Stochasticity in transcriptional expression of a negative regulator of Arabidopsis ABA network

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Abstract

Stably heritable spatiotemporal co/over-expression of distinct transcriptional regulators across generations is a desired target as they signal traffic in the cell. Here, the stability and expression pattern of *AtHB7* (*Arabidopsis* homeodomain-leucine zipper class I) cDNA was characterized in 220 random population of transformed tomato clones where no *AtHB7* orthologous has been identified in to date. Integration of p35S::*AtHB7* cassette was tested by the amplification of the stretches (700/425 bp) in the target by *NPT III/AtHB7* oligos. Transcriptional expression pattern for the amplicons of the specific transcripts in the leaf tissues of transformants were determined by qRT-PCR. Transgene copy number was negatively correlated with transgene expression level, yet a majority of transformants (78%) carried single-copy of transgene. About 1:3 of the lines containing two-copy inserts showed less transcript expression. Heterologous CaMV 35S promoter drove *AtHB7*, illuminated no penalty on transgene expression levels, stability or plant phenotype under drought stress. Integration and expression analysis of transcription factors is of great significance for reliable prediction of gene dosing/functions in plant genomes so as to sustain breeding under abiotic stress to guarantee food security.

Keywords HDZip · *AtHB7* · Tomato · 35S · Trans-gene expression stability · Drought

Introduction

To live, cells must be able to cope with the changes in their environment as well as their developmental flow (Hoffmann and Hercus 2000). Transcription factors (Tfs) are critical against this evolutionary force and are key regulators in how cells adapt to environmental cues along with fluctuating ambience in nature (Darnell et al. 2017). They bind to regulatory sites on DNA nearby to promoters and act as a clutch to provide shifts between active and inactive status of the gene expression in a cell (Takahashi and Nakagawa 2017).

Over the past 2 decades, hundreds of Tfs driving dynamic biological processes have been identified in eukaryotes (Kuang et al. 2018). A knowledge-based public web resource states that *Arabidopsis*, maize and sorghum nuclear genomes encode minimum 1510–1581, 3470 and 2137 Tfs, respectively (Yilmaz et al. 2009) which are classified under 40–60 families based on their similarity in their primary and/or

3-dimensional structures in the DNA-binding domains (Warren 2002).

Homeobox Tfs are found in all eukaryotic organisms and they have a wide phylogenetic distribution (Takahashi and Nakagawa 2017). Of the 89 Homeobox Tfs in *Arabidopsis* (whose functions still have not fully been able to be detected), 47 are characterized as regulating the protein-dimer configuration at the C-terminal end and a leucine zipper motif adjacent to this region (HD-Zip) (Schrack et al. 2004). HD-Zip genes exist by duplication and highly conserved during evolution. These genes were clustered under four classes (I–IV) by sequence similarity and common exon–intron patterns (Hanson et al. 2002). This domain found in animals has roles in determining the cell shape, cell size and surface properties (cohesiveness) and the region in which the cell is to be settle down after cell division (Hjellstrom et al. 2003). However, in plants HD-ZIP family is not responsible for these type of roles but for adaptation to environmental stresses (Ding et al. 2017). Among this family, *AtHB7* binds to the conserved CAT(N)ATTG region in the plants under water deficit and is an on/off switch for numerous downstream genes which are ABA dependent in plant growth. It negatively regulates plant development by

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repressing the transcription of ABA receptors in plants (Ré et al. 2014).

The rapid decrease of fresh water stocks and the loss of crops make the negative effects of drought stress on the plants very challenging (Mittler and Blumwald 2010). Genetic engineering aimed at cultivating drought-tolerant plants are the most effective and economical approach for resistance against fresh water scarcity problems (Ashraf 2010). Today, molecular technology allows the isolation of genes in stress signaling pathways, their transfer and their expression into the transgenic plants (Mittler and Blumwald 2010). For instance, breeding strategies for tomato based mainly on the genetic engineering techniques (which allows firmness, fruit flavor, yield and/or ancillary characters, stress tolerance, adaptation increase) rather than classical methods such as pedigree or backcross (Causse et al. 2006). However, technical difficulties in achieving stable and heritable transgenic lines make ectopic gene expression interpretations hard for researchers, which leads to drawbacks in the causal illustrations of the mechanisms (Li et al. 2009). This is one of the main limits for achieving proper genetic modification and creating acclimation networks.

The transformation of Tfs induced by drought and determination of expression patterns with stable and heritable integration would enrich understanding of abiotic stress adaptation in plants (Li et al. 2009). It helps developing higher stress tolerance and constitution of the basis of effective engineering strategies.

In the light of the knowledge and an attempt to create drought-tolerant tomato plants in a heterologous system, the *AtHB7* (800 bp cDNA), transferred to tomato plants (*Solanum lycopersicum* cv. UC82 MA) using the *Agrobacterium*-mediated gene transfer under the control of the CaMV35S promoter was performed here. Trans-gene integration, organization and selection of progenies on the basis of expression levels and trans-gene copy numbers was implemented.

Materials and methods

Plant material, recombinant vectors and cotyledon transformation

Transgenic tomato events were produced by isogenic line UC82 MA in Metapontum AGROBIOS S.R.L. (Metaponto, MT, Italy). Transformation of UC82 MA cotyledons was performed by *Agrobacterium tumefaciens* carrying pRG2-35S::*AtHB7* plasmid (modified by pBI121) according to Iannacone et al. (2008) (Fig. 1). Generated transformants were self-propagated to ensure the homozygosity of the *AtHB7* gene in the tomato genome. Out of 20 distinct transformations, 4 events (1–3, 5–8, 7–6, and 20) were selected to test

Fig. 1 Schematic of T-DNA. pRG2 expression vector generated by modifying the plasmid pBI121 is derived from the Bin19 (Accession number AF485783). The T-DNA region contains β -glucuronidase (*GUS*) reporter gene, expression cassettes for a neomycin phosphotransferase II—*nos* (*NPTII*) selection marker (*kan R*), right and left borders

trans-gene integrity and expression. Copy number and presence of the transgene for each transformation event was verified by Southern blot (Iannacone et al. 2008).

Growth conditions

Isogenic control plants (UC82 MA) and putative transgenics incubated at 28 ± 2 °C with 52.5% average humidity and 400 W sodium lights (25×) with $190 \mu\text{mol m}^{-2} \text{s}^{-1}$ solar radiation for 3 weeks in small pots. After 3 weeks, plants were transferred to bigger pots supplemented with NPK Fertilizer 20.20.20 (Valagro, Italy) [5.5% nitric nitrogen, 3.9% ammoniacal nitrogen ($\text{NH}_3\text{-N}$), 10.6% urea nitrogen, potassium oxide (K_2O), phosphoric anhydride (P_2O_5), 0.01% boron (B), 0.05% copper (Cu), 0.01% zinc (Zn) and low chlorine (Cl)] for another 2 weeks. Either 3- or 5-week-old tomato transgenics in macro- and micro-elements added fresh soil were used for further gene integration and expression analyses.

Verification of the transformants regarding target gene

To verify the integration of the transgene, genomic DNAs from the young leaves (300 mg) of a total of 220 individual putative transformants after 3 weeks old growth period were isolated with the method of Peterson et al. (1997). The purity

and concentrations were determined by measuring the optical density of DNA samples with the NanoDrop ND1000 Spectrophotometer (Thermo Fisher). The PCR program were 95 °C for 5 min, followed by 35 cycles of: 95 °C for 30 s, 58 °C for 45 s and 72 °C for 60 s and 72 °C for 5 min (96 universal peQStar) for the amplification of the 425 bp and 700 bp portions of the *AtHB7* and *NPTII* genes. Reaction was set up to 50 µl with the following conditions: Taq DNA Polymerase (5 U) (Invitrogen), 20 µM oligonucleotides and 100 µg genomic template for each gene. Primer combinations are given in Table 1.

Comparative real-time PCR for *AtHB7* trans-gene expression

To estimate the expression level of the transgene in four different transformation events after at most six generations of sexual propagation quantitative real-time polymerase chain reaction (qPCR) analysis was performed on different tomato progenies that were found the transgene is correctly integrated in. The mRNAs of total 48 transgenic tomato plants were isolated using the fresh leaves (100 mg) according to the method of Prescott and Martin (1987). The presence and quality of RNA molecules (1:10 dilution) was detected with the E-Gel 96 Mother Base™ instrument (75 V), with E-Gel® 48 ready gel (Invitrogen) and a Low Range Quantitative DNA Marker (E-Gel®, Invitrogen). RNA samples were screened for genomic contamination by 18S rRNA gene-specific primers (Table 1) after DNase (TurBO DNA-free™, AMBION, USA) treatment and stored in CELLSTAR® 96-well plates (Greiner, Germany). iScript™ cDNA Synthesis Kit (BIORAD, USA) was used to convert RNA samples to cDNA templates (> 1 µg) with random hexamers. Real-time PCR performed in CFX96™ Real Time System C1000 (BIORAD, USA) using dual-labeled TaqMan probes (Biosearch Technologies, Novato, CA) designed by Beacon

2.0 software (BIORAD, USA) (Table 1). *AtHB7* (Accession number X67032) and 18S housekeeping gene (internal control) specific oligonucleotide sequences (10 µM each) were used in the reaction. PCR program for real-time reaction was 50 °C for 2 min, and 95 °C for 2 min, followed by 45 cycles of: 95 °C for 15 s, 60 °C for 30 s. The specificity of the reaction was assessed by slope analysis of the amplification products for the relative amount of *AtHB7* transcript by the standard curve method. Sample no 153 was selected based on the pilot melt curve data and diluted [ST 1 (1:0)–ST 0.1 (1:10)–ST 0.01 (1:100) and ST 0.001 (1:1000)] as a standard. *AtHB7*/18S ratio was calculated for each sample by the $2^{-\Delta\Delta C_T}$ method via C_T values (Livak and Schmittgen 2001).

Stress tolerance tests

Seeds of the selected transgenic line besides with control UC82 MA were grown 4 weeks after germination in a controlled growth chamber at 28 ± 3 °C under above-mentioned growth conditions. Drought stress was applied by water withholding for 8 days at the whole plant level. Control plants were well watered every 2 days with tap water during experiments. For regeneration, plants were re-hydrated for 6 h. Pictures were captured right after every experimental condition.

Results

Through *Agrobacterium*-mediated cotyledon transformation in tomato, four independent T0 transgenic events [(1–3), (5–8), (7–6) and 20] were obtained carrying constructs targeting *AtHB7*. Out of four events, T1 generation of seeds for three events [(1–3), (5–8), (7–6)] was used in the study whereas seeds were obtained for six consecutive generations (T6) for event 20. In total, 220 transformants belonged to

Table 1 Primer pairs used in PCR and real-time qPCR reactions

Gene	Primer	Purpose	Sequence (5'–3')	Amplicon size	T_m
<i>AtHB7</i>	Forward	PCR	GACCGGAGATGGAGATGAAA		60.4
<i>AtHB7</i>	Reverse	PCR	TCTCGCTGAGAAACGAGTCA	425	60.4
<i>NPT II</i>	Forward	PCR	ATGACTGGGCACAACAGACA		57.3
<i>NPT II</i>	Reverse	PCR	AATATCACGGGTAGCCAACG	795	57.3
<i>18S rRNA</i>	Forward	PCR	GCATTTGCCAAGGATGTTTT		62.4
<i>18S rRNA</i>	Reverse	PCR	TAGCAGGCTGAGGTCTCGTT	347	56.3
<i>AtHB7</i>	Forward	qPCR	AACTACGACAACCTGGCTTCTCA		58.9
<i>AtHB7</i>	Reverse	qPCR	TGATTCATGATGTGTGCTGCTTAG	171	59.3
<i>18S</i>	Forward	qPCR	GAAACGGCTACCACATCCAAG		65.4
<i>18S</i>	Reverse	qPCR	CCCCGTGTTAGGATTGGGT	112	65.7
<i>18S</i>	Probe	qPCR	Fam AAGGCAGCAGGCGCGCAAATam		
<i>AtHB7</i>	Probe	qPCR	FamACCGCTTGATCTCCACTACACTGCCTTTam

Fig. 2 Development of putative tomato transgenics under ambient conditions. Transformants used in nucleotides extraction (a). Transgenic plant carrying the target gene (b). Isogenic control UC82 (c)

4 different transformation groups and different generations were analyzed to demonstrate the gene integration and the differences in transcript levels. The growth of control plants (UC82 MA) and putative transgenic plants used in these analyses under greenhouse conditions is given in (Fig. 2).

Trans-gene integration analysis of putative tomato transgenics

Out of 220, 45 transformants that were found to be positive regarding *AtHB7* from the first tests were amplified a second time with gene-specific primers. Another 45 T6 progeny generated from transformation event 20, which had allelic stabilization that were captured in previous experiments and definitely expected to have resistance, were amplified for kan R cassette existence. A total of 43 and 19 fragments were obtained for *AtHB7* and *NPT II*, respectively. Interestingly however, only 4 samples of T6 generations were found to be positive for the target genes. *AtHB7* (425 bp) and *NPT II* (795 bp) amplified fragments observed are shown in Fig. 3.

Transcriptional expression analysis of the target transgene

Relative expression pattern for the amplicons of the specific transcripts in the leaf tissues of putative tomato transgenics were determined by qRT-PCR. The expression analysis of the gene of interest *AtHB7*, was verified in 39 plants of different generations (T0, T1, T2, T6) coming from 4 different transformation events. Analysis by qRT-PCR was performed using TaqMan technology to determine the transcript accumulation in the leaves of the tomato plants. The

Fig. 3 Verification of transgenic plants by target gene amplification. Columns A, B, C and D represent PCR-amplified fragments of *AtHB7* (425 bp). Columns E, F, G and H are a partial segment of *kan R* cassette (795 bp). B: blank, UC: negative control UC82 MA, +: plasmid in which the target gene was cloned (628), M: Low Range Quantitative DNA Marker (E-Gel® Invitrogen). The lines show no signal was categorized as non-transgenics

expression level was estimated by the ratio between *AtHB7* and 18S (*AtHB7*/18S rRNA). The standard curve was created based on the relative expression levels (initial quantity of *AtHB7* and 18S cDNAs by serial dilutions of sample no. 153), extracted threshold cycle (C_T) values and standard deviation for the target gene as well as 18S housekeeping gene. The amplification specificity and PCR activity values were determined as efficiency (E) = 100% for 18S internal control and E = 91% for target gene *AtHB7*. Specific transcripts of each different RNA sample, consisting of three biological and technical replicates, were confirmed in the graph with values expressed by a separate peak. In 39 transgenic plants the ratio of *AtHB7*/18S rRNA expression was close to 3 and was highly heterogeneous among samples. Ten transgenic lines showed *AtHB7* expression at high levels: lines 76, 59, 19, 204, 167, 164, 165A, 149, 145B and 120. Average level of expression was observed in lines 14, 12, 6, 185, 184, 170, 163A, 163B, 165B, 154A, 154B and 122. Lines 130, 145A, 148, 153, 199, 11, 15A, 23, 29–34, 36A, 36B, 46, 53, 69, 72, 73A, 75, 115A, 115B, 116 and 117 developed low-level or undetectable expression of *AtHB7* (Fig. 4).

The effect of *AtHB7* transcript on drought stress tolerance

The effect of *AtHB7* trans-gene administration on tomato development and biomass under drought stress was observed

Fig. 4 Quantitative real-time PCR amplification profile for stable expression analyses of *AtHB7* transcription factor under the control of CaMV35S promoter in tomato plants. The fold change in the target gene was calculated by $2^{-\Delta\Delta C_T}$ method. Amplification efficiencies were calculated by a series of dilutions. UC82: isogenic control of tomato plant. 20., (1–3.), (5–8.), (7–6.): transformation event num-

bers. Events 1–3 and 20 carry a single copy of transgene, whereas (5–8) and (7–6) carry 2 copies of transgene. T₀, T₁, T₂, T₆ represent different generations of different transformation events. Error bars represent SE calculated from biological and technical triplicates. 18S transcript was used as reference gene

and documented during the experimental period. *AtHB7* overexpressing plants appeared to be much better developed than that of the control ones (Fig. 5). It was estimated

that *AtHB7* overexpressing plants have better regeneration capability under severe stress and produced up to 36% more biomass/regeneration capability than control plants.

Fig. 5 The association of *AtHB7* overexpression on tomato development and biomass under drought. After germination, the seedlings were planted in pots and grown with LemnaTec plant phenotyping systems for 4 weeks at 28 ± 3 °C. Drought was applied by water withholding for 8 days and plants were re-hydrated for 6 h. UC82, control;

AtHB7, transgenic tomato plants. *AtHB7* overexpressing plants appear to be much better developed than that of the controls. It was observed that *AtHB7* overexpressing plants have more regeneration capability than control plants

Discussion

Eukaryotic genes are regulated at both transcriptional and translational levels. Much of this control is carried out by Tfs at the transcriptional level in an inherently stochastic way for eukaryotic nuclear genes (Kærn et al. 2005). These Tfs have key roles to participate in activation of abiotic stress-induced genes and possibly initiate a set of subsequent adaptations in plants (Liu et al. 1998). *AtHB7* is a good example of this notion whose expression was detected in root tips, meristems and flowers of *Arabidopsis* and its upregulation is affected by drought (Olsson et al. 2004).

Creating the desired gene diversity in crop plants is the main goal of basic functional genomic research and molecular breeding (Xu et al. 2015). Moreover, identification and quantification of transcript accumulation are important tasks in agriculture for abiotic stress tolerance studies (Kosová et al. 2011). Even though gene transfer is an exceptional and trustworthy technique to select best lines under stress conditions by a vis-à-vis approach (Ashraf 2010), the stochasticity in trans-gene expression will be unavoidably introduced using this application after multiple generations (Kærn et al. 2005). Correspondingly, *AtHB7* here showed high instability across the samples belong to different transformation events. Six transgenic lines (149, 164, 165A, 167, 204 and 19) containing a single copy with a moderate-to-strong level of varied expression were identified. Even though efficiency of long-term gene expression is a basic determinant factor, transgenes tend to be susceptible to loss of expression as a result of gene loss or due to the constitutive promoters other than transcriptional/post-transcriptional gene silencing (Vaucheret and Fagard 2001; Li et al. 2009). A physiologically regulated promoter may preferentially be chosen here over a constitutive one in discarding these probabilities. However, *AtHB7* expression varied to a wide extent in different tomato lines under CaMV 35S promoter, and our data showed that the *AtHB7* transgene can be expressed stably through generations without any phenotypic penalty. Although constitutive promoters may have epigenetic gene silencing or unwanted downstream effect possibilities, there have been many works that used these promoters for such cases (Oh et al. 2009; Schmidt et al. 2013; Zale et al. 2016). Yet, inducible and/or tissue-specific promoters are in demand recently due to their advantageous features over constitutive ones (Jeong et al. 2013; Liu et al. 2017; Xu et al. 2018).

Although the rates and the causes of instability vary widely among species, environments and transformation systems, or the cell division itself (meiosis regulated gene silencing) allowing unstable transgenic lines (Li et al. 2009), the detection and removal of unstable transgenic events are the essential steps for commercial development in molecular

breeding (Li et al. 2009). Multiple trans-gene copy number, T-DNA structure and integration sites (heterochromatin formation), inverted repeats of the gene cassettes, DNA methylation or RNAi can also trigger silencing mechanisms (Leibbrandt and Snyman 2003; Yamasaki et al. 2008). The presence of multiple copies of transcripts that are coded by transgenes might cause homology-dependent transgene silencing due to transcriptional or post-transcriptional gene silencing. Studies on the correlations between transgene silencing and copy number have shown conflicting results; in most cases no correlation was observed (English and Jones 1998; Vain et al. 2002; Yamasaki et al. 2008; Khuong et al. 2013). However, in this work, number of inserted copies was determined by Southern blotting. Integration from one to two gene copies have been revealed for T-DNA used (data not shown). Events with a single copy number had substantially greater trans-gene expression level than events with double copy number. Expression stability decreased as generations increased in transgenic lines with single copy numbers (except for line 59) and belonged to two different events, which was expected. Two events (5–8) and (7–6), which have two copies of transgene, appeared to develop decreased gene expression. This means that multi-copy number did greatly affect trans-gene expression as expected. The best expression was determined in T2 and T6 generations of single-copy transgenics. Unstable expression of transgenes can be initially silenced or inactivated over time across generations and are of particular concern for commercial purposes (Matzke et al. 2000). High levels of stability may, therefore, be dictated by commercial needs (Li et al. 2009). Formation of transgene duplications and its association with the stability have been reported previously (Lechtenberg et al. 2003). Transgenes that are organized as inverted repeats can show low expression or complete silencing (Morino et al. 1999), yet this is not always the case (Meza et al. 2002; Lechtenberg et al. 2003). Therefore, using multiple trans-gene-carrying cassettes for genetic transformations is a more logical way against abiotic stresses whose response is mainly multifactorial and to get stable expression lines is never a trivial task (Halpin 2005).

The various stresses that occur during plant transformation and tissue culture are also known to cause genomic instability (reviewed in Phillips et al. 1994). High genomic instability of transgenics has been frequently observed in annual plants undergoing sexual propagation (Brunner et al. 2007). Tomato is one of these species since it is a perennial in its native habitat, but are grown mainly as annuals. Thanks to its high nutritional value (especially potassium, vitamins A and C) in the human diet (Causse et al. 2006), it is very popular vegetable crop worldwide. High consumption rates and accordingly its commercial value makes it crucial to reinforce sustainability of its agricultural practice by genetic modifications in current era full of pessimistic

scenarios on environmental problems (e.g., global warming, climate change and hunger predictions). It would therefore be important to transfer/assess abiotic stress resistance-linked genes in tomato cultivars.

Even though large-scale and long-term studies are difficult and expensive to conduct, examination of large number of transgenic events helps prevention of trans-gene instability. In addition, studies that evaluate stability after sexual propagation are needed, because instability might be reversed or amplified in association with meiosis (Li et al. 2009). Results here strongly suggest that instability of transgene expression, including in multi-copy transformants is low and, stable events can be readily identified.

In summation, stable transgenic lines need to be developed to effectively understand the underlying mechanisms to combat stress in plants. This work with its large-scale starting material (transformation events and transgenic line numbers) represented a number of stable *AtHB7* lines that are suitable to be used for further stress tolerance tests due to the role of the target gene in drought stress tolerance/ABA signaling.

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Compliance with ethical standards

Conflict of interest No conflict of interest does exist between third parties.

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