Asymmetric Redundancy of ZERZAUST and ZERZAUST HOMOLOG in Different Accessions of Arabidopsis thaliana

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ABSTRACT Divergence among duplicate genes is one of the important sources of evolutionary innovation. But, the contribution of duplicate divergence to variation in Arabidopsis accessions is sparsely known. Recently, we studied the role of a cell wall localized protein, ZERZAUST (ZET), in Landsberg erecta (Ler) accession, lack of which results in aberrant plant morphology. Here, we present the study of ZET in Columbia (Col) accession, which not only showed differential expression patterns in comparison to Ler, but also revealed its close homolog, ZERZAUST HOMOLOG (ZETH). Although, genetic analysis implied redundancy, expression analysis revealed divergence, with ZETH showing minimal expression in both Col and Ler. In addition, ZETH shows relatively higher expression levels in Col compared to Ler. Our data also reveal compensatory up-regulation of ZETH in Col, but not in Ler, implying it is perhaps dispensable in Ler. However, a novel CRISPR/Cas9-induced zeth allele confirmed that ZETH has residual activity in Ler. Finally, the synergistic interaction of the receptor-like kinase gene, ERECTA with ZET in ameliorating morphological defects suggests crucial role of modifiers on plant phenotype. The results provide genetic evidence for accession-specific differences in compensation mechanism and asymmetric gene contribution. Thus, our work reveals a novel example for how weakly expressed homologs contribute to diversity among accessions.

KEYWORDS Arabidopsis accessions genetic redundancy asymmetric gene expression ZERZAUST ZERZAUST HOMOLOG

How genetic variation translates into phenotypic variation is of immense scientific interest (Weigel 2012). Among others, gene duplication followed by functional divergence is an important source of evolutionary complexity and innovation in multicellular organisms (Ohno 1970; Lynch and Conery 2000; Lynch and Katju 2004). The Arabidopsis genome underwent several duplication events which resulted in large number of homologous genes and regions across the genome (Blanc and Wolfe 2004; Ambrosino et al. 2016; Panchy et al. 2016). The functional importance of homologs has been demonstrated in various aspects of plant signaling and metabolism (Briggs et al. 2006). But, whether and how the differentiation in duplicate gene expression contributes to accession variation in Arabidopsis is not known.

Studies have shown that divergence of many duplicate genes occurs by expression divergence among and within species (Gu et al. 2004; Li et al. 2005). This phenomenon expands gene regulatory networks and contributes to physiological and morphological diversity (Carroll 2000; Lynch and Conery 2000; Gu et al. 2004; Rensing 2014). In Arabidopsis, about two-thirds of duplicates were shown to exhibit expression divergence (Haberer et al. 2004). An evolutionary study on gene duplication revealed that duplicate genes show a high degree of variance in expression within species and suggested that this variation partly depends upon the biological function of the gene involved (Kliebenstein 2008). Another study also found high variance of duplicated gene expression between closely related A. thaliana and A. arenosa (Ha et al. 2009).
Functional redundancy among homologs is widespread in Arabidopsis, since several single loss-of-function mutants lack phenotype (Briggs et al. 2006). Homologous genes can be either fully or partially redundant. But, when two homologous genes show unequal genetic redundancy, a mutation in one of them causes a phenotype and the phenotype is enhanced when the other homolog is mutated as well. Interestingly, the defect in the other homolog doesn’t result in any phenotype on its own. For example, the receptor-like kinase gene BRASSINOSTEROID INSENSITIVE 1 (BRI1) is accompanied by its close homolog BRI1-LIKE1 (BRL1). Although brl1 lacks a mutant phenotype it enhances the severe dwarf phenotype of brl1 mutants (Caño-Delgado et al. 2004). This kind of unequal functional redundancy can be explained by divergence in duplicate expression, however, their perseverance in plant genome is under debate given the dispensable nature of the duplicate.

Genetic factors involved in plant morphogenesis will have crucial role in the differentiation of various Arabidopsis accessions. Tissue morphogenesis in Arabidopsis requires the cell wall-localized GPI-anchored β-1,3 glucanase ZERZAUST (ZET) (Vaddepalli et al. 2017). ZET was initially identified as a genetic component of the STRUBBELIG (SUB) signaling pathway along with QUIRKY, a C2 domain containing protein (Fulton et al. 2009). Absence of ZET results in a so-called strubbellig-like mutant (slm) phenotype characterized by abnormal integument initiation and outgrowth, aberrant floral organ and stem morphology, reduced plant height and irregular leaf shape.

Our previous studies have shown that mutations in SUB and QKY in Col background result in obvious slm mutant phenotypes similar to their respective mutants in Ler background (Fulton et al. 2009; Vaddepalli et al. 2011, 2014). But in the current work, we discovered that ZET acts differently in Col accession due to the presence of the close homolog ZERZAUST HOMOLOG (ZETH). Using genetic and gene expression tools, we show how ZET and ZETH diverged between the two common laboratory accessions Col and Ler in terms of expression and function. Furthermore, we try to understand the contribution of the weakly expressing redundant homolog on the morphological diversity of accessions.

MATERIALS AND METHODS

Plant work, Plant Genetics and Plant Transformation

Arabidopsis thaliana (L.) Heynh. var. Columbia (Col-0) and var. Landsberg (erecta mutant) (Ler) were used as wild-type strains. Plants were grown as described earlier (Fulton et al. 2009). The zet-1 mutant was described previously (Vaddepalli et al. 2017). T-DNA insertion lines were received from the GABI-KAT (zet-3, GABI-KAT-460C06) (Kleinboelting et al. 2012) and Wisconsin collections (zet-4, WiscDsLoxHs057_03H; zeth-1, WiscDsLoxHs066_12G) (Sussman et al. 2000). Plants were transformed with different constructs using Agrobacterium strain GV3101/pMP90 (Koncz and Schell 1986) and the floral dip method (Clough and Bent 1998). Transgenic T1 plants were selected on Hygromycin (20 µg/ml) or Glufosinate (Basta) (10 µg/ml) plates and transferred to soil for further inspection. The figures represent the phenotyping analysis performed on at least 4 to 6 plants for each genotype.

Recombinant DNA work

For DNA and RNA work standard molecular biology techniques were used. PCR-fragments used for cloning were obtained using Phusion high-fidelity DNA polymerase (New England Biolabs, Frankfurt, Germany) or TaKaRa PrimeSTAR HS DNA polymerase (Lonza, Basel, Switzerland). PCR fragments were subcloned into pLmLinus 28i (NEB).

All PCR-based constructs were sequenced. Primer sequences used for cloning and qRT PCR in this work are listed in S1 Table.

Cloning

Genomic fragments of ZETH were amplified from Col-0 and Ler backgrounds using primers ZETHCol_F/ZETHCol_R and ZETHLer_F/ZETHLer_R and sub cloned into pZET::TS:ZET (Vaddepalli et al. 2017) using Xmal/BamHI restriction sites to obtain pZET::TS:ZETHCol and pZET::TS:ZETHLer respectively. For CRISPR/cas9 ZETH construct, the egg cell-specific promoter-controlled CRISPR/Cas9 system was used as described (Wang et al. 2015). zet-1 plants were transformed with the CRISPR/cas9 ZETH construct by floral dip method. T1 plants were screened for exaggerated phenotype and ZETH locus was sequenced for identifying the mutation.

Quantitative RT-PCR analysis

Tissue for quantitative real-time PCR (qPCR) was harvested from plants grown in long day conditions. With minor changes, tissue collection, RNA extraction and quality control were performed as described previously (Box et al. 2011). RT-PCR was performed on Biorad CFX96 by using iQ SYBR Green Supermix (Bio-Rad) according to the manufacturer’s recommendations. All expression data were normalized against reference genes At5g25760, At4g33380, and At2g28390 by using the ΔΔ-Ct method (Czechowski 2007). Experiments were performed in biological and technical triplicates.

Data availability

Strains and plasmids are available upon request. The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article. Supplemental material available at FigShare: https://doi.org/10.25387/g3.8152808.

RESULTS

Molecular identification of ZETH

In Ler background, zet-1 carrying a loss-of-function mutation in ZET locus (At1g64760) (Figure 1A,B), shows a strong slm mutant phenotype (Vaddepalli et al. 2017) (Figure 1D,F). Except one amino acid in the signal peptide, ZET shows no difference between Col and Ler. We investigated the functionality of ZET in Columbia accession by analyzing two available T-DNA insertion lines (zet-3 and zet-4) (Figure 1A). We expected the T-DNA insertion in zet-4 to cause a mutant phenotype as it is predicted to result in a truncated ZET protein (Figure 1A,B). But, the plants surprisingly failed to display the twisted morphology, characteristic of slm mutants (Fig. S1B). We asked, if the observed accession-specific phenotypic differences could relate to a close homolog of ZET. An NCBI BLAST search with the ZET coding sequence revealed that ZET is most closely related to At2g19440 with 89% identity at the amino acid level (Fig. S1A). We named this gene ZERZAUST HOMOLOG (ZETH). ZET and ZETH form a subclade within the larger β clade of β-1,3 glucanase (BG) genes, which comprises 11 members (Doxey et al. 2007; Gaudioso-Pedraza and Benitez-Alfonso 2014). Sequence-based analysis of the evolutionary history revealed that ZET and ZETH duplication is specific to species within the Arabidopsis lineage (Fig. S2).

To assess ZETH activity in Col, we investigated a T-DNA line (zet-1), which is presumed to carry a truncated protein (Figure 1A,B). But, like zet-4, the zet-1 insertion line also failed to show a mutant phenotype (Fig. S1C). However, the zet-4 and zet-1 double mutant exhibited a strong phenotype (Figure 1G-I, S1D) suggesting that the two genes act redundantly. The double mutant resembled zet-1
plants except for appearing less bushy but with exaggerated twisting of flowers and increased sterility. Nevertheless, we could complement
the double mutant plants by introducing a construct encoding a translational fusion of ZET to the GFP variant T-Sapphire driven by the endogenous ZET promoter (pZET:TS:ZET: zet-4 zeth-1 4/4 T1 plants), ruling out the contribution of any other background mutation (Figure 1J). This construct was used in a previous study to complement zet-1 mutants in Ler background (Vaddepalli et al. 2017).

**Accesion-specific regulation of ZET and ZETH expression**

Next, we analyzed the expression pattern of ZET and ZETH in Col and Ler accessions to assess the cause for the mutant phenotype disparities between the two accessions. Our qPCR data from various tissues revealed that these genes are co-expressed (Figure 2A). Surprisingly, we found much lower levels of ZETH transcripts in comparison to ZET. Moreover, ZETH expression was even further reduced in Ler where it was barely detectable in rosette leaves, stems, or flowers. Additional qPCR tests revealed that ZET and ZETH expression levels in seedlings and flowers undergo compensatory regulation in the zeth-1 and zet-4 mutants in Col, respectively (Figure 2B). This result provides evidence for redundant functions of ZET and ZETH in the Col background and thus offers a convenient explanation for the lack of phenotype in single mutants in this accession. Interestingly, this compensatory regulation appears to be absent in flowers of Ler accession since ZETH expression was not detectably upregulated in zet-1 mutant.

**Ler carries a functional ZETH gene**

Despite minimal ZETH expression profiles in both accessions, appearance of prominent phenotypes only in Ler, when zet is mutated, can be attributed to any or all of the following reasons. It could be because of the low expression of ZETH, the lack of compensatory mechanism, or the Ler version of ZETH exhibiting a different amino acid composition when compared to Col which might affect its activity. The Ler/Col variants of ZET differ by only one amino acid at position 3 in the predicted signal peptide (change from an asparagine to a lysine) but there are nine amino acid differences between the Ler/Col variants of ZETH (Figure 3A). We wanted to test if these changes affect the activity of ZETH in Ler. For this purpose, we replaced the ZETH coding sequence 3’ to the predicted signal peptide with the equivalent Ler or Col variants of ZETH sequence in our complementing pZET::TS:ZET reporter (Vaddepalli et al. 2017). This resulted in zet-1 plants transgenic for the Ler or Col variants of ZETH, under the control of the native ZET promoter (pZET::TSZETHL/C zet-1).

**Figure 1 Molecular and genetic characterization of ZET and ZETH.** (A) Cartoon depicting the genomic organization of ZET and ZETH. Horizontal lines represent introns. Filled rectangles mark untranslated regions. The positions of EMS-, CRISPR-, and T-DNA-induced mutations are denoted by lines, arrow, and open triangles, respectively. (B) Schematic view of the predicted ZET protein. The signal peptide (SP), GH17 and CBM43/X8 domains are highlighted. The position and effects of the zet mutations are indicated. Phenotypic analysis (C-J). Comparison of (C, E) Ler, (D, F) zet-1, (G) Col and (H, I) zet-4 zeth-1. Note the aberrant flower (D, H), stem and silique (F, I) morphology of mutants. Mutant phenotype is strong in zet-4 zeth-1 double mutant (H, I). (J) zet-4 zeth-1 double mutant phenotype is complemented by pZET::TS:ZET (TG). Scale bars:1 mm.
ZETH acts in a dose dependent manner

Thus far, our results have established the functional role for the weakly expressed ZETH in both Col and Ler accessions, implying small amount of ZETH protein can have a noticeable impact. Next, we asked if this gene is acting in a dose-dependent manner. For this purpose, we checked the effect of ZETH gene copy number on zet mutant phenotype (Figure 4). Interestingly in the Ler background, zet-1 zeth-2/+ displayed an intermediate phenotype between the single zet-1 mutant and the double zet-1 zeth-2 mutant. The leaf petioles are somewhat elongated in zet-1 with narrow blades. This phenotype got slightly enhanced in zet-1 zeth-2/+ background, whereas zet-1 ZETH-2 double mutants show further worsening of the phenotype. The phenomenon of dosage-dependent enhancement of mutant phenotype was also observed for floral organs and was particularly obvious for siliques depending on the ZETH copy number. Surprisingly, in Columbia background the zet-4 zeth-1/+ mutant showed wild type morphology in all the organs tested except siliques which displayed shortening of length with no twisting. Despite these peculiarities among accessions, the observations indicate that mutation in zeth contributes to the overall exaggerated morphologies of double mutants in a dose dependent manner. Our results also imply that the extent of the ZETH effect on plant morphology is accession dependent.

ERECTA influences the zet-1 phenotype

Col and Ler accessions display obvious discrepancies in their phenotypic appearance. For example, Ler exhibits shorter stems and more compact inflorescences (Passardi et al. 2007). The phenotypic differences could be ascribed to genetic variability between the accessions and the contribution of accession-specific modifiers needs to be addressed. To further understand the accession specific effects on zet mutant phenotype, we crossed zet-1 (Ler) with zet-4 zeth-1/+ (Col). In the resulting F1 population plants with zet-1 (Ler) /zet-4 (Col) displayed WT phenotype as expected (Figure 5A and C), whereas zet-1 ZETH (Ler) /zet-4 zeth-1 (Col) displayed twisted flowers and siliques, but interestingly the stem twisting was absent and plant height was normal (Figure 5B and C). Major genetic difference between the Col and Ler accessions is the lack of ERECTA (ER) in the Ler background, which is present in the zet-1 ZETH (Ler) /zet-4 zeth-1 (Col) genotype. Previous work revealed that SUB and QKY show a synergistic interaction with ERECTA (ER) with respect to the control of plant height (Vaddepalli et al. 2011, 2014). We investigated the phenotype of zet-1 (Ler) plants transformed with pKUT196, a plasmid carrying 9.3 kb of Col-0 DNA spanning the entire genomic ER locus (Torii et al. 1996; Godiard et al. 2003). Like zet-1 ZETH (Ler) /zet-4 zeth-1 (Col) genotype, zet-1 ER transgenic plants also exhibited ameliorated plant height and stem twisting whereas aberrant floral organ phenotype appeared unaffected by the ER transgene (Figure 5). Our result exemplifies the influence of accession-specific modifiers on plant morphology.

DISCUSSION

Numerous studies have shown the compensation of gene loss by duplicate genes implying that close homologs give robustness to the plants against mutations (Hanada et al. 2009). Studies have also shown stronger reduction in duplicate expression and this expression divergence was noted as an important innovation for conservation of the duplicate gene (Ganko et al. 2007; Panchy et al. 2016). Despite acknowledging this interesting pattern, examples are missing that show functional relevance of gene duplicates, since retention of almost identical duplicates goes against the evolutionary instability of genetic redundancy (Lynch and Conery 2000). Here, our work reveals an unexpected variation of a weakly expressed gene and its homolog between accessions. Our results imply that divergence in duplicate expression may play a crucial role in accession-specific genetic variations.
Redundancy between duplicate genes by a compensation mechanism via feedback responsive circuit serves as an advantage for biological systems to deal with stochastic fluctuations in signaling pathways (Kafri et al. 2006, 2009). In the Col background, an overall higher expression level of ZETH, in combination with a compensatory upregulation in zet-4 mutants seems to account for their wild-type appearance. Although this phenomenon is absent in Ler, the residual expression of ZETH in Ler seems to be above the threshold, otherwise the zet-1 zeth-2 double mutant would have resembled the single mutant zet-1. Furthermore, understated changes in expression pattern may further enhanced by tissue level sampling. Although segregating mutants in Col background appear to be fine morphologically, we may have missed subtler cellular phenotypes in our analysis. All the experiments were performed in controlled lab conditions. There is also a possibility that ZETH may express at higher levels under different conditions and reveal a novel function. Indeed, the role of SUB in coordinating cell proliferation and differentiation during leaf development was revealed only at high ambient temperature of 30°C (Lin et al. 2012).

Initially, we assumed ZETH in Ler background as a pseudogene, since a previous RNA-seq analysis has found that ZETH transcripts were undetectable in young Ler flowers (Jiao and Meyerowitz 2010). Although our qRT-PCR results showed ZETH expression, the functionality was still in question given the very low expression pattern in all the tissues tested. But, surprisingly our results indicate that weakly expressed ZETH is functionally very relevant. Our results also highlight the importance of gene specific analysis, since in large scale studies differentiating between identical duplicate sequences is challenging. Thus, duplicate genes with low expression levels are often overlooked, as the focus goes inadvertently on highly expressed genes.

If minimal ZETH expression in Col was enough for wild type appearance of zet-4 mutants, then the high ZET expression implies that it may have additional roles apart from development. Transcriptome analysis performed on sbsms showed that several ZET responsive genes are related to biotic and abiotic stress responses (Fulton et al. 2009). Since ZET is localized to cell wall, it is intriguing to speculate that the high expression of ZET may have a role in dealing with imminent stress situations which demand instant response. Future experiments could test this possibility by assessing if zet is more susceptible to stress compared to zeth or vice versa. Interestingly, ZET transcript level was shown to be altered in Arabidopsis plants infected with Fusarium oxysporum (Fallath et al. 2017). Thus, further analysis of ZET may reveal its potential role in adaptation, apart from morphogenesis.

Duplicate genes provide mutational robustness to living organisms. In yeast and Caenorhabditis elegans, functional compensation by the
A duplicated gene displayed higher robustness to gene perturbation than singletons (Gu et al. 2003; Conant and Wagner 2004). But, ZETH showed a highly reduced expression pattern compared to ZET. Such a reduction in expression was proposed to facilitate the retention of duplicates and the conservation of their ancestral functions (Qian et al. 2010). In this scenario, loss of either of the duplicate genes renders the total expression level lower than normal which would hamper the function. This also inhibits functional divergence of duplicated genes and helps in rebalancing gene dosage after duplication. Interestingly, with ZET this phenomenon was observed only in Ler, where mutating ZET was enough for the manifestation of phenotype, but not in the Col background. These results indicate that the divergent behavior of duplicates may vary depending on the accession and the specific gene pair under study. Since the ER locus was able to partially alleviate zet-1 mutant phenotype, the influence of accession-specific differences on gene contribution needs to be considered. Further, it would be interesting to know if there exists a correlation between accession-specific modifiers and expression divergence of certain duplicates.

A study on homologs revealed significant diversity in expression pattern among different accessions of Arabidopsis (Kliebenstein 2008). For instance, background specific regulation and unequal genetic redundancy has been observed for BR1 (Caño-Delgado et al. 2004; Zhou et al. 2004). In another example, the sucrose transporter AtSUC1 was shown to have differential tissue expression pattern depending on the accession it was tested (Feuerstein et al. 2010). The observed accession-specific disparities in ZETH expression levels may relate to differences in its cis-regulatory region, either caused by DNA polymorphisms, as was found for the tomato fvs2.2, rice qSH1, or Arabidopsis FLOWERING LOCUS (FT) loci (Konishi et al. 2006; Cong et al. 2008; Schwartz et al. 2009; Liu et al. 2014), or by epigenetic variation (Durand et al. 2012). Thus, our work provides an interesting example for the diversification of cis and/or trans regulatory elements between two Arabidopsis accessions. A correlation between duplicate divergence and evolution of cis-regulatory elements and networks was observed (Arsovski et al. 2015). But, how the transcriptional networks regulate the levels of the duplicated genes and their role in the context of evolution is largely unexplored. Whole genome analysis and comparison of Ler with reference sequence of Col revealed immense sequence differences between the two accessions with hundreds of diversified regions and unknown accession specific genes (Zapata et al. 2016). But how these differences contribute to natural variation in terms of phenotype and adaptation is a challenging task and requires novel approaches. Thus, our study gives an example of how variation in duplicates involved in morphogenesis could serve as a great tool to understand their contribution in natural variation among different accessions of Arabidopsis.
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Figure 5 The zet-1 phenotype in the presence of functional ERECTA (ER). (A, B, D-F) Morphology of flowers (upper panel), stems (central panel) and siliques (bottom panel). (A) zet-1 (Ler)/zet-4 (Col). Morphology looks essentially wild type. (B) zet-1 ZETH (Ler)/zet-4 zeth-1 (Col). Note the irregular flower and silique morphology. Stem morphology is essentially normal. (C) Plant height comparisons of six-week-old zet-1 ZETH (Ler)/zet-4 zeth-1 (Col) and zet-1 (Ler)/zet-4 (Col) respectively. (D) Wild-type Ler. (E) zet-1. (F) Transgenic zet-1 ER. Flower and silique morphology is irregular. Stem shows wild type morphology. (G) Plant height comparisons of six-week-old zet-1 ER transgenic plants in comparison to wild type and mutant reference lines. Note the rescue of plant height in zet-1 ER plants. Scale bars: (A, B and D-F) 0.5 mm.
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