The Second Intron Is Essential for the Transcriptional Control of the Arabidopsis thaliana GLABRA3 Gene in Leaves

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The GLABRA3 gene is a major regulator of trichome patterning in Arabidopsis thaliana. The regulatory regions important for the trichome-specific expression of GL3 have not been characterized yet. In this study, we used a combination of marker and rescue constructs to determine the relevant promoter regions. We demonstrate that a 1 kb 5’ region combined with the second intron is sufficient to rescue the trichome mutant phenotype of gl3 egl3 mutants. Swap experiments of the second intron suggest that it is not sufficient to generally enhance the expression level of GL3. This implies that the second intron contains regulatory regions for the temporal and spatial regulation of GL3. The corresponding GUS-marker constructs revealed trichome-specific expression in young trichomes.

Keywords: Arabidopsis, trichomes, patterning, GLABRA3, transcriptional regulation

INTRODUCTION

Arabidopsis trichomes are single epidermal cells that develop on the surfaces of most aerial organs. Trichomes are regularly distributed on rosette leaves, cauline leaves, sepals and the stem without any obvious reference to other morphological structures (Hulskamp et al., 1994; Hulskamp, 2004; Balkunde et al., 2010). The distribution of trichomes is regulated by gene regulatory network containing genes promoting or inhibiting trichome fate. The positive regulators include the WD40 factor TRANSPARENT TESTA GLABRA1 (TTG1) (Koornneef, 1981; Galway et al., 1994; Walker et al., 1999), the bHLH factors GLABRA3 (Koornneef et al., 1982; Payne et al., 2000; Bernhardt et al., 2003) and the redundantly acting ENHANCER OF GLABRA3 (EGL3) (Bernhardt et al., 2003), and the R2R3MYB factors GLABRA1 (GL1, trichome system) (Oppenheimer et al., 1991) and MYB23 (Kirik et al., 2001, 2005). In addition, several redundantly acting negative regulators, the R3MYB proteins, mediate cell–cell communication by moving between cells (Wada et al., 1997; Schellmann et al., 2002; Kirik et al., 2004a,b; Kurata et al., 2005; Digiuni et al., 2008; Tominaga et al., 2008; Wang et al., 2008; Wester et al., 2009). The trichome promoting genes and the negative regulators are engaged in a regulatory feed back loop that is described in the activator-inhibitor model (Hulskamp, 2004; Pesch and Hulskamp, 2009): The activators TTG1, GL3/EGL3, and GL1/MYB23 form an activator complex, in which TTG1 and the R2R3MYB protein bind to GL3 or EGL3. This complex activates the R3MYBs that in turn can move into neighboring cells where they repress the activators. In addition a so-called activator-depletion mechanism has been postulated according to which TTG1 is mobile in the epidermis and due to its binding to
GL3 protein it is trapped in trichomes and depleted in the neighboring cells (Bouyer et al., 2008; Pesch and Hulskamp, 2009; Balkunde et al., 2011).

For a better understanding of the regulatory network it is important to analyze the transcriptional regulation of the key gene GL3. In rosette leaves, GL3 in situ hybridization experiments have shown that GL3 is expressed in developing trichomes. The expression analysis of a 2.5 kb 5′-promoter fragment driving the GUS reporter gene revealed a similar expression pattern (Zhang et al., 2003; Zhao et al., 2008) suggesting that the promoter fragment is sufficient for GL3 function in leaves. As a 1 kb 5′-promoter fragment can rescue gl3 mutants it is likely that this 1 kb fragment contains all regulatory sequences essential for trichome patterning (Bernhardt et al., 2005). Recently, the 2.5 kb 5′-promoter driving the GL3 cDNA was shown to rescue the trichome and root hair phenotype of gl3 egl3 double mutants though the rescue was not complete (Zhao et al., 2012).

In this study, we aimed to identify the relevant promoter regions of the GL3 gene in the context of trichome patterning. We could not confirm that the 2.5 kb 5′-promoter fragment drives GUS expression in trichomes (Zhang et al., 2003). We also show that a 1 kb 5′-promoter region is not sufficient for rescue. As this upstream region was previously shown to rescue the trichome phenotype when combined with the genomic region of GL3 containing introns and the 3′-1 kb downstream region (Bernhardt et al., 2005) we speculated that introns or the 3′ region contains additional regulatory sequences. A detailed analysis of the function of all introns revealed that intron 2 is essential for rescuing the gl3 mutant trichome phenotype and that it is sufficient for rescue in combination with the 1 kb 5′-promoter fragment. We exchanged the second intron with intron sequences leading to a generally enhanced expression without rescuing the gl3 egl3 phenotype. This implies that the intron sequences contain regulatory sequences for the temporal and spatial regulation of GL3 rather than for an un-specific up-regulation of the GL3 levels. Finally, we show that the relevant promoter sequences mediate a trichome specific expression of the GUS marker gene.

**MATERIALS AND METHODS**

**Molecular Biology**

The 35S promoter cassette of the vector pAMPAT-GW (GenBank accession no. AY436765, Pesch et al., 2015) was exchanged with the 898 bp 5′ sequence immediately upstream of the start codon of GL3 gene using Ascl and XhoI [pAMPAT-GW-GL3(5′-1 kb)]. The 1051 bp 3′ fragment was cloned into the PmeI site of pAMPAT-GW-GL3(5′-1 kb) to create the pAMPAT-GW-GL3(5′-1 kb):LR recombination cassette(3′-1 kb) vector. All genomic fragments of GL3 were cloned into pDONR201 by BP reactions (Invitrogen). Deletions of single introns within the genomic sequence of GL3 were introduced by PCR based site directed mutagenesis. The entry clone carrying the GL3 gene with the second intron was generated using the following strategy: an entry clone carrying the genomic GL3 was cut with EcoRV and KpnI generating a GL3 fragment that includes the second intron. We exchanged the second intron with intron sequences leading to a generally enhanced expression without rescuing the gl3 egl3 phenotype. This implies that the intron sequences contain regulatory sequences for the temporal and spatial regulation of GL3 rather than for an un-specific up-regulation of the GL3 levels. Finally, we show that the relevant promoter sequences mediate a trichome specific expression of the GUS marker gene.

| Genotype                  | Plant number | Leaf number | Average trichome number |
|---------------------------|--------------|-------------|-------------------------|
| Col-0                     | 20           | 3           | 74.1 ± 17.0             |
| eg3-77439                 | 20           | 3           | 91.2 ± 15.4             |
| gl3-3                     | 20           | 3           | 68.3 ± 5.5              |
| gl3-3 eg3-77439           | 20           | 3           | 78.0 ± 6.0              |
| pGL3:GL3 (genomic):3′-1 kb| 33           | 3           | 19.1 ± 10.2             |
| pGL3:GL3 (genomic:Δintron1):3′-1 kb | 36    | 3           | 30.6 ± 8.7              |
| pGL3:GL3 (genomic:Δintron2):3′-1 kb | 100  | 3           | 0 ± 0                   |
| pGL3:GL3 (genomic:Δintron3):3′-1 kb | 10   | 3           | 0 ± 0                   |
| pGL3:GL3 (genomic:Δintron4):3′-1 kb | 20   | 3           | 28.1 ± 18.1             |
| pGL3:GL3 (genomic:Δintron5):3′-1 kb | 16   | 3           | 55.1 ± 27.4             |
| pGL3:GL3 (genomic:Δintron6):3′-1 kb | 14   | 3           | 18.2 ± 17.7             |
| pGL3:GL3 (genomic)        | 20           | 3           | 32.0 ± 25.4             |

**Table 1**

Trichome number of the third and fourth true leaf in gl3-3 eg3-77349 mutants transformed with different GL3 rescue constructs.
FIGURE 1 | Schematic description genomic GL3 constructs and intron deletions. All constructs were cloned into the pAMPAT vector backbone. The length of promoter, exon, and intron elements are proportional to the respective sequence length. Ex, exon; In, intron.

and third intron of GL3. This fragment was exchanged against the corresponding GL3 fragment without introns in the entry clone carrying the coding sequence of GL3. Thereafter, the third intron was deleted by PCR based site directed mutagenesis. Coding and genomic sequences of GL3 were introduced into pAMPAT-GW-GL3(5′-1 kb):LR recombination cassette:(3′-1 kb) by LR recombination with the respective entry clones to generate the various intron deletion constructs. Plants were transformed using the floral dip method described previously (Clough and Bent, 1998).

Plant Materials and Growth Conditions
Plants were grown on soil at 24°C with 16 h of light per day. All Arabidopsis thaliana used in this study were of the Columbia (Col-0) ecotype. The gl3-3 mutant line has been described previously (Jakoby et al., 2008). egl3-77439 corresponds to the TAIR accession 1008704039.

Expression Analysis
Total RNA was extracted from 10-day-old true leaves using the RNeasy Mini Kit (Qiagen, Cat No./ID: 74106) and first-strand cDNA was then synthesized from the total RNA (1 µg) using the RevertAid H Minus 1st strand cDNA synthesis (Thermo) as described by manufacturer's instruction. Real-time polymerase chain reactions (PCR) contained 1 µl of primer mix (10 µM), 1 µl cDNA template (10-fold dilution),10 µl 2 x SYBR Green master PCR mix and 8 µl water to a total of 20 µl. cDNA concentrations in different samples were normalized with reference to AtAct2. Gene-specific primers are listed in Supplementary Table S1.

Morphological and Histochemical Analysis
GUS stainings were essentially done as previously (Sessions et al., 1999; Schroeder et al., 2016). After staining for 16 h at 37°C, tissues were cleared and leaves were inspected by light microscopy and pictures taken using the DISKUS software (Carl H. Hilgers -Technisches Büro, Germany). Trichome numbers were determined on the third and fourth fully expanded leaf of soil-grown seedlings.

RESULTS

5′-Promoter Region of GL3 Is Not Sufficient for Proper Expression and Rescue
It has been previously reported, that a 2.5 kb 5′-promoter fragment of GL3 fused to GUS reveals trichome specific expression in leaves and that a fusion to the GL3 cDNA can rescue the trichome phenotype in gl3 egl3 double mutants (Zhang et al., 2003; Zhao et al., 2012). In addition, it was shown that a genomic GL3 fragment including 1kb of the 5′-promoter was sufficient to rescue the gl3 trichome phenotype (Bernhardt et al., 2005). To test, whether the 1 kb 5′-promoter fragment is sufficient for trichome-specific expression or whether the introns are also important we created a pGL3(1 kb):GUS line. We observed ubiquitous GUS expression in young leaves (Supplementary Figures S1A–D). In older leaves, expression levels were close to background (Supplementary Figures S1E–G). In parallel, we performed rescue experiments by expressing the GL3 cDNA under the control of the 1 kb 5′-promoter and 1 kb downstream of STOP codon (termed as 3′-1 kb) in the gl3-3 egl3-77439 double mutant. The gl3-3 single mutant shows about 50% reduction in trichome number whereas the egl3-77439 mutant shows a significant reduction of about 10% similar as reported for the egl3 allele in the Ler background (Table 1 and Supplementary Figures S2A,B) (Zhang et al., 2003). The gl3-3 egl3-77439 double mutant is completely glabrous and one would expect that rescued lines should exhibit the egl3 77439
The Second Intron of GL3 Is Essential for Rescue

Our data indicated that the 5′ promoter region and the 3′-1 kb are not sufficient for rescuing the trichome phenotype of gl3-3 egl3-77439 double mutant. We therefore tested the possibility that introns are relevant for the proper regulation of GL3 during trichome formation. Toward this end, we created a gateway construct containing 1 kb of the 5′ promoter region and 1 kb of the 3′-1 kb [called pGL3:GL3(genomic):3′-1 kb] such that the coding region can be replaced by recombination (Figure 1). This construct was used to study the rescue ability in gl3 egl3 mutants in the T1 generation. As expected, we found a range of rescue phenotypes in the T1 generation. The average rescue efficiency was used as a reference for subsequent analysis (Table 1). Next, we created a series of constructs each lacking one of the six introns (Figure 1). We found a clear rescue with constructs missing the third, fourth, or fifth intron. The deletion of the first or the sixth intron resulted in a weaker rescue of trichome formation. No rescue was observed in plants carrying the pGL3:GL3 (genomic:Intron 2):3′-1 kb construct (Table 1) indicating that the second intron is essential. We therefore focused in the following on the function of the second intron.

To test the relevance of the 3′-1 kb region we studied the rescue in lines harboring the pGL3:genomic GL3 construct (Figure 1). These lines showed a rescue of the trichome phenotype. Thus, the 3′-1 kb of GL3 is not necessary for trichome rescue.

Analysis of the Function of the Second Intron of GL3

In order to demonstrate that the second intron together with the 1 kb 5′-promoter fragment is sufficient for the transcriptional regulation of GL3 in the leaf, we expressed the GL3 cDNA containing the second intron at its original site under the 1 kb 5′-promoter [pGL3:GL3(Intron 2)] in gl3 egl3 mutants (Figure 2). The majority of T1 lines showed rescue of the trichome phenotype (Table 2). In an attempt to map potential relevant regions in the second intron, we compared two deletion constructs missing either the 5′ 125 nt (pGL3:GL3(Intron 2 delta 3-125) or 454 nt at the 3′end [pGL3:GL3(Intron 2 delta 126-579)] of the second intron. Only the construct containing

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**TABLE 2** Rescue of gl3-3 egl3-77439 trichome phenotype.

| Genotype                      | T1 lines showing trichome rescue (rescued lines/total number of lines) |
|-------------------------------|--------------------------------------------------------------------------|
| gl3-3 egl3-77439              | 0/20                                                                     |
| pGL3:GL3                      | 0/20                                                                     |
| Col-0                         | 20/20                                                                    |
| egl3-77439                    | 20/20                                                                    |
| pGL3:GL3 (genomic)            | 16/17                                                                    |
| pGL3:GL3 (intron 2)           | 18/20                                                                    |
| pGL3:GL3 (1–125 nt of intron 2)| 9/10                                                                    |
| pGL3:GL3 (126–579 nt of intron 2)| 0/15                                                                  |
| Intron 2 (forward) pGL3:GL3    | 0/16                                                                    |
| Intron 2 (reverse) pGL3:GL3    | 0/16                                                                    |
| pGL3:GL3 (intron 1 of UBO10)  | 0/11                                                                    |
| pGL3:GL3 (intron 1 of Cor15a10)| 0/20                                                                    |
| 35S:GL3                       | 13/15*                                                                   |

*Indicates irregular trichome pattern.
the 125 nt at the 5′ end rescued the gl3 egl3 mutant trichome phenotype (Table 2) suggesting that this fragment contains all regulatory sequences. In a next step, we assessed whether the position of the intron 2 is important. Toward this end we placed intron 2 in front of the 5′ promoter in both directions (Figure 2). Neither construct was able to rescue the gl3 egl3 trichome mutant phenotype (Table 2) indicating that intron 2 does not act as a transcriptional enhancer element. This suggested to us that its position in the transcribed region is important for its function. One well-characterized regulatory mechanism that requires the intron within the transcribed sequence in its correct orientation is intron mediated enhancement (IME) (Rose, 2002; Gallegos and Rose, 2017). To address this possibility, we created two constructs in which the second intron of GL3 was replaced by introns for which their ability to mediate IME is well characterized (Figure 2). The first intron of UBQ10 resulted in a 2- to 10-fold higher GL3 expression as compared to wild type Col (Figure 3). Insertion of the first intron of the Cor15a gene lead to wild-type levels or up to about two-fold increased GL3 expression (Figure 3). By comparison, constructs containing intron 2 or the first 125 nt of intron 2 could enhance GL3 expression up to three-fold (Figure 3). However, neither the UBQ10 nor the Cor15a constructs rescued the trichome phenotype (Table 2).

These results suggest that it is not sufficient to merely increase the GL3 expression by replacing intron 2. It is therefore conceivable that intron 2 is important for the proper regulation of the temporal and spatial expression of GL3.

Expression Analysis of pGL3: GL3(intron 2)-GUS

Our analysis revealed that a 1 kb promoter fragment combined with intron 2 in the transcribed region of GL3 is sufficient for complete rescue. To study the expression pattern mediated by this construct we fused the GUS marker gene directly after the second intron. As the signal levels were very low when using X-Gluc as a substrate, we used the more sensitive magenta-Glc-A as a substrate (Schroeder et al., 2016). We detected GL3 expression in young leaves in all stages of trichome development Figures 4A,B. In addition we noted weak expression in epidermal pavement cells in young leaves (Figure 4A). In older leaves with young trichome stages at the leaf bases and mature trichomes at the tip of the leaf trichomes exhibited much stronger expression then the mature trichomes (Figure 4B). Low levels of GL3 were maintained during further leaf growth but disappeared in fully mature leaves (Figures 4C,D).

DISCUSSION

In this study, we examined which regions of the GL3 gene are important for the transcriptional regulation in the context of trichome development. While previous data suggested that the 1 kb 5′ sequences together with 1 kb 3′ sequences might be sufficient for rescuing the trichome phenotype (Zhang et al., 2003; Bernhardt et al., 2005; Zhao et al., 2008) we show that the presence of the second intron is essential and that the insertion of only the second intron in the coding region is sufficient for full rescue in combination with a 1 kb 5′ region. Our data also suggest that intron 2 contains regulatory sequences for the temporal and/or spatial expression of GL3 as high expression levels mediated by the UBQ10 intron cannot rescue the trichome mutant phenotype. Consistent with this, the intron 2 of the GL3 gene lies in a region that is hypersensitive to DNase digestion. DNase hypersensitive sites are well-established to indicate regions

http://plantdhs.org/plotdhs/TAIR10_Chr5:16530075-16530411
FIGURE 4 | GL3 expression as revealed by pGL3:GL3(intron2)-GUS. Glucuronidase activity was detected in the first or second true leaves using magenta Glc-A as a substrate. (A) Incipient leaf with six trichomes at different stages 4 days after germination (DAG). (B) Young leaf with young stained trichomes at the leaf base and weakly stained older trichomes at the leaf tip 6 DAG. (C) Ten days old leaf with weakly stained trichomes. (D) Fourteen days old leaf with mature unstained trichomes. Scale bars in (A–C) are 100 µm. Scale bar in (D) is 200 µm.

of active transcriptional elements (Keene et al., 1981; McGhee et al., 1981) and DNase hypersensitivity of DNA regions in intron 2 support therefore a regulatory role.

This raises the question, how and by which factors intron 2 is regulated to mediate trichome specific expression. One likely scenario would be the regulation by trichome patterning genes, in particular GL1, GL3/EGL3, and TTG1. However, previous studies had shown that the transcriptional regulation of GL3 does not seem to require any of the known trichome activator genes GL1, GL3, or TTG1. The total expression level of GL3 is not reduced or absent in gl1, gl3, or ttg1 mutants as judged by RT-PCR experiments (Payne et al., 2000). A possible negative auto-regulation was postulated because overexpression of GL3 can suppress its own expression (Morohashi et al., 2007). This regulation is likely to be direct as GL3 protein binds to the 5' region immediately upstream of the transcriptional start site in chromatin-immunoprecipitation experiments (Morohashi et al., 2007). In contrast to the GL2 and CPC promoters the recruitment of GL3 to this promoter region was independent of GL1 (Morohashi et al., 2007). Although these data suggest that GL3 regulation does not involve GL1 and TTG1 this possibility is not ruled out as the exact temporal-spatial expression of GL3 might be important and not the overall level as measured in PCR experiments. A possible role of transcription factors that are involved in trichome patterning in the regulation of the second intron of GL3 is suggested by several conserved MYB and WRKY (WBOX) binding sites in the second intron of GL3 (Supplementary Figures S3–S5 and Table S2). Intron 2 and in particular the first 125 nt fragment is generally highly conserved (Supplementary Figure S4) in several Brassicaceae species including Arabidopsis lyrata, Capsella rubella, and Arabis alpina (Supplementary Table S2 and Figures S3–S5). Strikingly, the relative position of several MYB and WRKY binding sites (WBOXes) is conserved.

Therefore, these binding sites are potentially relevant for the regulation of GL3 in Arabidopsis. For future studies of the temporal and spatial regulation of GL3 it will be helpful that we could map one relevant region down to a fairly small fragment of only 125 nt containing conserved binding sites.
AUTHOR CONTRIBUTIONS
AF, BZ, SH, MP, and AS designed, planned and performed the experiments and analyzed the data. MH supervised the project and wrote the manuscript.

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