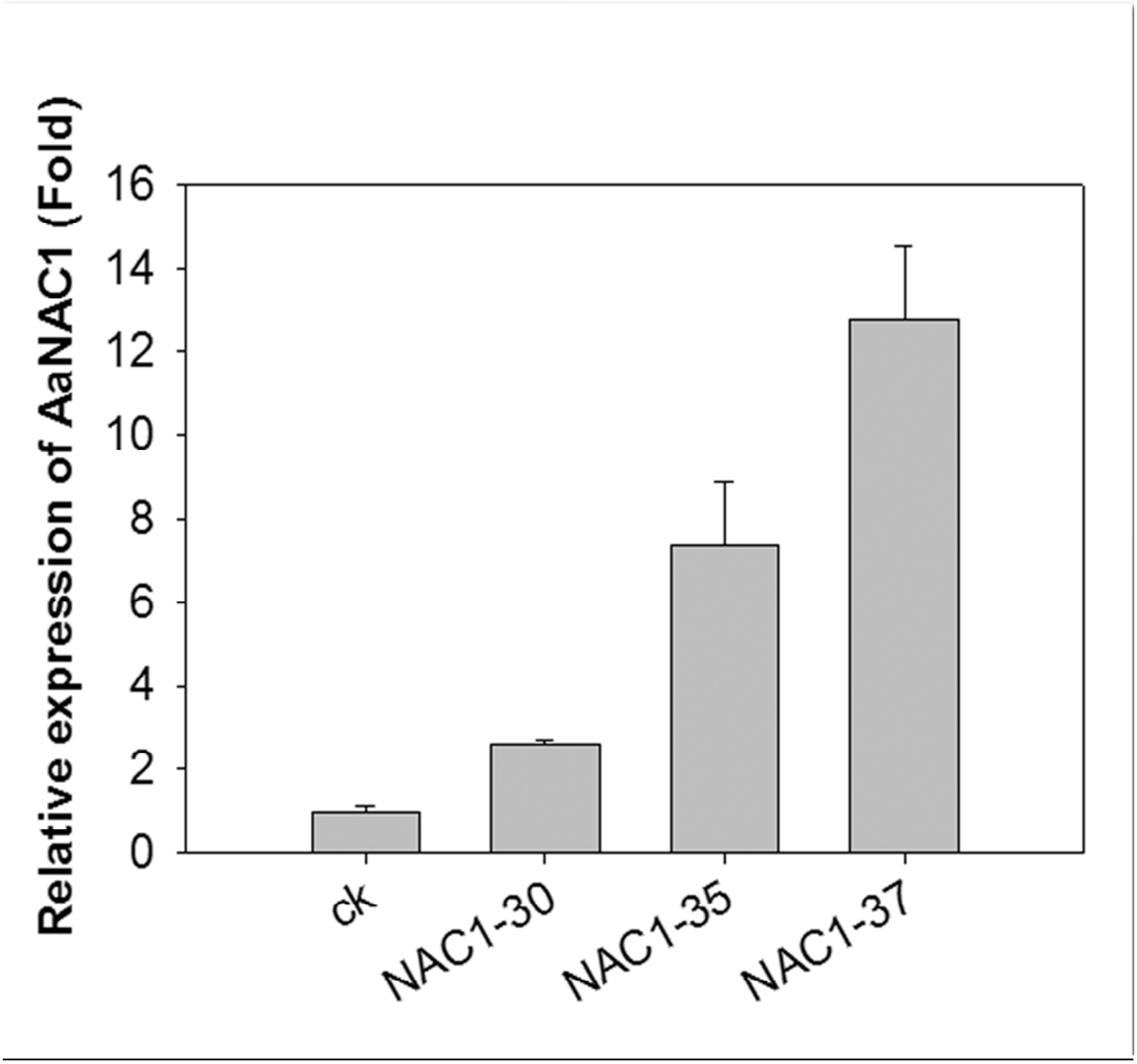

Figure 4

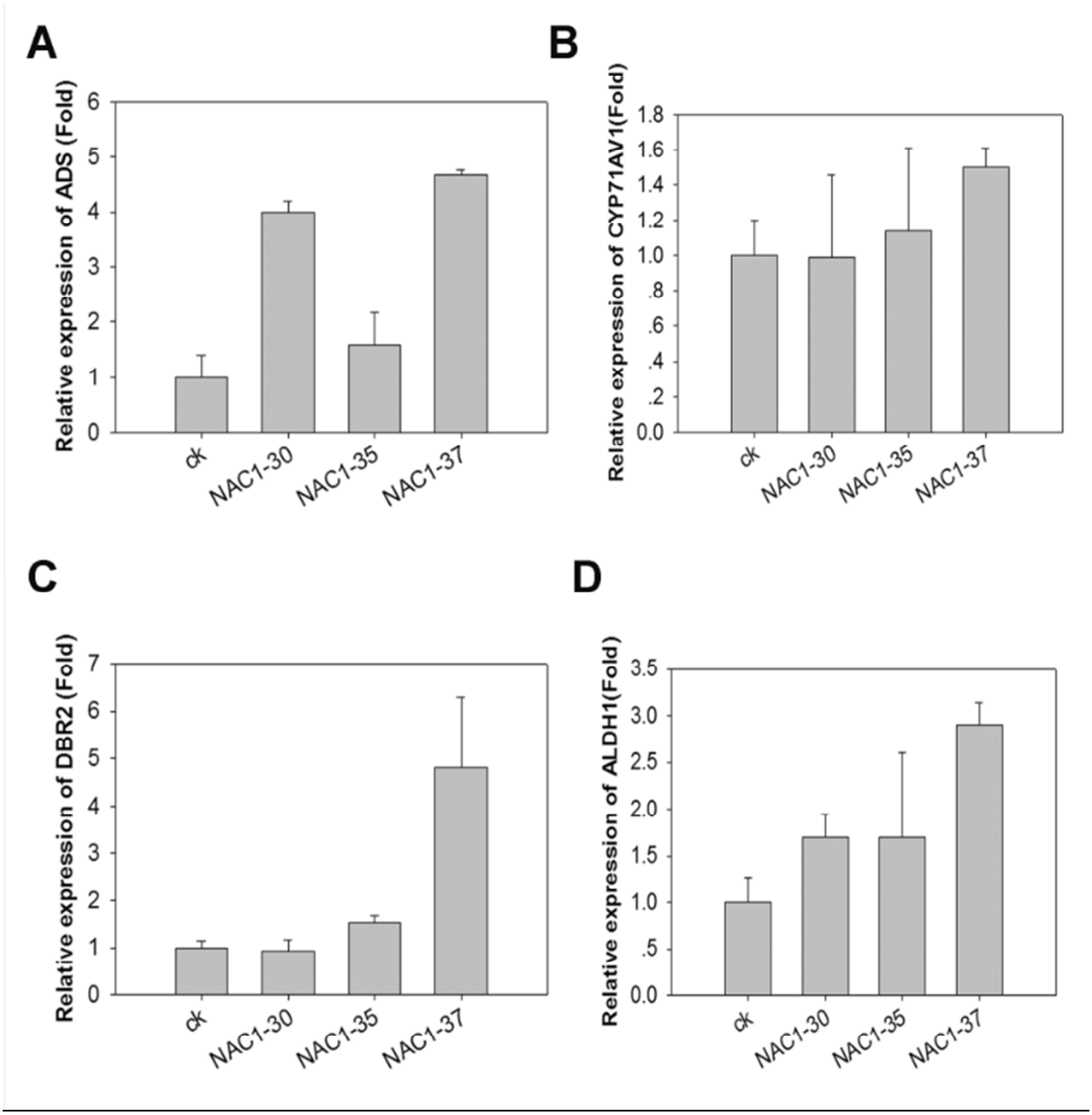

Figure 5

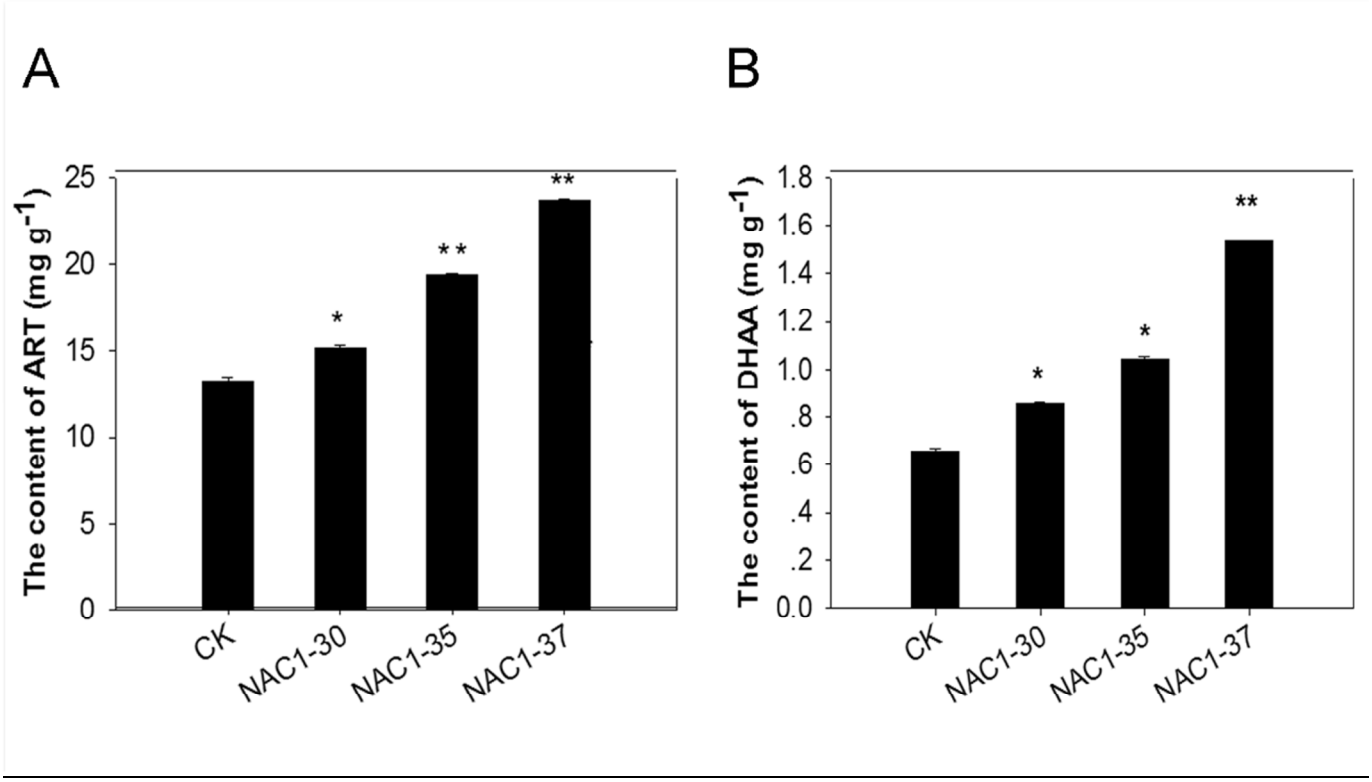

Figure 6

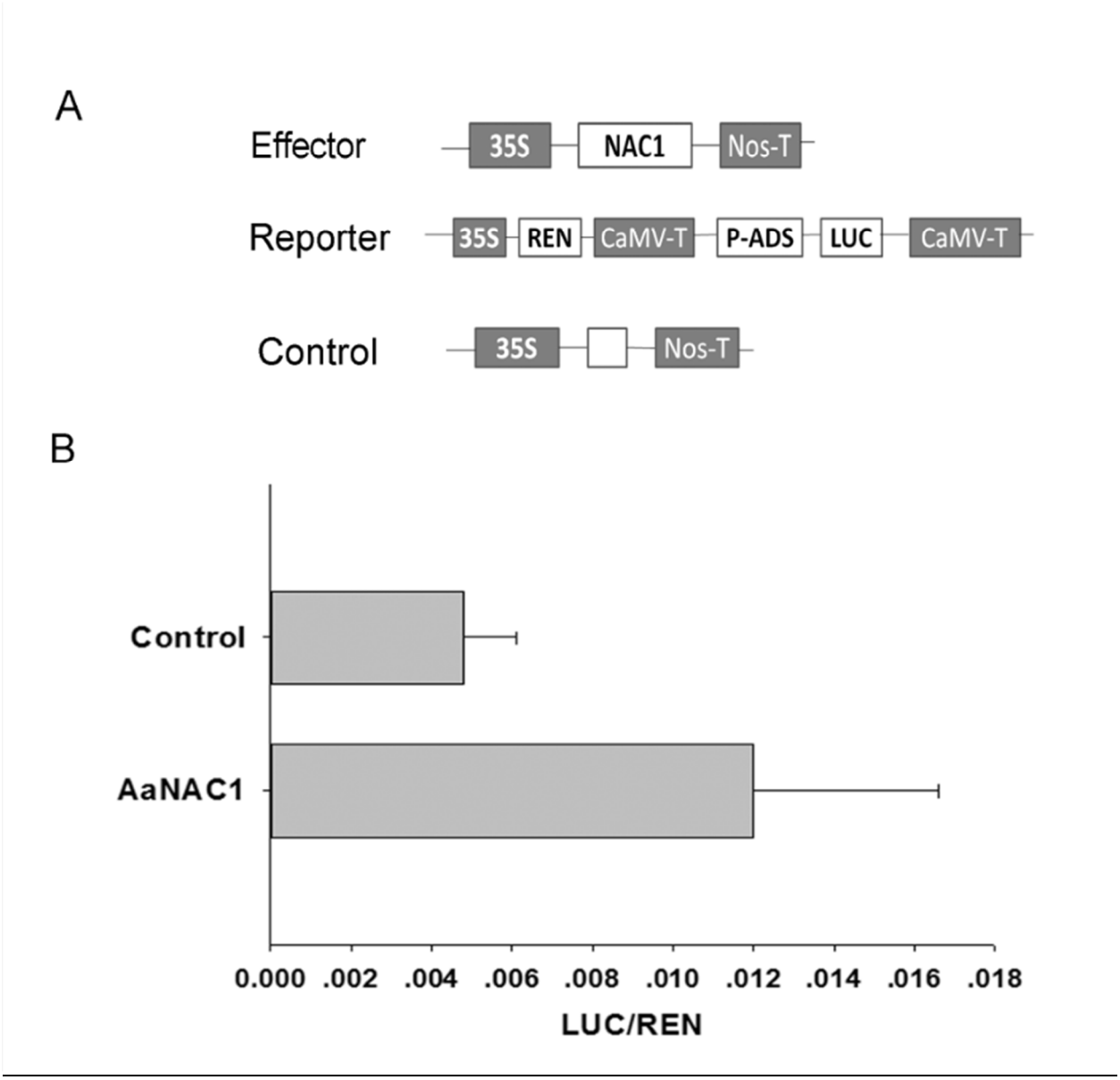

Figure 7

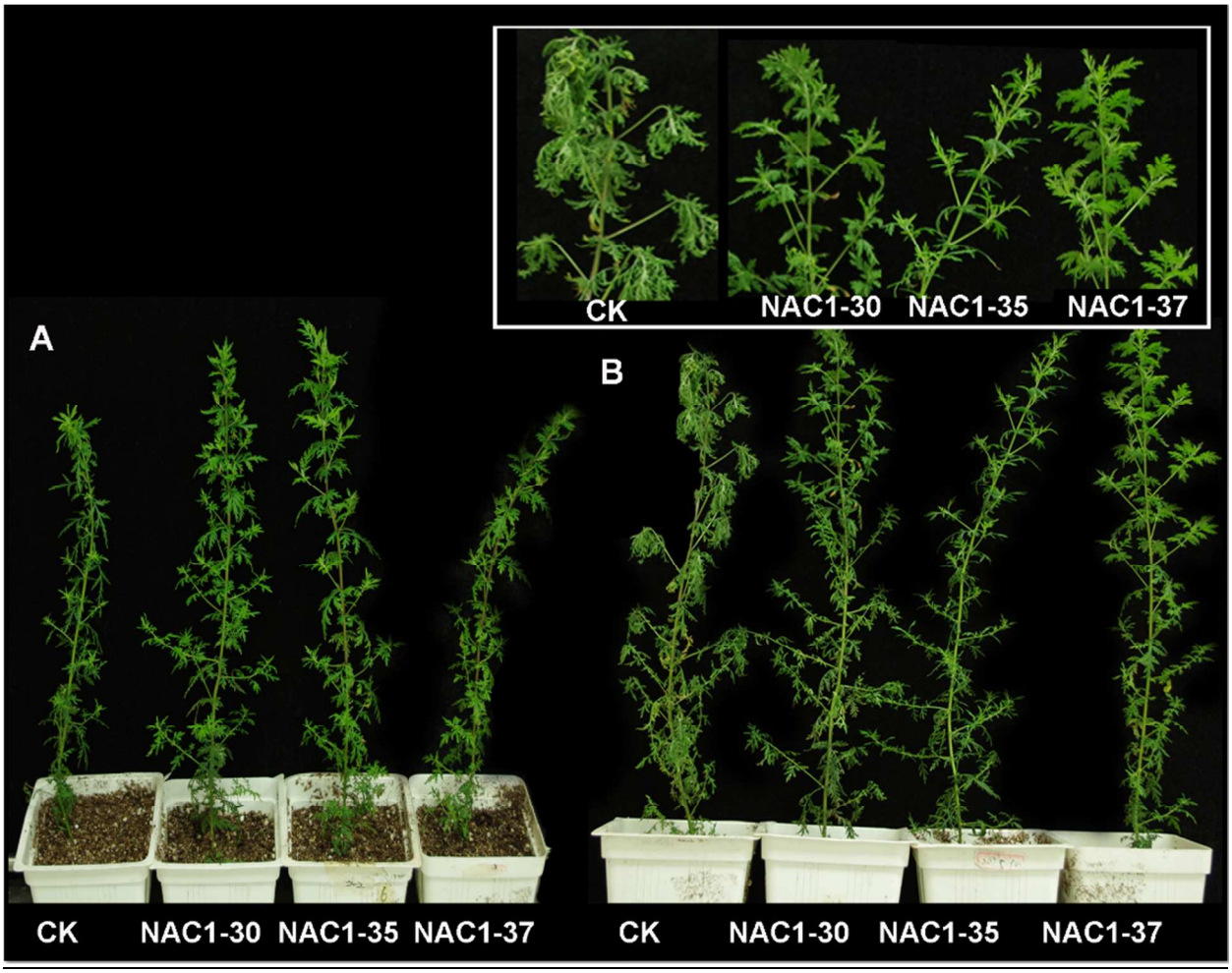

Figure 8

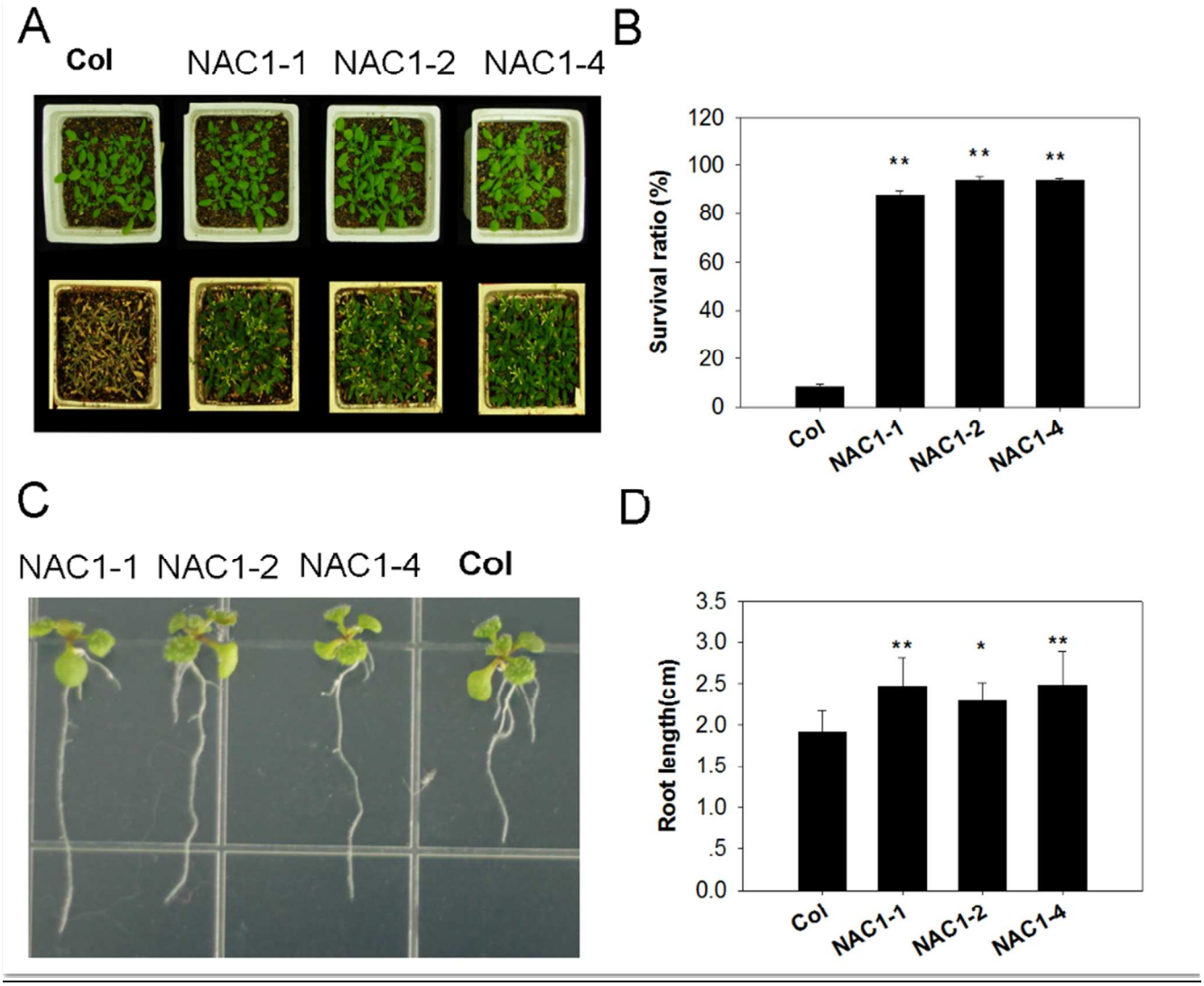

Figure 9

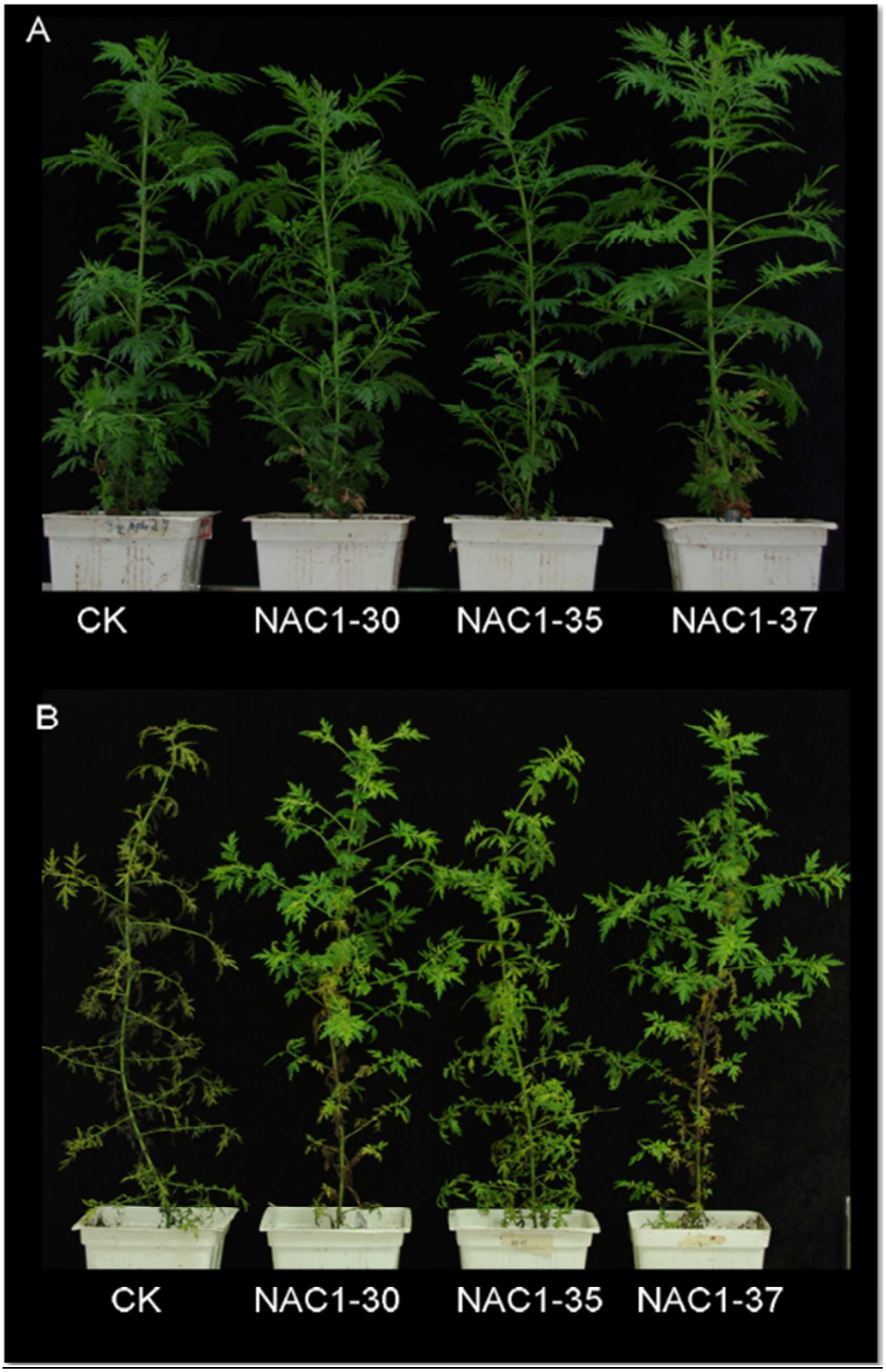

Figure 10