DETORQUEO, QUIRKY, and ZERZAUST Represent Novel Components Involved in Organ Development Mediated by the Receptor-Like Kinase STRUBBELIG in Arabidopsis thaliana

Lynette Fulton¹, Martine Batoux¹, Prasad Vaddepalli¹, Ram Kishor Yadav¹, Wolfgang Busch², Stig U. Andersen², Sangho Jeong³, Jan U. Lohmann², Kay Schnitz¹

¹Entwicklungsbiologie der Pflanzen, Wissenschaftszentrum Weihenstephan, Technische Universität München, Freising, Germany, ²Max Planck Institute for Developmental Biology, Department of Molecular Biology, AG Lohmann, Tübingen, Germany, ³Section of Cell and Developmental Biology, University of California San Diego, La Jolla, California, United States of America, ⁴Center for Organismal Studies, University of Heidelberg, Heidelberg, Germany

Abstract

Interacellular signaling plays an important role in controlling cellular behavior in apical meristems and developing organs in plants. One prominent example in Arabidopsis is the regulation of floral organ shape, ovule integument morphogenesis, the cell division plane, and root hair patterning by the leucine-rich repeat receptor-like kinase STRUBBELIG (SUB). Interestingly, kinase activity of SUB is not essential for its in vivo function, indicating that SUB may be an atypical or inactive receptor-like kinase. Since little is known about signaling by atypical receptor-like kinases, we used forward genetics to identify genes that potentially function in SUB-dependent processes and found recessive mutations in three genes that result in a sub-like phenotype. Plants with a defect in DETORQUEO (DOQ), QUIRKY (QKY), and ZERZAUST (ZET) show corresponding defects in outer integument development, floral organ shape, and stem twisting. The mutants also show sub-like cellular defects in the floral meristem and in root hair patterning. Thus, SUB, DOQ, QKY, and ZET define the STRUBBELIG-LIKE MUTANT (SLM) class of genes. Molecular cloning of QKY identified a putative transmembrane protein carrying four C2 domains, suggesting that QKY may function in membrane trafficking in a Ca²⁺-dependent fashion. Morphological analysis of single and all pair-wise double-mutant combinations indicated that SLM genes have overlapping, but also distinct, functions in plant organogenesis. This notion was supported by a systematic comparison of whole-genome transcript profiles during floral development, which molecularly defined common and distinct sets of affected processes in slm mutants. Further analysis indicated that many SLM-responsive genes have functions in cell wall biology, hormone signaling, and various stress responses. Taken together, our data suggest that DOQ, QKY, and ZET contribute to SUB-dependent organogenesis and shed light on the mechanisms, which are dependent on signaling through the atypical receptor-like kinase SUB.

Introduction

How intercellular communication mechanisms coordinate the activities of cells during organogenesis is an important topic in biology. In higher plants shoot apical meristems and floral meristems are the ultimate source of above-ground lateral organs, such as leaves, flowers, and floral organs [1]. Meristems are organised into three distinct meristematic or histogenic layers, called L1, L2, and L3 [2], and cells of all histogenic layers contribute to organogenesis [3,4]. The L1 layer gives rise to the epidermis while the L2 and L3 layers contribute to internal tissues. In Arabidopsis ovules, for example, the integuments that eventually develop into the seed coat are entirely made up of L1-derived cells, while L2 cells generate the inner tissue [5].

Classic studies have demonstrated that meristematic layers communicate [6,7], but it is only recently that the biological relevance and the molecular mechanisms are being elucidated [8–11]. For example, work on the receptor-like kinase (RLK) BRASSINOSTEROID INSENSITIVE 1 (BRI1) has provided evidence that the epidermis both promotes and restricts organ
Author Summary

Plant organs, such as flowers or leaves, are made up of distinct cell layers. Although communication across these cell layers is essential for organ development, we have only recently gained some insight into the underlying mechanisms. Receptor-like kinases are cell-surface receptors that perceive and relay intercellular information. In Arabidopsis, the receptor-like kinase STRUBBELIG is required for inter-cell-layer communication during floral development, amongst other functions; little is known, however, concerning its exact signaling mechanism. Here, we identified three new genes called DETORQUEO, QUIRKY, and ZERZAUST. Plants defective in any of these genes strongly resemble the strubbelig mutant, both at the whole-organ and cellular levels. Thus, all four genes may share or contribute to a common signaling pathway essential for plant morphogenesis. Analyses revealed complex interactions between the genes, indicating that each has additional and distinct activities. We provide the molecular nature of QUIRKY; the encoded protein is likely membrane-localised and predicted to require Ca²⁺ for activity. In light of analogous animal models, we speculate that QUIRKY facilitates transport of molecules to the cell boundary and may support a STRUBBELIG-related extra-cellular signal. These results open new inroads into a molecular understanding of inter-cellular communication during flower development.

growth [12]. Furthermore, microsurgical experiments indicated that the epidermis also maintains cell division patterns in subtending layers [13]. These are but two examples that highlight the importance of the epidermis and inwards-oriented signaling in this inter-cell-layer cross-talk required for correct organ size and shape. At the same time, radial outwards-oriented signaling also takes place during organogenesis. Known scenarios include transcription factors or small proteins that are synthesized in inner layers and move outwards into overlaying cell layers in a controlled fashion [14–17]. The so far best-characterised case of such a movement underlies radial patterning of the root [18,19]. In addition, the epidermally-expressed RLKs CRINKLY4 (CR4) from corn or its Arabidopsis homolog ACR4 are necessary for epidermis development and may receive signals from underlying cell layers [20–24].

Inter-cellular communication during floral morphogenesis in Arabidopsis also depends on signaling mediated by the leucine-rich repeat transmembrane receptor-like kinase (LRR-RLK) STRUBBELIG (SUB) [25]. Analysis of sub mutants indicated that SUB is required for proper shaping of floral organs such as carpels, petals and ovules. At the cellular level SUB participates in the control of cell shape and/or the orientation of the cell division plane in floral meristems and ovules. In addition, SUB, also known as SCRAMBLED (SCM), affects specification of hair cells in the root epidermis [26,27]. Recent evidence suggests that the SUB protein is confined to interior tissues in floral meristems, developing ovules and young roots although SUB mRNA is monitored throughout those organs [28]. In particular functional SUB:EGFP fusion protein is absent from cells that show a mutant phenotype in sub mutants, but can either be found in adjacent cells, as in floral meristems and ovules, or in cells that are separated from mutant cells by two cell diameters, like in the root. The non-cell-autonomous effects of SUB were corroborated by an analysis of sub-1 plants expressing a functional SUB:EGFP transgene under the control of different tissue-specific promoters. Thus the data indicate that SUB undergoes posttranscriptional regulation, acts in a non-cell-autonomous fashion and mediates cell morphogenesis and cell fate across clonally distinct cell layers in an inside-out fashion [28]. The SUB protein is a member of the LRRV/STRUBBELIG-RECEPTOR FAMILY (SRF) family of receptor-like kinases [29,30]. It is predicted to carry an extracellular domain with six leucine-rich repeats, a transmembrane domain, and a cytoplasmic intracellular domain with the juxtamembrane and kinase domains. Interestingly, phosphotransfer activity of the SUB kinase domain is not essential for its function in vivo [25] and thus SUB seems to belong to the family of atypical or “dead” receptor kinases [31,32].

Very little is known regarding signaling through atypical receptor-like kinases in plants [31]. In addition, it remains to be understood how cellular morphogenesis is coordinated across cell layers [8–11]. It is therefore of great interest to investigate the molecular basis of SUB signaling and function. Here we present the identification and analysis of three genetic factors that may relate to SUB signaling. Our results show that mutations in QUIRKY (QKY), ZERZAUST (ZET), and DETORQUEO (DOQ) result in a sub-like phenotype. Molecular cloning of QKY revealed that the predicted QKY protein is likely a transmembrane protein with four C2 domains indicating a role for QKY in Ca²⁺-dependent signaling. Global gene expression profiling of the mutants corroborates the morphological analysis but also suggests additional and distinct roles for each gene. Furthermore, the data indicate that SUB signaling plays previously unknown roles in cell wall and stress biology.

Results

Isolation of sub-Like Mutants

We applied a forward genetic approach to isolate additional factors of the SUB signaling pathway, based on the hypothesis that mutations in some of the genes that are part of the SUB pathway should result in sub-like (slm) mutant phenotypes. We thus screened M2 families of an ethylmethane sulfonate-mutagenized Ler population for slm mutants (see Materials and Methods). In this experiment we identified two new sub alleles [25] as well as several novel mutants with sub-like phenotypes (Figures 1–3). These fell into three different complementation groups, which map to distinct positions on chromosome 1 (Table 1). We termed two of the genes DETORQUEO (DOQ) and ZERZAUST (ZET), respectively. DETORQUEO refers to a Latin term that means “to twist out of shape”. ZERZAUST is a German term for “disheveled”. We also isolated three mutant alleles of QUIRKY (QKY), which plays a role in fruit dehiscence (L.F., unpublished results; S.J. and Martin F. Yanofsky, unpublished observations) and the numbering of qky alleles was coordinated. Thus, our genetic approach resulted in the identification of three loci, DOQ, QKY, and ZET, mutations in which result in a sub-like phenotype and that, together with SUB, define the STRUBBELIG-LIKE MUTANT (SLM) class of genes.

Comparison of sub-1, doq-1, zet-2, and qky-8 Phenotypes

We identified one mutant allele of DOQ and two and three independent alleles of ZET and QKY, respectively (Table 1). All sub, doq, zet and qky alleles were recessive and behaved in a Mendelian fashion (not shown). The various zet and qky mutants did not noticeably differ in their respective phenotypes and the three qky alleles are likely to be nulls (see below). Thus, zet-2 and qky-8 or qky-9 were used as reference alleles for further analysis. In addition, we used the well-characterised sub-1 mutant for comparison [25]. This mutation likely represents a null-allele since it results in a stop codon and a predicted shorter SUB protein lacking the transmembrane and intracellular kinase domains.
Thus, it is expected that SUB-dependent signaling across the plasma membrane is blocked in sub-1 mutants.

At the macroscopic level sub-1 mutants are known to be affected in several above-ground organs [25] (Figures 1–4) (Tables 2, 3). Inflorescences are characterised by reduced height, an irregularly twisted stem, and an aberrant phyllotaxis of flowers. Flowers open prematurely and show a large percentage of twisted and often notched petals. Furthermore, all flowers exhibit twisted carpels and about 70 percent of sub-1 ovules showed aberrant initiation of the outer integument (Figure 2) (Table 2). This results in outer integuments with gaps that often resemble ''multifingered clamps'' or ''scoops''. Also ovules with a fully developed outer integument show defects. In particular the distal or micropylar cells of the outer integument can show aberrant size and shape. In addition, about 40 percent of sub-1 plants show at least one leaf per rosette with twisted petioles.

At the cellular level sub-1 exhibits aberrant cell shape and robustly scorable numbers of periclinal, rather than anticlinal cell division planes in cells of the L2 layer of stage 3 floral meristems [25] (Figure 3) (Table 3). Interestingly, in this analysis we could also observe a previously unnoticed low number of periclinal divisions in the L1 of sub-1 floral meristems. Furthermore, sub/scm mutants develop root hairs at discordant positions in the epidermis [26,27]. This defect in root hair patterning can be followed by expressing the bacterial β-glucuronidase gene under the control of the Arabidopsis GLABRA2 (GL2) promoter (GL2::GUS) [27,33] (Figure 3). This reporter conveniently labels the regular files of non-hair cells in the epidermis of wild-type roots and exhibits an irregular expression pattern in sub/scm mutants.

Upon examination, many aspects of the phenotypes of sub-1 and the other sub mutants were comparable. The doq-1, qky-8 and zet-2 mutants showed aberrant floral phyllotaxis and flowers with...
twisted petals (Figure 1). In addition, we observed premature floral bud opening in doq-1 and zet-2. Petals of zet-2 mutants showed notches similar to sub-1 petals but zet-2 floral organs were generally more misshapen. In rosettes, nearly all plants showed examples of leaf petiole twisting. In particular, qky-8 mutants showed leaf twisting comparable to sub-1, whereas doq-1 rosette leaves showed in addition elongated petioles and narrow blades. In contrast, zet-2 rosette leaves did not show major defects at the gross morphology level. Irregular twisting of siliques and stems was apparent in doq-1, qky-8 and zet-2 mutants, although the twisting in doq-1 siliques and stems was more subtle. Plant height was most affected in qky-8 while doq-1 and zet-2 showed only a slight reduction. Mature ovules of doq-1 mutants were mildly but consistently affected (Figure 2) (Table 2). The outer integument did not fully extend to the funiculus (reduced campylotropy). In addition, its distal cells were shorter and of irregular shape. Mature ovules of qky-8 and zet-2 closely resembled ovules of sub-1 mutants with respect to gaps and altered cell shape in the outer integument. Interestingly, zet-2 showed a slightly higher percentage of malformed outer integuments. As was the case for sub [25] the inner integument in all other slm single mutants appeared to be unaffected.

Further, doq-1, qky-9 and zet-2 mutants were investigated for cellular defects in the root epidermis and in L1/L2 cells of stage 2 to 4 floral meristems (Figure 3) (Table 3). In 4 day old main roots, all three mutants showed misregulation of GL2::GUS reporter expression comparable to sub-1, indicating that root hair patterning was similarly affected. The three mutants also exhibited cell shape and periclinal cell division plane defects in the L1 and L2 cells of floral meristems. Floral meristems of doq-1 showed a higher percentage of those defects while in zet-2, cell separation and disintegration could also be observed. The latter finding indicates that ZET is also required for cell viability.

Taken together the analysis of the slm single mutants revealed that there is a large functional overlap of the corresponding genes and that individual SLM genes participate in subsets of SUB-dependent processes, such as ovule development, cellular behavior of L2 cells of stage 3 floral meristems, and root hair patterning. The results also suggest, however, that SLM genes have additional functions unrelated to each other. For example, ZET has a particular function in cell survival in floral meristems as has DOQ in the regulation of leaf shape.

Double Mutant Analysis

To investigate further the genetic relationship between SLM genes we generated all possible double-mutant combinations and analysed the respective mutant phenotypes. The results are summarized in Figures 4 and 5 and Table 2.

**sub-1 doq-1**

Petals of sub-1 doq-1 double mutants mostly resembled doq-1 petals (Figure 4A), however, stem twisting was more similar to sub-1. Other aspects of the sub-1 doq-1 phenotype, such as silique twisting, plant dwarfism and rosette leaf petiole twisting were more exaggerated when compared to either single mutant. Ovules of sub-1 doq-1 plants showed an increase in outer integument defects, although not as strong as in some other double mutant combinations. In addition, about 11% of ovules of sub-1 doq-1 plants showed defects in inner integument morphology (Figure 5C) (Table 2), with gaps of variable sizes and finger-like protrusions. The fertility of sub-1 doq-1 plants was severely reduced (Figure 5R).

**sub-1 qky-8**

Petal and carpel twisting in sub-1 qky-8 double mutants was similar to that shown by each single mutant (Figure 4G). In contrast, twisting of siliques, stems, and leaf petioles was more pronounced as was the reduction in plant height (Figure 4I–L). In addition, ovule development was more heavily affected compared to the single mutants (Figure 5D–F) (Table 2), with 13% of ovules showing inner integument defects and a corresponding reduction in fertility (Figure 5R). About 5% of ovules were hardly recognizable as such, but rather resembled a mass of cells with integument-like outgrowths (Figure 5E).
Figure 3. Analysis of cellular defects in 4-day old main roots and stage 3 floral meristems of sub-1, doq-1, qky and zet-2 mutants. (A–E) Expression of the GL2::GUS reporter in whole-mount main roots. (A) Wild-type Ler root. The reporter is detected in regular files of non-hair cells. (B–E) GL2::GUS reporter expression is patchy. (F–K) Mid-optical longitudinal sections through stage 3 floral meristems obtained by confocal microscopy of propidium-iodide-stained specimen. (F) Wild-type Ler. Note the regular arrangement of cells in the L1 and L2. (G) sub-1. The arrow marks a periclinal cell division event. (H) doq-1. The region marked by the square (i) is shown at higher magnification in (Hi). The arrows highlight aberrant oblique and periclinal cell divisions in the L1 and L2, respectively. (I) qky-8. The region marked by the square (i) is shown at higher magnification in (Ii). The arrow labels a periclinal cell division in the L2. (J–K) zet-2. (J) The region marked by the square (i) is shown at higher magnification in (Ji). The arrows highlight periclinal cell divisions. A cell undergoing cell separation is indicated. (K) Disintegrating cells are marked. Abbreviations: cd, cell disintegration; cs, cell separation; L1, L1 cell layer; L2, L2 cell layer. Scale bars: (A–E) 25 μm, (F–K) 20 μm.

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Table 1. Summary of sub-like mutants identified.

| Mutant         | Gene Symbol | slm identifier | Chromosome | Boundary markers                      | Map interval (AGI)* |
|----------------|-------------|----------------|------------|--------------------------------------|---------------------|
| detorqueno-1   | DOQ         | slm8           | 1          | F1003(48ID)
|                |             |               |            | NF21M122                             | 0.75 Mb–3.21 Mb     |
| zerzaust-1     | ZET         | slm26          | 1          | CER453151(58ID)
|                |             |               |            | F13011(164ID)                        | 22.29 Mb–24.13 Mb   |
| zerzaust-2     |             | slm72          | 1          |                                      |                     |
| quirky-7       | QKY         | slm3           | 1          | 27.99(RsaI)
|                |             |               |            | F25A48(RgII)                         | 27.99 Mb–28.09 Mb   |
| quirky-8       |             | slm17          |            |                                      |                     |
| quirky-9       |             | slm38          |            |                                      |                     |

*Values represent approximate AGI positions of molecular markers listed.

1Developed within this study.

2Source: TAIR database and attributions therein.

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Overall, the sub-1 zet-2 double mutants showed the most disturbed morphology (Figure 4M–R). Perianth organs were twisted, narrower and notched, while carpels were heavily twisted. As a result the overall structure of the flower was irregular. The stem phenotype was knotted rather than twisted. Leaf morphology was misshapen with more pronounced leaf petiole twisting and plants showed prominent dwarfism. Consistent with this exaggerated phenotype ovules of sub-1 zet-2 plants showed severe defects with 21% of ovules resembling a mass of cells with integument-like
outgrowths (Figure 5H) (Table 2). About 18% of sub-1 zet-2 ovules exhibited short gapped outer integuments and similarly malformed inner integuments (Figure 5I). Again, fertility was reduced in sub-1 zet-2 plants (Figure 5R).

ddq-1 qky-8

Perianth organs and carpels in ddq-1 qky-8 mutants were more twisted as compared to the parental lines (Figure 4S). Twisting of leaf petioles, stems, and siliques was also more pronounced as was dwarfism. Ovules of ddq-1 qky-8 mutants showed a less exaggerated phenotype compared to some of the other double mutant combinations. Still, a large proportion of ddq-1 qky-8 ovules showed gaps in the outer integument (Figure 5J–L) (Table 2) and aberrant inner integument morphology was seen in 9% of ovules (Figure 5L). Fertility was strongly reduced in ddq-1 qky-8 double mutants (Figure 5R).

ddq-1 zet-2

Perianth morphology in ddq-1 zet-2 mutants was about equivalent to that observed in sub-1 zet-2 and ddq-1 qky-8 flowers (Figure 4Y). Twisting of stems appeared slightly more pronounced compared to each single mutant while siliques were drastically more twisted. Plant height was comparable to zet-2 single mutants. Overall a higher percentage of ddq-1 zet-2 ovules showed gaps in the outer and inner integuments (Figure 5M–O), with 4% of ovules exhibiting severe malformations comparable to sub-1 zet-2 mutants (Table 2). The reduction in fertility was comparable to that of ddq-1 qky-8 double mutants (Figure 5R).

qky-8 zet-2

The qky-8 zet-2 double mutants largely phenocopied zet-2 single mutants in above-ground morphology (Figure 4Ae–Aj) including ovules (Figure 5P,Q) (Table 2). One exception to this was leaf petiole twisting as this aspect was most similar to the phenotype in qky-8 single mutants.

In summary, the pleiotropic phenotypes of slm single and double mutants complicated the double mutant analysis. Although an exaggerated phenotype was often observed in a double mutant combination it was usually difficult to decide whether a double mutant displayed an additive or synergistic phenotype, or whether a particular mutation was epistatic to another. Overall, the double mutant analysis reinforced the notion that SLM genes likely do not act in a single linear pathway but have both overlapping and separate functions.

### Table 2. Ovule defects observed in sub and slm single and double mutants.

| Genotype         | Enclosed structure | <50% oi absent | ≥50% oi absent | Severe malformation | Defective ii |
|------------------|--------------------|----------------|----------------|---------------------|-------------|
| sub-1            | 60.4%              | 21.3%          | 16.8%          | 0%                  | 0%          |
| doq-1            | 96.8%              | 3.2%           | 0%             | 0%                  | 0%          |
| qky-8            | 69.3%              | 16.8%          | 13.9%          | 0%                  | 0%          |
| zet-2            | 52.4%              | 24.2%          | 23.4%          | 0%                  | <1%         |
| sub-1 doq-1      | 39.1%              | 45.3%          | 15.6%          | 0%                  | 10.9%       |
| sub-1 qky-8      | 41.6%              | 13.2%          | 40.3%          | 4.9%                | 12.5%       |
| sub-1 zet-2      | 26.8%              | 22.0%          | 28.6%          | 20.9%               | 17.6%       |
| doq-1 qky-8      | 39.8%              | 39.8%          | 20.4%          | 0%                  | 9.4%        |
| doq-1 zet-2      | 27.3%              | 44.7%          | 24.0%          | 4.0%                | 13.3%       |
| qky-8 zet-2      | 50.0%              | 35.6%          | 14.4%          | 0%                  | 1.9%        |

oi, outer integument; ii, inner integument.

| Mutant | L1   | L2    | CW sep. | Cell dis. | Samples (N) |
|--------|------|-------|---------|-----------|-------------|
| Ler    | 0%   | 2.6%  | 0%      | 0%        | 38          |
| sub-1  | 3.3% | 46.7% | 0%      | 0%        | 30          |
| doq-1  | 28.6%| 64.5% | 0%      | 0%        | 31          |
| qky-7  | 4.0% | 52.5% | 0%      | 0%        | 25          |
| zet-2  | 0%   | 43.7% | 24.2%   | 18.7%     | 64          |

1Cell wall separation.
2Cell disintegration.

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Phenotypic Analysis of doq, qky, and zet Plants Carrying a 35S::SUB Transgene

To further explore the genetic relationship between SLM genes we first tested whether SUB expression was responsive to other genes of this group. We investigated SUB expression in inflorescence apices and stage 10–12 flowers (see below) from several slm mutants by quantitative real time PCR (qRT-PCR). As can be seen in Figure 6A...
only very moderate changes in SUB expression were detected with a slight but consistent reduction of SUB expression in flowers of doq-1, qky-8 and zet-2 mutants. In contrast, SUB transcript levels were unaltered in those tissues and mutants when assessed by a transcriptome analysis (see below). Given that SUB is expressed at very low levels to begin with [34] we reasoned that the observed mild effects may not be the result of direct effects but rather be a consequence of indirect influences due to tissue sampling or the altered morphology of the mutants. To test the relevance of the effects we asked whether rendering SUB independent of its normal transcriptional regulation, by ectopically expressing SUB using the cauliflower mosaic virus 35S promoter, could result in phenotypic rescue of doq, qky and zet mutants. We analysed the phenotypes of 35S::SUB doq-1, 35S::SUB qky-8 and 35S::SUB zet-2 plants (Figure 6B). In particular we scored stem twisting, flower morphology and silique twisting of individual transgenic T1 lines. Transgene functionality was demonstrated by the wild-type appearance of 35S::SUB sub-1 plants. Despite demonstrating comparable transgene expression (Figure 6C), the other tested combinations did not show phenotypic rescue (35S::SUB doq-1: 42 T1 lines scored, 35S::SUB qky-8: >100, and 35S::SUB zet-2: >100), indicating that SUB does not act as a downstream target gene of DOQ, QKY or ZET. Taken together our

Figure 5. Comparison of ovule morphology in different slm double mutants. SEM micrographs of stage-4-V ovules are depicted. (A–C) sub-1 doq-1. (D–F) sub-1 qky-8. (G–I) sub-1 zet-2. (J–L) doq-1 qky-8. (M–O) doq-1 zet-2. (P, Q) qky-8 zet-2. Each double mutant shows a range of ovule phenotypes. (A, D, J, M and P) Mild phenotypes are depicted. Note the irregular size and shape of cells at the distal outer integument (arrow heads). (B, C, G, K, N and Q) Stronger ovule phenotypes are observed whereby specimens have half-formed outer integuments. (C, F, L, O) Inner integuments can also appear as finger-like protrusions. The qky-8 zet-2 double mutant is an exception, whereby inner integument defects are rarely observed. (E, H) Severely malformed ovules can also appear as integument-like and nucellus-like structures emerging from placental tissue. (R) Fertility was assayed as the average seed number per silique (n = 20). Abbreviations: fu, funiculus; ii, inner integument; mp, micropyle; oi, outer integument. Scale bars: 20 μm.
data suggest that SUB is not directly regulated at the transcriptional level by the other SLM genes.

**Genome-Wide Transcriptome Analysis and Global Expression Profiling**

To shed light on the molecular nature of SUB-dependent processes and to systematically investigate the similarities between the SLM genes at the mechanistic level, we applied a transcriptome analysis. Since mutants with defects in genes of overlapping functions should also exhibit overlapping alterations in transcript profiles we compared wild type (Ler) with plants of two sub alleles, as well as doq-1, zet-2 and qky-8 mutants using the Affymetrix ATH1 GeneChip platform (see Materials and Methods). The analysis of two independent sub alleles was aimed at obtaining a

**Figure 6. Analysis of SUB expression in the slm plant lines.** (A) Quantitative expression analysis of SUB in the slm mutants. Steady-state mRNA levels were measured in apex and flower tissues by quantitative real-time PCR. UBC21 expression was used for normalization. (B) Phenotypes of 35S:SUB slm transgenic lines. A 35S:SUB:myc construct was transformed into each slm mutant background. T1 plants were analysed for rescue of flower, silique and stem mutant phenotypes. Note only sub-1 mutants exhibit rescue by SUB overexpression. (C) Transgenic 35S:SUB:myc T1 plants represented in (A) were assayed for SUB transgene expression using semi-quantitative RT-PCR (top panels). GAPC [118] served as internal control (bottom panels). Scale bars: 0.5 mm.

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robust set of SUB-responsive genes for the different pairwise comparisons and the list of SUB-responsive genes represents the overlap of misexpressed genes in sub-1 and sub-3 mutants [25]. Since we had observed tissue-specific differences in the phenotypes of slm mutants, which suggested tissue-specific sub-functions for the corresponding genes, we tried to capture these differences also in the sampling for transcriptome analysis. To this end, we sampled inflorescence apices plus flowers up to stage 9 (apex data set), representing mostly proliferative tissues, while stage 10–12 flowers were collected to represent maturation stages (flower data set). The samples thus covered SUB-related aspects such as the control of cell shape/division plane in L2 cells of floral stage 3 meristems, and the regulation of petal, carpel and ovule development.

Since traditional analysis tools to identify differential gene expression in whole genome transcriptome data are not well suited for comparisons of multiple samples we developed a meta analysis tool based on Z-score statistics (see Materials and Methods). It offers a variety of benefits for the simultaneous analysis of multiple pairwise comparisons, such as normalization for the overall biological effect observed in the individual experiments, increased sensitivity, and the possibility of querying data using Boolean logic. Using this tool we identified 89 and 193 significantly misexpressed genes, respectively, in the apex and flower samples of SUB plants (Table 4). The other mutants were characterised by higher numbers of misexpressed genes (Table 4) indicating that DOQ, QKY, and ZET affect more processes than SUB. More importantly, we systematically analyzed the overlap of misexpressed genes from individual slm mutants compared to wild type. The results of all pairwise comparisons are given in Table 5. For example, a pairwise comparison between sub and daoq-1 in the apex sample revealed a 42 gene overlap. This corresponded to 47% of genes misexpressed in sub and about 17% of genes aberrantly expressed in daoq-1. Comparable values were observed for sub versus gky-8 and sub versus zet-2 comparisons. The overlap with SUB-dependent genes was even more pronounced in the flower data set and in the sub-daoq-1 comparison we found 118 common genes, representing 61% and 17% of the genes misexpressed in sub and daoq-1, respectively. The sub gky-8 and sub zet-2 comparisons revealed higher values with 76% and 81% of SUB-responsive genes being found to overlap. Thus, a significant number of SUB-responsive genes are also sensitive to DOQ, QKY, and ZET function. Similar pairwise comparisons were made between daoq-1, gky-8 and zet-2 (Table 6).

Again there was considerable overlap of misexpressed genes between different mutants. Most strikingly, in stage 10–12 flowers 67% and 71% of the QKY-responsive genes are also misregulated in daoq-1 and zet-2, respectively. This finding implies that the activity of more than two-thirds of QKY-responsive genes also depends on DOQ and ZET function in stage 10–12 flowers. Taken together the microarray data corroborate the morphological analysis of the single mutants and are compatible with the notion that the different SLM genes share overlapping functions.

To further study the complexity of the functional relationship among SLM genes we carried out additional pairwise comparisons, examined the identity of genes present in the overlap lists, and identified transcripts which are misregulated in all four mutants (i.e., a sub, daoq-1, gky-8 and zet-2 “quadruple” overlay) (Figures 7B, Tables 5,6; Datasets S1, S2). While doing so we uncovered striking trends in the transcriptome data. For example, we found that more than 30% of the misregulated genes found in any sub/slmp over/underlapping the identified in the apex samples are indeed shared by all mutants (Figure 7A). More significantly, 13 of the 14 transcripts identified as common SLM responsive in the apex were consistently elevated in expression in all mutants, suggesting that a core function of the SLM genes is to repress this set of genes in proliferating tissue (Figure 7B). Strikingly, this trend was reversed in differentiating tissue represented by the floral samples. Here the misregulated genes shared by all mutants made up more than 65% of transcripts identified in pairwise comparisons (Figure 7A) and 88 of the 93 genes were reduced in expression (Figure 7B). In addition to supporting the notion of functional overlap between the SLM genes, this analysis also demonstrated that diversification of SLM function observed in different tissues is based on a dramatic switch in the underlying molecular mechanism. While signaling through SLM proteins in proliferating tissue mainly causes repression of target gene transcription, SLM-mediated signals lead to a transcriptional activation of downstream genes in differentiating cells, suggesting that SUB and other SLM proteins interact with a radically different regulatory environment. This notion is supported by another striking observation: while we found strong similarities between different genotypes within one tissue we observed very little overlap in misregulated genes between the same genotype in the two tissues analysed (Figure 8). For example, only four misregulated transcripts were shared in apex and flower samples of sub mutants. Similar observations were made for the other single and pairwise comparisons. In addition, the core target gene sets of apex and flower, derived from quadruple comparisons, did not share any transcripts.

To investigate the nature of SLM-dependent processes we assessed the known or predicted functions of the apex and flower core sets of SLM-responsive genes by searching The Arabidopsis Information Resource (TAIR), the literature and making use of the AtGenExpress expression atlas [34] with the help of the AtGenExpress Visualisation Tool (AVT, http://jsp.weigelworld.org/expviz/) (Tables 7, 8). In both core sets we observed a strong representation of genes that are involved in processes, such as cell wall biosynthesis and function, hormone signaling and abiotic and biotic stress responses. For example, two genes in the apex core set encode the inositol oxygenase family enzymes MIOX2 and MIOX4 required for biosynthesis of uridine-diphospho-glucuronic acid (UDP-GkA), a precursor for various cell-wall matrix polysaccharides [35]. In addition, GERMIN-LIKE PROTEIN 6 (GLP6) is a member of a gene family encoding putative extracellular GERMIN-LIKE proteins [36] of which play a role in biotic stress responses and cell wall biology. The flower core set of SLM-responsive genes also included several genes coding for cell wall proteins, such as EGSI [37], At2g05340 (Glycine-rich protein), At5g03350 and At5g18470 (lectin family proteins), and At2g43370 and At4g01700 (chitinases). In another example that relates to hormone signaling, five of the 13 upregulated apex core set genes were inducible by jasmonates (JA) while the flower core set was characterized by a large group of genes that relate to different aspects of signaling mediated by salicylic acid (SA) (see Discussion). SLM-responsive genes whose

| Table 4. Genes misexpressed in sub and slm mutants. |
| Mutant profiles (Apex) | Genes<sup>1</sup> | Mutant profiles (Flower) | Genes<sup>2</sup> |
|---|---|---|---|
| sub | 89 | sub | 193 |
| daoq-1 | 255 | daoq-1 | 698 |
| gky-8 | 158 | gky-8 | 352 |
| zet-2 | 330 | zet-2 | 497 |

<sup>1</sup>Genes misexpressed in apex tissues sampled (up to flower stage 9).
<sup>2</sup>Genes misexpressed in flowers (stages 10–12).
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expression is sensitive to JA encoded for example JAZ1, a central negative regulator of JA signaling [38,39], ERD5 a mitochondrial proline dehydrogenase [40], AtLEA5/SAG21 [41,42], and AtTTPG, a class III trehalose-6-phosphate phosphatase involved in the biosynthesis of trehalose [43,44]. All of these genes are believed to contribute to the plant’s response to dehydration, cold and oxidative stress [40,41,44–50]. We also observed SLMDependency of genes involved in the homeostasis of the auxin indole-3-acetic acid (IAA) and the production of the Arabidopsis phytoalexin camalexin and indole glucosinolates. Glucosinolate metabolites play important roles in plant nutrition, growth regulation and the interactions of plants with pathogens and insect herbivores [51,52]. In the apex core set we identified IAA-LEUCINE RESISTANT-LIKE GENE 6 (ILL6), which belongs to a gene family encoding IAA amino acid conjugate hydrolases involved in the release of the active auxin from corresponding inactive amino acid conjugates [53,54]. CYP83B1 and CYP79B2 encode cytochrome P450 monooxygenases that regulate the pool of indole-3-acetaldoxime (IAOx) [55–58] a key branching point of primary and secondary metabolic pathways and a precursor of indole glucosinolates, camalexin [59], and IAA (reviewed in [51,52]). In addition, the flower core set contained two downregulated cytochrome P450 genes, CYP71A15 and CYP71B15, which promote distinct steps in camalexin biosynthesis [60–63].

Molecular Identification of QKY

As a first step to molecularly define the SLM pathway, the QKY gene was identified by map-based cloning (Figure 9 and Materials and Methods). In all three EMS-induced qky alleles we could identify single point mutations in At1g74720 (Figure 9A). Further, two distinct T-DNA insertions in this locus result in inactive amino acid conjugates [53,54].

| Apex Overlap profiles | Genes | % sub<sup>2</sup> | % slm<sup>3</sup> | Flower Overlap profiles | Genes | % sub<sup>2</sup> | % slm<sup>3</sup> |
|-----------------------|-------|----------------|----------------|-------------------------|-------|----------------|----------------|
| sub_ dog-1            | 42    | 47.2%          | 16.5%          | sub_ dog-1              | 118   | 61.1%          | 16.9%          |
| sub_ qky-8            | 40    | 44.9%          | 25.3%          | sub_ qky-8              | 147   | 76.2%          | 41%            |
| sub_ zet-2            | 48    | 53.9%          | 14.5%          | sub_ zet-2              | 156   | 80.8%          | 31.4%          |

1Denotes a comparison (overlap profile) made between two mutant transcriptome profiles.
2Percentage of sub misexpressed genes.
3Percentage of slm misexpressed genes represented in listed comparisons.

Percentages are calculated based on mutant gene list totals (Table 4).

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Table 6. Comparisons of genes co-misexpressed in slm mutants.
of the number of C2 domains still present in the predicted truncated proteins. In summary, the results suggest that QKY is a membrane-bound Ca\textsuperscript{2+}-signaling factor.

**Discussion**

**DOQ, QKY, and ZET Contribute to SUB-Dependent Organ Development**

In this work we set out to genetically identify additional components of the SUB-dependent mechanism regulating ovule development and floral morphogenesis. To this end we identified second-site mutations that resulted in a sub-like phenotype, reasoning that such mutations should reside in genes that either act directly in the SUB signaling pathway, or, that SUB and these genes affect parallel pathways that converge on a common molecular or developmental target. A defined set of criteria was applied in the identification of candidate mutants. At the developmental level, candidates were chosen that showed a sub-like ovule phenotype and twisting of petals, carpels, siliques and the stem. In addition, they were analysed for two types of sub-like cellular defects: an increased frequency of periclinal cell divisions in the L1/L2 layers of stage 3 floral meristems and a defect in root hair patterning. Mutations in three genes, DOQ, QKY, and ZET, satisfied all criteria and were considered as sub-like mutants.

Although slm mutants were phenotypically very similar there were some noteworthy distinctions. For example, doq-1 was generally characterised by a weak ovule phenotype and mild silique/stem twisting. At the same time, doq-1 showed the highest frequency of periclinal cell divisions in the L1/L2 layers of stage 3 floral meristems. Furthermore, rosette leaves of doq-1 plants had elongated petioles and narrow leaf blades, features not observed in other slm mutants. These findings indicate that DOQ is less important for ovule, silique and stem development compared to other SLM genes, but plays a more prominent role in the regulation of cell shape/division plane in the young floral meristem and leaf development. In contrast ZET does not seem to be involved in leaf development as zet mutants exhibited apparently normal leaves. This clearly distinguishes ZET from the other SLM genes, which all seem to be important for leaf development. Plants with a defect in ZET activity also showed more defects in perianth development when compared to other slm mutants. Cell separation and disintegration observed in stage 3 floral meristems of zet mutants indicated that ZET may function in cell adhesion and viability, roles that seem to be unique among SLM genes.

Since the phenotypes of sub and the other slm mutants overlap we assessed the genetic relationship among SLM genes by testing whether the expression of SUB is sensitive to SLM activity. This was apparently not the case, at least not in a relevant manner, since SUB expression was unaltered in the respective other slm mutants in our transcriptome analysis and showed only minor downregulation in slm flowers when assayed by qRT-PCR. In addition, a functional 35S:SUB transgene was unable to rescue the phenotype of doq, qky and zet mutants, including the floral defects. To further investigate the genetic interactions between SLM genes we performed a comprehensive double mutant analysis. The
pleiotropic and variable phenotypes of slm single and double mutants complicated this task. Although an exaggerated phenotype was often observed in double mutants it was sometimes difficult to decide whether this represented an additive or synergistic phenotype. In addition, the various effects were often tissue dependent and thus precluded conclusions at the whole-plant level. One exception was qky-8 zet-2 where zet-2 appears to be largely epistatic to qky-8. This result indicates that ZET either acts upstream of QKY in a genetic pathway, or that ZET generally acts prior to QKY. Focusing on ovule development simplified our analysis, since a stringent set of morphological criteria could be applied. In sub-1 zet-2, sub-1 qky-8, and doq-1 zet-2 double mutants a mass of cells with integument-like structures often developed in place of true ovules. Furthermore, all double mutants, with the exception of qky-8 zet-2, often showed aberrant inner integuments, in contrast to slm single mutants that only displayed defects in the outer integument. Taken together, these synergistic effects indicate that the SLM genes contribute to similar aspects of ovule development and show that QKY and ZET function is still present in ovules of sub plants. In addition, the analysis supports the notion that DOQ may play a more peripheral role in ovule development in comparison to SUB, QKY, and ZET. The exact genetic relationship between SLM genes during this process remains to be elucidated.

The phenotypic analysis of slm single mutants suggested that SLM genes variably contribute to several common functions but it also showed that they have distinct roles, both of which depend on tissue context. To characterise the molecular mechanisms that are under control of SLM genes and to narrow down the overlap of SLM gene function, we used transcript profiling of mRNA isolated from two different tissues. Our Z-score based meta-analysis revealed 89 and 193 largely non-overlapping SUB-responsive genes in the apex and flower data sets, respectively (Table 4). That we consistently observed larger numbers of affected transcripts in the flower data set might reflect the relatively higher importance of SLM genes for later stages of flower development, but it could also be explained by differences in the nature of the samples. While the flower sample only contained tissue from two well-defined floral stages, the apex sample contained the shoot apical meristem as well as flowers from stage 1 to stage 9 and thus was much more diverse. Consequently, the sensitivity of the array to detect changes that are limited to only a few developmental stages was reduced.
When comparing the number of misregulated transcripts across the various genotypes, we found that fewer genes were affected in sub mutants than in the other slm mutants. This observation suggests that DOQ, QKY and ZET play broader roles than SUB. The result might have been expected for ZET, as zet mutants show the most severely affected flowers of all slm mutants, while sub, qky and dog mutant flowers, however, are more alike and thus their morphology is less congruent with a more dramatic change at the molecular level. Meta-analysis revealed a consistent overlap in misregulated genes between sub and the other slm mutants within a given tissue and even allowed us to identify a set of core targets shared by all four SLM genes. Interestingly, these transcripts were consistently up- or down-regulated in all genotypes, while the direction of the change was dependent on tissue context. These results demonstrate not only that SLM genes affect common processes with a high tissue specificity, but also that the molecular mechanisms employed by SLM genes in the different tissues are distinct. In addition, the identification of common targets that show consistent behaviour across all genotypes underlines the sensitivity and specificity of our meta-analysis.

### QKY Encodes a Putative Transmembrane Protein Involved in Ca^{2+}-Mediated Signaling

Sequence analysis of QKY suggests that QKY is the first described plant representative of the previously described MCTP family [65] and that it might act as a membrane-bound protein involved in a process regulated by Ca^{2+} and phospholipids. While the function of animal MCTPs is unknown, human MCTP2 is a membrane protein located to intracellular vesicular structures [65]. Interestingly, the C2 domains of human MCTPs were found to bind Ca^{2+} with high affinity but lacked any phospholipid binding capacity [65]. Other membrane-bound proteins with multiple C2 domains are the synaptotagmins and ferlins [70,72]. Synaptotagmins contain two C2 domains and an amino-terminal transmembrane domain while most ferlins carry between four and six C2 domains and a carboxy-terminal transmembrane domain. Members of these two protein families function during regulated exocytosis, a process in which specific vesicles are signaled to fuse with the plasma membrane. Processes that rely on regulated exocytosis include neurotransmitter release at synapses and plasma membrane repair, a basic cellular process that mends physical injuries inflicted upon the plasma membrane [73–75]. During the repair process lysosomes [76] or specialised vesicles, such as the enlargosomes [77,78], fuse with the plasma membrane providing new membrane material and facilitating resealing.

The synaptotagmins Sys 1 and 2 are required for Ca^{2+}-regulated synaptic vesicle exocytosis during neurotransmitter release into the synaptic cleft [79,80,70] while Syt VII promotes lysosomal exocytosis during plasma membrane repair in fibroblasts [76]. The C. elegans ferlin FER-1 is localized to the membranes of membranous organelles (MOs) and promotes the fusion of MOs with the plasma membrane during the development of crawling spermatozoa [81,82]. Mutations in dysferlin result in progressive muscular dystrophies [83–85] and dysferlin appears to be required for Ca^{2+}-dependent sarcolemma resealing during membrane repair in skeletal muscle fibres [84,86].

Given the predicted domain topology of QKY one could speculate that QKY, and possibly other SLM genes, could participate in the control of vesicle trafficking. In principle, the predicted membrane localisation of QKY also allows for the possibility that the SUB and QKY proteins interact directly. A role of QKY in vesicle trafficking would help to explain the non-cell-autonomy of SUB function in inter-cell-layer signaling [28]. Thus, in one possible scenario SUB could directly or indirectly regulate

| Gene/Keyword | Gene Identifier | Description |
|--------------|----------------|-------------|
| **Genes upregulated** |
| MIOX2 | At2g19800 | Myo-inositol oxygenase family gene / biosynthesis of nucleotide sugar precursors for cell-wall matrix |
| MIOX4 | At4g26260 | Myo-inositol oxygenase family gene / biosynthesis of nucleotide sugar precursors for cell-wall matrix |
| GLP6 | At5g39100 | Germin-like family protein / cell wall biology, pathogen response, nutrient reservoir |
| JAZ1 | At1g19180 | JASMONATE-ZIM-DOMAIN PROTEIN 1 / nuclear-localized protein negatively regulating jasmonate signaling, inducible by JA |
| ERF5 | At3g30775 | EARLY RESPONSIVE TO DEHYDRATION 5. Proline dehydrogenase / osmotic stress responsive, inducible by JA |
| AT3IP2 | At3g57520 | ARABIDOPSIS THALIANA SEED IMBIBITION 2 / glycosyl hydrolase, catalysis of raffinose family oligosaccharides, inducible by JA |
| AtLEA5 | At4g02380 | LATE embryogenesis abundant-like protein 5 / response to cold, oxidative stress, reactive oxygen species, inducible by JA |
| AtTTPG | At4g22590 | Trehalose-6-phosphate phosphatase / trehalose biosynthetic process, sugar-based signaling, inducible by JA |
| IL6 | At1g44350 | AA-LEUCINE RESISTANT (ILR)-LIKE GENES 6 / IAA-α-amino acid hydrolase |
| CYP79B2 | At1g39950 | Cytochrome P450 family/tryptophan metabolism / converts Trp to indo-3-acetaldoxime (IAOx), a precursor to IAA, camalexin and indole glucosinolates |
| CYP83B1 | At1g31500 | Cytochrome P450 monoxygenase / biosynthetic pathway of indole glucosinolates |
| Nodulin MtN21-like | At2g39510 | Nodulin MtN21 family protein / function unknown. |
| Unknown | At1g74055 | Unknown protein |
| **Genes downregulated** |
| AtDof5.8 | At5g66940 | Dof-type zinc finger domain-containing protein / putative transcription factor, possibly involved in vascular development |

1 Gene descriptions were derived from TAIR and attributions therein. doi:10.1371/journal.pgen.1000355.t007

**Table 7.** List of genes co-misexpressed in slm mutant apex tissues.
Table 8. List of genes co-misexpressed in *slm* mutant flowers.

| Gene/Keyword | Gene Identifier | Description |
|--------------|-----------------|-------------|
| **Genes upregulated** | | |
| ATHB40 | At4g36740 | Homeodomain leucine zipper class I (HD-Zip I) protein/ transcription factor |
| GLTP3 | At3g21260 | GLYCOLIPID TRANSFER PROTEIN 3 |
| Glycine-rich | At2g05540 | Glycine-rich protein |
| Lactoylglutathione lyase | At1g15380 | Lactoylglutathione lyase family protein |
| SUS3 | At4g02280 | sucrose synthase 3/putative sucrose synthase activity |
| **Genes downregulated** | | |
| AGP5 | At1g35230 | Arabinogalactan-protein 5 |
| AIG1 | At1g33960 | AVRRPT2-INDUCED GENE 1/ exhibits RPS2- and avrRpt2-dependent induction early after bacterial infection |
| ANAC036 | At2g17040 | Arabidopsis NAC domain containing protein 36/ transcription factor |
| ATARD3 | At2g26400 | acireductone dioxygenase (ARD/ARD')family protein 3, putative |
| ATGH9B17 | At4g39000 | ARABIDOPSIS THALIANA GLYCOSYL HYDROLASE 9B17/ O-glycosyl hydrolase activity |
| ATNUDT6 | At2g04450 | ARABIDOPSIS THALIANA NUDIX HYDROLASE HOMOLOG 6/ DNA metabolism, putative pathogen response protein |
| COBL5 | At5g59500 | COBRA-LIKE PROTEIN 5 Precursor/ phytochelatin synthetase, putative |
| CYP71A13 | At2g30770 | CYTOCHROME P450 MONOOXYGENASE 71A13/IAA and camalexin biosynthetic process, pathogen defence response |
| CYP71B15S | At3g26830 | CYTOCHROME P450 71B15S family protein |
| CYP96A2 | At4g32170 | CYTOCHROME P450 96A2 |
| ECS1 | At1g31580 | ECOTYPE SPECIFIC 1/ plant cell wall-associated protein. |
| EDS1 | At3g49090 | ENHANCED DISEASE SUSCEPTIBILITY 1 / SA-mediated signaling, defense against pathogens |
| EDS5/SID1 | At4g39030 | ENHANCED DISEASE SUSCEPTIBILITY 5 / MATE-transporter family protein / SA biosynthesis, defense against pathogens |
| EXP3 | At2g18660 | Expansin family protein |
| ABA | At5g13200 | GRAM domain-containing protein/ABA-responsive protein-related, function unknown |
| Ankyrin | At5g54610 | Ankyrin repeat family protein |
| Auxin-response | At5g35735 | Auxin-responsive family protein |
| Calcium-ion binding | At3g47480 | Calcium-binding EF hand family protein |
| Calcium-ion binding | At5g39670 | Calcium-binding EF hand family protein |
| Calcium-ion binding | At3g45770 | Calcium-binding EF hand family protein |
| Chitinase | At2g43570 | Chitinase protein, putative |
| Chitinase | At4g01700 | Chitinase protein, putative |
| Chitinase | At2g43570 | Chitinase protein, putative |
| DELTOL-PP | At5g3872 | Dehydrodolichyl diphosphate synthase (DEDOL-PP), putative |
| DELTA-VPE | At3g20210 | Delta vacuolar processing enzyme/seed coat development |
| Disease resistance | At1g72900 | Disease resistance protein (TIR-NBS class), putative |
| Disease resistance | At1g72920 | Disease resistance protein (TIR-NBS class), putative |
| Glucosidase | At3g03640 | Beta-glucosidase |
| GRX480 | At1g28480 | Glutaredoxin family protein 5/ regulates protein redox state, JA-mediated signaling pathway, SA-mediated signaling pathway |
| HIN1-related | At1g65690 | Harpin-induced protein-related/HIN1-related protein |
| ICS1/SID2/EDS16 | At1g74710 | isochorismate synthase 1/ salicylic acid biosynthetic process |
| Lectin family | At5g03350 | Legume lectin family protein, putative |
| Lectin family | At5g18470 | Curculin-like (mannose-binding) lectin family protein |
| Lipase | At5g50505 | GDSL-motif lipase/hydrolase family protein |
| Lipid transfer protein | At3g26000 | Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein |
| Lipid transfer protein | At5g54500 | Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein |
| LRR protein | At2g24160 | Leucine rich repeat protein family, putative disease resistance protein |
| Methionine metabolism | At4g21850 | Methionine sulfoxide reductase domain-containing protein |
| MIF protein | At3g51660 | Macrophage migration inhibitory factor (MIF) family protein |
| Gene/Keyword | Gene Identifier | Description |
|--------------|-----------------|-------------|
| NIMIN1       | At1g02450       | Transcriptional regulator/ negative regulator of SA-dependent signal transduction, modulates PR gene expression |
| NRAMP5       | At4g18379       | NRAMP METAL ION TRANSPORTER 5 |
| Oxidoreductase| At5g24530       | ZOG-Fe(II) oxygenase family protein |
| PBP1         | At5g54490       | PINOIID-BINDING PROTEIN 1/ responsive to auxin stimulus |
| PBS3/GDG1    | At5g13320       | AVRPPHB SUSCEPTIBLE 3/ Required for accumulation of salicylic acid, activation of defense responses and resistance |
| PCC1         | At3g22231       | PATHOGEN AND CIRCADIAN CONTROLLED 1/regulated by the circadian clock, responsive to pathogens |
| Plastocyanin domain | At4g30590 | Plastocyanin-like domain-containing protein |
| PR1          | At2g14610       | PATHOGENESIS-RELATED GENE 1/ pathogen defense response, SAR, response to vitamin B1 |
| PR2          | At3g57260       | PATHOGENESIS-RELATED GENE 2/ glycosyl hydrolase family 17 protein, cold responsive, SAR |
| PR4          | At3g04720       | PATHOGENESIS-RELATED GENE 4/ hevein-like protein precursor, responsive to virus and ethylene stimulus, SAR |
| PR5          | At1g75040       | PATHOGENESIS-RELATED GENE 5, thaumatin-like protein/ SAR, response to UV-B, regulation of anthocyanin biosynthetic process |
| Protease     | At3g51330       | Aspartyl protease family protein |
| Protein binding | At1g10340 | Ankyrin repeat family protein |
| Protein binding | At4g13435 | Zinc finger (C3HC4-type RING finger) family protein/ankyrin repeat family protein |
| Protein kinase | At4g1890 | Protein kinase family protein |
| Protein kinase | At4g23150 | Protein kinase family protein |
| Receptor     | At2g37710       | Receptor lectin kinase/ responsive to SA stimulus |
| RLK1         | At5g60900       | RECEPTOR-LIKE PROTEIN KINASE 1 |
| RLK6         | At4g23130       | RECEPTOR-LIKE PROTEIN KINASE 6 |
| SAL2         | At5g64000       | 3’(2’),5’-bisphosphate nucleotidase |
| Self-incompatibility | At1g26795 | Self-incompatibility protein-related |
| SUB1         | At3g56710       | SIGMA FACTOR BINDING PROTEIN 1 |
| TAT3         | At2g24850       | TYROSINE AMINOTRANSFERASE 3/ responsive to wounding and JA treatment |
| Transporter  | At3g10780       | emp24/gp25L/p24 family protein/ putative transporter protein |
| UBC31        | At1g36340       | UBIQUITIN-CONJUGATING ENZYME 31 |
| WAK1         | At1g21250       | WALL ASSOCIATED KINASE 1/ response to salicylic acid stimulus |
| WAKL8        | At1g16260       | WALL ASSOCIATED KINASE-LIKE 8 |
| WRKY33       | At2g38470       | WRKY-type DNA binding protein/ defence response to bacterium and fungus |
| WRKY46       | At2g46400       | WRKY-type DNA binding protein |
| WRKY70       | At3g56400       | WRKY-type DNA binding protein/ transcriptional repressor, JA mediated signaling pathway, SA mediated signaling pathway, SAR |
| WRKY53       | At4g23810       | WRKY-type DNA binding protein/ positive regulation of transcription, regulation of defense response |
| WRKY38       | At5g25750       | WRKY-type DNA binding protein/ defence response, SAR |
| ZAT10        | At1g27730       | Salt tolerant zinc finger/ transcriptional repressor activity, cold, water deprivation, salt stress, wounding, ABA and chitin responsive |

1 Gene keywords and gene identifiers are shown.
2 Gene descriptions were derived from TAIR and attributions therein.
ABA, abscisic acid; DR, disease resistance; JA, jasmonic acid; PR, pathogenesis-related; SA, salicylic acid; SAR, systemic acquired resistance. doi:10.1371/journal.pgen.1000355.t008
Figure 9. Molecular characterization of QKY. (A) Molecular organization of the QKY locus on chromosome 1. The horizontal black line highlights genomic DNA. The red arrow indicates the orientation and extent of the QKY open reading frame. The small black boxes mark the 5’ and 3’ untranslated regions, respectively. The orange arrow and box denote the end of the preceding and the start of the next annotated open reading frames, respectively. The positions of the various point mutations or T-DNA insertion sites are marked. (B) Predicted domain topology of the QKY protein. The sequence and numbering of the C2 domains, and the altered C-termini of the mutant protein variants, are given. The italic capital letters represent the de novo residues present in the predicted mutant QKY proteins from the two T-DNA lines. Stars mark the premature stops in the predicted mutant proteins. The green box denotes the phosphoribosyltransferase C-terminal region (PRT_C). Abbreviations: aa, amino acids; N, amino terminus; C, carboxy terminus; TM, transmembrane domain.

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QKY which in turn might affect vesicular transport of factors mediating the non-cell-autonomy of SUB-dependent signaling. Further work needs to address this exciting hypothesis.

Many SLM-Responsive Genes Relate to Cell Wall Biology, Hormone, and Stress Responses

To obtain additional insights concerning the molecular processes influenced by SLM function we investigated the known and predicted functions of SLM-responsive genes. The core sets of direct or indirect SLM targets included many genes encoding proteins relating to cell wall biology and a notable enrichment of genes that are inducible by hormones, such as JA and SA, and presumed to play a role in the plant’s response to various stresses. For example, a group of genes misregulated in slm mutants encode proteins with a known or predicted role in the biosynthesis and/or function of the cell wall, such as MIOX2/4, GLPs6, and putative glycine-rich proteins, lectin family proteins, and chitinases. Defects in SLM-activity also resulted in a deregulation of the basal expression of JA-inducible genes with a presumed role in abiotic stress responses to cold or dehydration. The altered expression of genes responsible for camalexin and glucosinolate metabolism, secondary metabolites involved in the defense against pathogens, indicates that SLM-activity appears to play a role in biotic stress responses as well.

The notion that SLM activity somehow relates to stress is reinforced by the interesting finding that a major group of SLM-responsive genes in flowers relate to various aspects of salicylic acid (SA)-dependent signaling. SA is synthesized upon exposure of plants to abiotic stresses, such as ozone or UV-C light, and plays an important role in pathogen defense (for reviews see for example [87–91]). Three of the SLM-responsive genes are known to be required for the pathogen-induced production of SA: RCSI/SID2/EDS16 encodes an isochorismate synthase that synthesizes SA [92,93], EDS5/SID1 encodes a MATE family transporter potentially involved in the transport of SA intermediates [94], and PBS3/GDG1 encodes an adenylate-forming enzyme required for SA accumulation [95,96]. Other SLM-dependent genes are involved in further aspects of SA signaling. EDS1 encodes a protein with a lipase-like domain [97], contributes to rapid SA accumulation in response to many SA-inducing stimuli, and is part of a central regulatory node of SA signaling. NPR1/NIM1 constitutes another, though SLM-independent, central regulator of SA signaling. At low SA levels NPR1 is present in the cytoplasm as a homo-multimeric complex. Increased SA levels result in the dissociation of NPR1 oligomers into monomers, which enter the nucleus where they interact with specific TGA transcription factors. These NPR1-TGA complexes regulate expression of defense genes, such as PATHOGENESIS-RELATED (PR) genes and WRKY-type transcription factor genes. Interestingly, we found NIMIN1 and GRX480, both of which act in the NPR1 pathway, to be downregulated in slm mutants. NIMIN1 encodes an interactor of NPR1 that counteracts NPR1 activity [98]. GRX480 codes for a glutaredoxin that interacts with TGA2 [99] and is possibly involved in cross-talk between SA and JA signaling. Another important class of regulators implied in plant immunity, the regulation of PR genes and SA-mediated signaling are WRKY transcription factors. Interestingly, we found five WRKY genes to be downregulated in slm mutants and three of them are known direct targets of NPR1: WRKY33, WRKY53, and WRKY70 [100]. WRKY33 is a positive regulator of systemic acquired resistance (SAR) [100] while WRKY70 is involved in the cross-talk between SA and JA responses by acting as a negative regulator of JA-inducible genes [101,102]. In addition, WRKY70 is required for resistance against several bacteria, fungi and an oomycete [100,101,103,104]. Another SLM-responsive WRKY gene, WRKY33, is a positive regulator of resistance against the necrotrophic fungi Botrytis cinerea and Alternaria brassicicola [105]. Fitting in the overall picture was the observation that expression of five PR genes was reduced in slm mutants, including PR1, PR2, and PR5 whose activities often undergo coordinated changes in response to various stimuli including SA [106]. In addition, PR1 is likely a direct target of a NPR1/TGA complex [107,108].

Many of the SLM-responsive genes involved in SA-signaling and pathogen defense are normally transcribed at basal levels while their expression is induced several fold in the case of pathogen
attack and increased levels of SA. Our transcriptome analysis of slm mutants suggests that SLM function is required for basal steady-state expression of SA-responsive genes. Thus, SLM function could be involved in priming components of the cellular defense machinery, thereby enabling floral cells to respond faster to invading pathogens. In this scenario SLM genes would contribute to basal resistance.

Another scenario is based on the hypothesis that QKY may play a role in membrane traffic. Since membrane repair or cell wall synthesis require extensive vesicle trafficking [74,109] SLM-dependent processes could be involved in the regulation of the composition of the plasma membrane or the cell wall and alterations in the two cellular compartments of slm mutants could be interpreted as wounding stress. For example, it was suggested that the SLM- and SA-responsive WALL-ASSOCIATED RECEPTOR KINASE1 (WAK1) gene transmits changes in the cell wall to the interior of the cell. In addition, WAK1 protects plant cells against negative aspects of the pathogen response [110]. The RLK THESEUS1 (THE1) has been proposed to act as a sensor of cell wall integrity [111]. It was shown that in the absence of proper cellulose synthesis THE1 modulates the activity of genes affecting pathogen defense, cell wall cross-linking and stress responses. Although THE1 and the SLM genes do not share common transcriptional targets, they affect a functionally related spectrum of genes. Thus, it is conceivable that one aspect of SLM function relates to the surveillance of cell wall integrity or the repair of plasma membranes.

The two proposed models are not mutually exclusive and it will be an exciting challenge to further dissect the biological function of SLM genes in development, cell biology, and stress response.

Materials and Methods

Plant Work and Genetics

Arabidopsis thaliana (L.) Heynh. var. Columbia (Col-0) and var. Landsberg (erecta mutant) (Lei) where used as wild-type strains. The sub-1 mutant was described previously [25]. Plants were grown in a greenhouse under Philips SON-T Plus 400 Watt fluorescent bulbs on a long day cycle (16 hrs light). Dry seeds were sown on soil (Patzer Einheitserde, extra-gesteift, Typ T, Patzer GmbH & Co. KG, Sinntal-Jossa, Germany) overlying perlite, stratified for 4 days at 4°C and then placed in the greenhouse. Plant trays were covered for 7–8 days to increase humidity and support equal germination. Lei seeds mutagenized with ethylmethane sulfonate (EMS) were obtained from Lehle Seeds (Round Rock, TX, USA). 60'000 M2 plants, corresponding to about 7'500 M1 plants, were screened for plants exhibiting a sub-like phenotype. All sub-like mutants described in this paper were outcrossed three times to Lei prior to further analysis. Two qky T-DNA insertion mutants (line SALK_140123 and SALK_043901) [112] were obtained from the ABRC (http://www.arabidopsis.org). The GL2::GUS line [33] in Lei was crossed into slm mutants for analysis of root hair specification.

Recombinant DNA Work

For DNA and RNA work standard molecular biology techniques were used [113]. PCR-fragments used for cloning were obtained using either PhiUltra high-fidelity DNA polymerase (Stratagene) or TaKaRa PrimeSTAR HS DNA polymerase (Lonza, Basel, Switzerland). All PCR-based constructs were sequenced. Information regarding all primers used in this study is given in Supporting Table S1.

Generation of 35S::SUB plants

Transformation of sub-1, dog-1, qky-8 and zet-2 mutants with the 35S::SUB construct was performed using the floral dip method [116] and Agrobacterium tumefaciens strain GV3101 [117]. Transgenic T1 plants were selected on 50 µg/ml Kanamycin plates and subsequently transferred to soil for phenotypic inspection. About 41 percent of the independent 35S::SUB sub-1 T1 plants scored showed a wild-type phenotype (51/126 scored). This indicates that the c-myc tag at the carboxy-terminus of SUB may result in a reduction of SUB functionality. To assay transgene expression RNA was isolated from inflorescences using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany). First-strand cDNA was synthesized from 2 µg of total RNA using Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase (New England Biolabs, Frankfurt, Germany). Semi-quantitative PCR was performed using Taq DNA polymerase (New England Biolabs, Frankfurt, Germany) and a transgenic-specific primer pair (5'TRT- SUBcmyc_F, 3'SUBcmyc_R). Between 24 and 32 thermal cycles were tested. The GAPC gene was used as positive control [118].

Map-Based Cloning of QKY

To map the QKY locus at high resolution, an F2-mapping population was generated. F1 plants from qky/qky (Lei) and QKY/QKY (Col) crosses were allowed to self-pollinate, and the F2 progeny were screened for qky alleles. The F2 progeny were screened for qky individuals based on twisted inflorescence morphology. DNA was isolated and used for PCR-based amplification of molecular markers. Indel polymorphism data was derived from the Monsanto L. er sub-1 mutant predefined map interval to 103 kb, as defined by markers F25A4(BglII) and 27.99(Rsal). Candidate genes were analysed via T-DNA insertion mutant analysis and/or sequence determination revealing that At1g74720 carried mutations in various qky alleles. To confirm qky allelic mutations, nucleotide sequences were obtained from both strands of PCR-amplified fragments. In all three EMS-induced qky alleles transitions result in stop codons. The qky-7 allele carries a G to A transition at position 2229 (genomic DNA, relative to the start ATG triplet (+1)) resulting in a shorter predicted protein.
Whole-Genome Transcriptome Analysis and Global Expression Profiling

Tissue was harvested from 21-day plants grown in continuous light at 25°C. Wild type and mutants were in the Ler background. The inflorescence apex plus flowers up to stage 9 were kept separate from later stage 10–12 flowers (the oldest 3–4 closed flower buds). The tissue was immediately frozen in liquid nitrogen and stored at −80°C. RNA was extracted with the Plant RNAeasy Mini kit (Qiagen). 1 μg of total RNA was used for probe synthesis with the MessageAmp II-Biotin Enhanced kit (Ambion) according to the manufacturer’s instructions. Affymetrix ATH1 GeneChips were hybridized, washed, and stained as described [129]. The geRNA implementation in the Bioconductor and R software packages was used for background correction, quantile normalization and expression estimate computation [130,131] (www.biocductor.org) [132]. Expression estimates were transformed to linear scale and the fold change (FC) was calculated for each gene in each mutant-wildtype comparison by dividing the expression estimate of the mutant condition by the value of an according control. Comparisons were done on a single replicate basis for all possible pair wise comparisons. A Z-score for each fold change was calculated by dividing the difference of log₂ transformed FC and the mean of the FC population by the standard deviation of the FC population (Z = \frac{\log_2(FC) - \bar{FC}}{\sigma_{FC}}). Genes were considered as differentially expressed if they displayed a Z-score>1 in a single comparison and an average Z-score>2 over all comparisons or, if they displayed a Z-score<-1 in a single comparison and an average Z-score over all comparisons <-2.

Accession Numbers

The QKT cDNA sequence was deposited at GenBank under the accession number JF209045. The expression profile data have been deposited to the EMBL-EBI ArrayExpress repository under the accession E-MEXP-1592.

Supporting Information

Dataset S1 This list details the 14 genes that show significant changes in expression level in all Ler versus sub-like mutant (slm) comparisons. This list thus comprises the apex core target gene list. Found at: doi:10.1371/journal.pgen.1000355.s001 (0.03 MB XLS)

Dataset S2 This list details the 93 genes that show significant changes in expression level in all Ler versus sub-like mutant (slm) comparisons. This list thus comprises the flower core target gene list. Found at: doi:10.1371/journal.pgen.1000355.s002 (0.07 MB XLS)

Table S1 List of primers
Found at: doi:10.1371/journal.pgen.1000355.s003 (0.08 MB DOC)

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Author Contributions

Conceived and designed the experiments: LF MB RKY JU L KS. Performed the experiments: LF MB PV RKY WB SUA SJ. Analyzed the data: LF PV WB KS. Wrote the paper: LF JU KS.
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