Arabidopsis O-fucosyltransferase SPINDLY regulates root hair patterning independently of gibberellin signaling

Krishna Vasant Mutanwad*, Isabella Zangl and Doris Lucyshyn*

Institute for Molecular Plant Biology, Department of Applied Genetics and Cell Biology, University of Natural Resources and Life Sciences

Muthgasse 18, 1190 Vienna, Austria

ORCID IDs: 0000-0003-2856-1953 (KVM), 0000-0002-5196-7444 (IZ), 0000-0001-8558-1219 (DL)

*correspondence: doris.lucyshyn@boku.ac.at, krishna.mutanwad@boku.ac.at

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Summary statement

A novel gibberellin independent function of O-fucosyltransferase SPY in modulating epidermal tissue patterning in Arabidopsis roots.

Abstract

Root hairs are able to sense soil composition and play an important role for water and nutrient uptake. In Arabidopsis thaliana, root hairs are distributed in the epidermis in a specific pattern, regularly alternating with non-root hair cells in continuous cell files. This patterning is regulated by internal factors such as a number of hormones, as well as external factors like nutrient availability. Thus, root hair patterning is an excellent model for studying the plasticity of cell fate determination in response to environmental changes. Here, we report that loss-of-function mutants in the Protein O-fucosyltransferase, SPINDLY (SPY) show defects in root hair patterning. Using transcriptional reporters, we show that patterning in spy-22 is affected upstream of the GLABRA2 (GL2) and WEREWOLF (WER). O-fucosylation of nuclear and cytosolic proteins is an important post-translational modification that is still not very well understood. So far, SPY is best characterized for its role in gibberellin signaling via fucosylation of the growth-repressing DELLA protein REPRESSOR OF ga1-3 (RGA). Our data suggests that the epidermal patterning defects in spy-22 are independent of RGA and gibberellin signaling.
**Introduction**

Post translational modifications (PTM) dynamically modulate various physiological and morphological events throughout the life span of plants (Millar et al., 2019). O-Glycosylation of nuclear and cytosolic proteins is one such PTM, and plants carry two O-glycosyltransferases responsible for these modifications: the Protein O-fucosyltransferase (POFUT) SPINDLY (SPY), and the O-GlcNAc Transferase (OGT) SECRET AGENT (SEC) (Hartweck et al., 2002; Olszewski et al., 2010; Zentella et al., 2016; Zentella et al., 2017). These proteins regulate significant events in plants, from embryo development to the determination of flowering time and flower development (Hartweck et al., 2002; Hartweck et al., 2006). *spy* mutants were initially identified due to their resistance to the gibberellin (GA) biosynthesis inhibitor paclobutrazol, leading to constitutively active GA signaling (Jacobsen and Olszewski, 1993; Swain and Olszewski, 1996). Further studies reported that SPY and SEC are involved in GA signaling via modification of the growth repressing DELLA protein RGA (REPRESSOR OF ga1-3) (Silverstone et al., 2007; Zentella et al., 2016; Zentella et al., 2017). *spy* mutants display various phenotypic traits, such as early flowering, early phase transitions, partial male sterility, abnormal trichome formation and disordered phyllotaxy (Silverstone et al., 2007). Recently, SEC also was reported to be involved in delaying flowering time in Arabidopsis (Xing et al., 2018). The majority of the studies thus have focused on the role of O-glycosylation in aerial tissue development and the subsequent phenotypes are often attributed to its participation in GA signaling. SEC and SPY are also active in roots, however their impact on root development and morphogenesis is largely unexplored (Hartweck et al., 2006; Silverstone et al., 2007; Swain et al., 2002).

Tissue morphology and cellular organisation are decisive for root development in *Arabidopsis thaliana*. Epidermal tissue is comprised of hair-forming trichoblast cells and non-hair-forming atrichoblast cells (Dolan et al., 1993; Löfke et al., 2015; Scheres and Wolkenfelt, 1998). The arrangement of the hair and non-hair cells is established around the single ring-like layer of cortex cells. A hair cell arises at the junction between and is connected to two cortical cells, while a non-hair cell is usually adhered to only a single cortex cell. Moreover, hair cells are generally separated by non-hair cells between them (Balcerowicz et al., 2015; Dolan et al., 1994; Salazar-Henao et al., 2016). Various transcription factors like GLABRA2 (GL2), WEREWOLF (WER) and CAPRICE (CPC) are responsible for determination of epidermal cell patterning in Arabidopsis. GL2 and WER regulate the establishment of non-hair cells (Lee and Schiefelbein, 1999; Masucci et al., 1996), whereas CPC activity is required for the formation of hair cells (Wada et al., 1997). GL2 expression is promoted by WER via the formation of a multiprotein complex comprised of TRANSPARENT TESTA GLABRA (TTG1), GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) (Bernhardt et al., 2003; Schiefelbein et al., 1999; Masucci et al., 1996).
Further, GL2 establishes non-hair cell fate by suppressing the expression of root hair-promoting basic Helix-Loop-Helix (bHLH) transcription factors like ROOT HAIR DEFECTIVE 6 (RHD6), RHD6-LIKE1 (RSL1), RSL2, Lj-RHL1-LIKE1 (LRL1), and LRL2 (Balcerowicz et al., 2015; Masucci and Schiefelbein, 1996). On the contrary, in root hair cells, expression of WER is strongly reduced. This allows CPC or its paralogs ENHANCER OF TRY AND CPC 1 (ETC1), ETC3 or TRYPTICHON (TRY) to take its place in the TTG1/EGL3/GL3 complex, resulting in negative regulation of GL2 and de-repression of root hair promoting genes, thus establishing root hair cell fate (Lee and Schiefelbein, 2002; Salazar-Henao et al., 2016).

Root hair development is dynamically controlled by environmental factors like reactive oxygen species (ROS) and pH (Monshausen et al., 2007). Furthermore, availability of mineral nutrients like inorganic phosphate (Pi) and iron (Fe) in the surroundings also modulates the development and morphology of root hairs (Janes et al., 2018; Müller and Schmidt., 2004; Salazar-Henao et al., 2016). Similarly, phytohormones like auxin, ethylene and brassinosteroids are known to influence root hair patterning and development (Balcerowicz et al., 2015; Borassi et al., 2020; Kuppusamy et al., 2009; Liu et al., 2018; Shibata and Sugimoto, 2019). However, a role of gibberellin (GA) in epidermis morphology, root hair formation and development has not been described as yet, nor a potential role of the O-glycosyltransferases SPY and SEC in this context.

spy mutants have been previously reported to display an extra layer of cortex cells, the middle cortex (MC), a phenotype associated with high level ROS signaling (Cui and Benfey, 2009; Cui et al., 2014). Beyond this, root tissue morphology of spy and sec mutants is largely unexplored. Hence, we initiated the investigation of the role of SPY and SEC in root development and tissue patterning, also in relation to GA signaling. Here, we show that epidermis morphology and root hair patterning is altered in spy, but not in sec mutants. Using a set of reporter constructs, we established that SPY regulates patterning upstream of WER. However, we did not find any evidence for an involvement of GA signaling, indicating that SPY regulates root hair patterning independently of DELLA proteins and GA-signaling.

Results

The Arabidopsis Protein O-fucosyltransferase mutant spy-22 has larger root apical meristems

In order to investigate the involvement of O-glycosylation in Arabidopsis root development we analysed various morphological phenotypes of the T-DNA insertion lines spy-22 and sec-5 in comparison to wild type Col-0. SPY and SEC regulate GA signaling by modifying the DELLA protein RGA (Silverstone et al., 2007; Zentella et al., 2016; Zentella et al., 2017) and spy-mutants display constitutive GA-signaling phenotypes (Jacobsen and Olszewski, 1993). GA deficient mutants like ga1-3 are reported to have a reduced root apical meristem (RAM) size
To analyse if O-glycosylation is involved in GA-dependent regulation of RAM size, we measured the RAM of 7-day old seedlings, as the region from quiescent centre till the uppermost first cortical cell which is twice as long as wide (Feraru et al., 2019). We observed that spy-22 mutants displayed a significantly longer meristem (347.6 +/- 34.65 µm) compared to the wildtype Col-0 (283.6 +/- 31.92 µm) and sec-5 (282.4 +/- 27.51 µm) (Fig. 1A, B). On counting the number of epidermal cells in the meristem, we found that the number of cells correlated with meristem size, showing a higher number of cells in spy-22 (39.10 +/- 4.599) compared to Col-0 (29.05 +/- 3.965) and sec-5 (28.92 +/- 5.008) (Fig. S1). This result is in line with the effect of increased GA-signaling on cell division and meristem size (Achard et al., 2009).

Additional to cell number, also the patterning and distribution of atrichoblasts (non-hair) and trichoblast (hair) cells of the epidermis is crucial in determining the size of the meristematic region in Arabidopsis (Löfke et al., 2013). While analysing our mutants, we observed that the difference between atricho- and trichoblast cell sizes was reduced in spy-22 mutants compared to wild-type and sec-5. To quantify that, we measured the lengths of the last four consecutive cells in adjacent (trichoblast and atrichoblast) cell files in the epidermis marking the transition from the root meristem to the differentiation zone (Löfke et al., 2015). We noted that the atrichoblast cells in Col-0 and sec-5 (16.21 +/- 4.30 µm and 18.05 +/- 3.62 µm respectively) were significantly longer than trichoblast cells (11.70 +/- 2.81 µm and 12.38 +/- 2.95 µm respectively). In spy-22, atrichoblast cells (15.92 +/- 4.08 µm) were only slightly longer than cells in corresponding trichoblast files (13.49 +/- 4.30 µm) (Fig. 1C, D). This difference was clearly reflected in a lower ratio of atrichoblast/trichoblast cell length in spy-22 (1.27) compared to Col-0 (1.44) and sec-5 (1.53) (Fig. 1E). Taken together, we observed both an increase in cell number, as well as an altered distribution of atrichoblast/trichoblast cell length in spy-22, resulting in an increase of root meristem size.

spy mutants display ectopic root hairs

The atypical atricho-to trichoblast morphology in spy-22 led us to explore the consequences of this observation on root hair development in fully differentiated epidermis cells. In spy-22, we frequently observed appearance of two trichoblast cells developing root hairs adjacent to each other, indicating ectopic root hair formation, while in Col-0 and sec-5 root hair cell files were always separated from each other by a non-hair cell file (Fig. 2A). The underlying cause for the appearance of ectopic root hairs in spy-22 was further analysed with the help of reporter lines. We used cell type specific promoter-YFP fusions as described (Marquès-Bueno et al., 2016) to monitor the expression of transcription factors implicated in root hair patterning at different stages of development. We initially targeted WER which is involved at an early stage of non-hair cell determination and is expressed strongly in atrichoblast cells and weakly in
trichoblasts (Lee and Schiefelbein, 1999). On crossing the WER::4xYFP reporter with spy-22 and sec-5, we observed an uneven signal distribution within single cell files in spy-22 (Fig. 2B). We also crossed our lines to GL2::4xYFP, which in the wild type is exclusively expressed in the atrichoblasts in the cell division and transition zone. While in Col-0 and sec-5 a regular pattern of reporter gene expression was observed, GL2 expression in spy-22 was very patchy, potentially underlying the formation of ectopic trichoblasts within non-hair cell files and vice versa (Fig. 2C). We next employed a reporter that is active in differentiated root hair cells, to determine if expression patterns in the meristematic and transition zone match the patterning of developed root hairs in the differentiation zone. EXP7 is expressed specifically in root hair cells. In EXP7::4xYFP spy-22 we observed non-hair cells without signal within YFP-positive root hair cell files and vice versa, an aberration in reporter expression which we did not detect in the Col-0 or sec-5 background (Fig. 2D). Taken together, these results suggest that SPY regulates root hair patterning upstream of WER. Furthermore, a cross between spy-22 and wer-1 exclusively forms trichoblast cells, with every epidermal cell in spy-22 wer-1 displaying root hairs in all cell files, like seen in wer-1 (Fig. 3).

In wild-type conditions, it is known that hair cells develop at the junction of two cortex cells whereas a non-hair cell is in contact with only a single underlying cortex cell (Dolan et al., 1994). In spy-22, we observed that root hair cells were frequently adhered to only a single underlying cortex cell (Fig. S2A). Additionally, it was previously shown that spy-mutants generate an additional layer of root cortex cells, which has been attributed to constitutively increased ROS signaling (Cui and Benfey, 2009; Cui et al., 2014). This middle cortex between the cortex and the endodermis was also clearly visible in spy-22 (Fig. S2B). When crossing our lines with SCR::4xYFP to visualize specifically the endodermis, we could confirm the increase in middle cortex formation and clearly distinguish ectopic cell file formation from the endodermis, like seen before (Cui and Benfey, 2009), but there is no indication for a defect in endodermis formation in spy-22 (Fig. S2C).

Epidermal cell patterning and ectopic root hair formation in spy-22 is independent of gibberellin signaling

So far, the best-characterised target of SPY is the DELLA protein RGA, which undergoes a conformational change upon O-fucosylation that enhances the interaction with downstream transcription factors, in some cases inhibiting their binding to DNA (Zentella et al., 2017). As a result, spy mutants show constitutively active GA signaling. So far, GA signaling has not been described to play a role in epidermal cell patterning in Arabidopsis thaliana, hence we aimed to understand whether the epidermal patterning of spy-22 was influenced by increased GA signaling. For initial experiments we treated spy-22, sec-5 and Col-0 with 10µM GA₃ and measured the tricho– and atrichoblast cell length in the root meristem transition zone. The
distribution pattern remained similar to untreated seedlings, as reported in (Fig. 1C). The difference in length of trichoblast cells (13.60 +/- 4.21 µm) and atrichoblast cells (16.15 +/- 3.38 µm) was smaller in spy-22 when compared to Col-0 and sec-5 (Fig. 4A), with a lower atrichoblast/trichoblast ratio (1.3) in spy-22 also after GA₃ treatment (Fig. 4B), at a ratio comparable to the untreated seedlings (compare Fig. 1E and 4B). We also observed that the overall root length was not influenced by supplementing additional 2 µM or 10 µM GA₃. We did not see any effect on the RAM length after growing seedlings on 2 µM and 10 µM GA₃ supplemented plates for 7 days (Fig. S3). Next, we determined GL2::4xYFP expression in Col-0, spy-22 and sec-5 grown on 10 µM GA₃ and analysed the cell file patterning in the cell division and transition zones. We quantified this phenotype by counting the number of patterning defects (which we defined as the appearance of atrichoblast cells in trichoblast cell files and vice versa) per seedling (Fig. 4C). We observed that Col-0 displayed on average 1.47 patterning defects per seedling, with 7/19 seedlings showing no patterning defects. After treatment with 10 µM GA₃, frequencies of patterning defects did not significantly change, with an average of 2 per seedling (Fig. 4D). Similarly, there was no significant change in patterning defects in GL2::4xYFP sec-5 in untreated controls (2.7 patterning defects per seedling) compared to 10µM GA₃-treated seedlings (2.6 patterning defects per seedling) (Fig. 4D). GL2::4xYFP spy-22 displayed the highest number of patterning defects per seedling (8.1 per seedling) and this did not change significantly upon treatment with 10 µM GA₃ (7.6 patterning defects per seedling). These results suggest that exogenous application of gibberellin does not influence epidermal patterning in the genotypes analysed.

The plant hormone ethylene regulates root hair initiation and development (Feng et al., 2017). Treatment of Arabidopsis with ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) induces formation of ectopic root hairs in non-hair positions (Zhang et al., 2016). Moreover, ethylene is known to influence root growth by preventing GA accumulation in roots (Shani et al., 2013). Considering such phytohormonal crosstalk during root development, we explored the possibility of ethylene signaling inducing ectopic epidermal patterning in spy-22. GL2::4xYFP seedlings were grown for 7 days on ½ MS plates supplemented with 1 µM ACC or 100 nM aminoethoxyvinylglycine (AVG), a known ethylene biosynthesis inhibitor before analysing GL2 expression in Col-0, spy-22 and sec-5 (Fig. 5A). We found that the number of patterning defects in all the lines remained unaffected in presence of ACC and AVG (Fig. 5B). Additionally, we also subjected the EXP7::4xYFP lines in Col-0, spy-22, sec-5 background to ACC and AVG treatment. We could clearly observe increased root hair length in response to ACC treatment. The appearance of hair cells in non-hair cell files upon ACC treatment and formation of non-hair cells in hair cell files upon AVG treatment, as described by Zhang et al., 2016, was observed at a low frequency (Fig. S4) in Col-0 and sec-5 background, while
patterning defects in spy-22 were largely unchanged upon the treatments. These results suggest that ethylene does not affect SPY-dependent regulation of root hair patterning.

Gibberellin signaling in Arabidopsis is regulated via its ability to mediate the degradation of DELLAs, a family of growth inhibitors. In the current working model, the degradation of DELLAs de-represses DELLAs interacting proteins which in turn positively regulate growth (Bao et al., 2020; Davière and Achard, 2016). Most of the available literature on DELLAs is based on work in the Ler-background. In order to mimic an environment with reduced GA signaling also in our mutant lines in Col-0 background, we deleted 17 amino acids of the DELLA domain of RGA as described by (Dill et al., 2001), preventing its recognition by the GA receptor GID1. This resulting RGA::\(\Delta\) RGA construct was transformed into Col-0, rendering the transformants insensitive to GA and thus constitutively repressing the DELLAs interacting proteins. The resulting plant lines displayed similar phenotypes like described before in the Ler background, including smaller leaf and rosette size, darker leaves, and reduced inflorescence axis length (Fig. S5). We then crossed this line into sec-5 and spy-22, in order to test whether reduced GA signaling impacts on root development and root hair patterning. We found that RGA::\(\Delta\) RGA Col-0 roots (1.09 +/- 0.26 cm) were significantly shorter than Col-0 roots (1.34 +/- 0.24 cm). A similar tendency was also seen in RGA::\(\Delta\) RGA sec-5 roots (1.17 +/- 0.21 cm) compared to sec-5 (1.32 +/- 0.21 cm), while RGA::\(\Delta\) RGA spy-22 roots were only slightly shorter (1.15 +/- 0.17 cm) compared to spy-22 (1.28 +/- 0.21 cm) (Fig. 6A,B). We did not see any significant difference in the RAM length of RGA::\(\Delta\) RGA lines in all backgrounds compared to the Col-0, spy-22 and sec-5 parent lines (Fig. 6C,D).

The influence of reduced GA signaling on epidermal tissue patterning in the late meristem was studied by measuring the cell lengths of four consecutive epidermal cells in neighbouring cell files (Fig. 7A). There was no significant difference between the cell lengths of atrichoblasts in RGA::\(\Delta\) RGA Col-0 (21.73 +/- 6.44 µm) and atrichoblasts in Col-0 (20.48 +/- 5.59 µm). Likewise, trichoblast cells in RGA::\(\Delta\) RGA Col-0 (15.97 +/- 3.83 µm) were similar in length to Col-0 trichoblasts (14.85 +/- 3.59 µm) (Fig. 7B). The ratio of atrichoblast/trichoblast cell length in RGA::\(\Delta\) RGA Col-0 (1.40) was thus unchanged compared to Col-0 (1.44) (Fig. S6). We found similar results when comparing cell lengths of RGA::\(\Delta\) RGA sec-5 and sec-5, as well as RGA::\(\Delta\) RGA spy-22 and spy-22 (Fig. 7B). The ratio of atrichoblast/trichoblast in case of RGA::\(\Delta\) RGA spy-22 (1.23) was lower, like seen in the spy-22 parent line (1.21) (Fig. S6).

Examination of RGA::\(\Delta\) RGA Col-0 and RGA::\(\Delta\) RGA sec-5 roots demonstrated that root hair patterning is similar to that of Col-0 and sec-5 respectively, showing no discernible ectopic root hair formation, and RGA::\(\Delta\) RGA spy-22 still displayed ectopic root hairs resembling the parent line spy-22 (Fig. 7C). To further assess our findings regarding GA dependent root
development, we analysed the phenotypes of the GA biosynthesis mutant *ga1-4* and the *della* quintuple mutant, *gai-t6 rga-t2 rgl1-1 rgl2-1 rgl3-1*, both in Ler. At 7 days, *ga1-4* showed a shorter RAM, whereas the *della* quintuple mutant displayed a RAM size similar to that of Ler wild type (Fig. S7A, B). The reduced RAM of *ga1-4* was complemented by a reduced overall root length (Fig. S7C). To further analyse the involvement of GA in epidermal patterning, atrichoblast and trichoblast cell lengths in the late meristem were measured (Fig. S7D). We found that atrichoblasts were significantly longer than trichoblasts in all three lines (Fig. S7E), like seen in Col-0 and *sec-5*, but not *spy-22* (Fig. 1D, E). The *della* quintuple mutant shows increased GA signaling, like observed in *spy-22*. However, *della* mutants did not display ectopic root hair formation as seen in *spy-22*. Also, the GA deficient *ga1-4* showed regular root hair patterning without ectopic hairs, similar to Ler wild type (Fig. S7F). Taken together, we did not find evidence that epidermal cell patterning defects in *spy-22* are dependent on GA signaling.

**Discussion**

Root hairs are essential for the uptake of water and nutrients, as they can sense nutrients in the soil and react by increasing the root surface in a very flexible way. Root hair patterning is therefore regulated by internal as well as environmental factors, allowing for a high degree of plasticity in the developmental program. Thus, many different pathways feed into the regulation of cell fate determination in the epidermis, including a number of hormones such as auxin, ethylene and brassinosteroids (Balcerowicz et al., 2015; Borassi et al., 2020; Kuppusamy et al., 2009; Liu et al., 2018; Shibata and Sugimoto, 2019). Root hair patterning in *Arabidopsis* has been studied extensively and represents a very useful model system for analysis of plasticity in cell fate determination. In recent years, a number of tools have been made available to monitor the establishment of hair- and non-hair cell files in the root apical meristem, including a set of transcriptional reporters labelling specific cell types (Marquès-Bueno et al., 2016). Here, we present evidence that O-fucosylation is involved in establishing root hair cell patterning. Using a number of transcriptional reporters, genetics and phenotypical analysis, we show that root hair cell patterning is impaired in the O-fucosyltransferase mutant *spy-22*. Monitoring the expression of WER by using a transcriptional reporter suggests that the patterning defect in *spy-22* is established already early on during epidermal cell fate determination, potentially due to defects in cortex development or cell-to-cell communication between cortex and epidermis, as these processes regulate cell type specific WER expression levels (Fig. 8). The atypical receptor-like kinase SCRAMBLED (SCM) plays an important role in signaling from the cortex to the epidermis and further on to WER in this context (Gao et al., 2019; Kwak et al., 2005). Further experiments targeting the function, localization or turn-over of SCM might help determining how SPY participates in cell-to-cell communication at this
stage, or alternatively in upstream signaling events in the cortex. Other potential targets of SPY include the transcription factor JACKDAW (JKD), that is expressed in the cortex and regulates epidermal cell fate in a non-cell autonomous way or other regulators of SCM, such as QKY (Hassan et al., 2010; Song et al., 2019).

Post-translational modification by attachment of O-fucose or O-GlcNAc is still not very well understood in plants. The best studied target is the gibberellin signaling repressor RGA, where O-GlcNAc and O-fucose have opposite effects on its activity, probably by inducing conformational changes (Zentella et al., 2016; Zentella et al., 2017). Accordingly, spy mutants show many phenotypes that can be associated with gibberellin signaling, such as paclobutrazol resistance, early flowering, or elongated growth (Olszewski et al., 2010; Silverstone et al., 2007). spy-5 showed an increase in trichome formation (Perazza et al., 1998; Silverstone et al., 2007), which has also been linked to constitutive GA signaling, as trichome initiation is positively regulated by GA (Chien and Sussex, 1996; Kim et al., 2018). Trichome and root hair formation share a similar genetic regulatory network with some of the genes involved being active both in leaf and root epidermis (Ishida et al., 2008). In our study, we did not find an indication that consequences of altered O-fucosylation on root epidermal patterning would require gibberellin signaling, as exogenous application of GA did not affect root hair patterning (Fig. 4). Ethylene not only regulates root hair initiation but also controls root growth by inhibiting GA accumulation by stabilizing the DELLA proteins (Achard et al., 2003; Shani et al., 2013). We observed that ectopic GL2 patterning of spy-22 mutants was independent of ethylene signaling (Fig. 5). RGA::ΔRGA lines with impaired GA signaling consistently produced shorter roots compared to parent backgrounds (Fig. 6A,B). RGA regulates GA signaling in the elongation zone (Shani et al., 2013), hence an altered RGA activity in the elongation zone could be responsible for the reduced root lengths. We did not observe root hair patterning defects in RGA::ΔRGA lines, whereas RGA::ΔRGA spy-22 still displayed patterning defects similar to spy-22 (Fig. 7C). Further analysis of the GA deficient mutant ga1-4 and della quintuple mutants with upregulated GA signaling, revealed that none of these lines displayed abnormal root hair patterning (Fig. S7F). These observations further confirm that ectopic root hair formation in spy-22 mutants is indeed independent of GA signaling. The observed increase in cell numbers of spy-22 meristems (Fig. S1) is probably independent of the patterning defect, but further studies are necessary to address if this increased cell division is dependent on GA-signaling.
Overall, we suggest a model, where SPY regulates root hair cell fate determination by affecting the spatial order of WER-expression, which then signals down to patchy expression of GL2 and EXP7, leading to ectopic root hair formation (Fig. 2). Thus, O-glycosylation potentially regulates the function of upstream regulators such as SCM or the cell-to-cell communication from cortex to the epidermis (Fig. 8), but further studies are necessary to reveal the direct targets of SPY in this context.

Methods

Plant material and growth conditions

All mutant lines used in this study were obtained from the Nottingham Arabidopsis Stock Centre NASC. Col-0 and Ler ecotype of Arabidopsis thaliana were referred to as wild-type controls. T-DNA insertion lines of spy-22 (SALK_090582) and sec-5 (SALK_034290) and previously published reporter lines WER::4xYFP (N2106117), GL2::4xYFP (N2106121) and EXP7::4xYFP (N2106118) (Marquès-Bueno et al., 2016) in Col-0 background, as well as ga1-4 (N3105) and the della quintuple mutant gai-t6 rga-t2 rgl1-1 rgl2-1 rgl3-1 (N16298), both in Ler background were used. After surface sterilisation with 70% ethanol, the seeds were plated onto ½ Murashige and Skoog medium (2.15 g/L MS Salts, 0.25 g/L MES, pH 5.7, 1% agar). After stratification in the dark at 4°C for 2 days, they were vertically grown in long day conditions (16 hours light / 8 hours dark) at 22°C.

Hormone treatments

For GA treatment, seeds were surface sterilized with 70% ethanol and transferred to ½ MS medium containing 2 µM or 10 µM GA₃ (for root and RAM length measurements) and 10 µM GA₃ (for patterning experiments), stratified in the dark at 4°C for 2 days and vertically grown in long day conditions (16 hours light / 8 hours dark) at 22°C for 7 days.

For ethylene treatments, seeds were surface sterilized with 70% ethanol and transferred to ½ MS medium containing 1µM ACC or 100 nM AVG, stratified in the dark at 4°C for 2 days and vertically grown in long day conditions (16 hours light / 8 hours dark) at 22°C for 7 days.

For experiments involving ga1-4, all lines were surface sterilised with 70% ethanol and treated with 10 µM GA₃ for 7 days at 4°C in dark to enable germination. Subsequently, the seeds were thoroughly rinsed with sterile distilled water, transferred to ½ MS medium and vertically grown in long day conditions (16 hours light / 8 hours dark) at 22°C for 7 days.
Microscopy

For imaging, a Leica TCS SP5 confocal microscope with an HCX PL APO CS 20.0x0.70 IMM UV objective was used. Seedlings were mounted in Propidium iodide (PI) (0.02 mg/mL) for staining the cell wall prior imaging. DPSS561 Laser was used to excite PI at 561nm (emission 584-735nm with standard PMT), and an Argon Laser at 30% intensity was used to excite YFP at 514nm (emission 524-552 with HyD detector). Z Stacks were taken for visualizing root hairs and Maximum Projections were made using the Leica LAS AF lite software.

Phenotyping and Image quantification

Measurements and quantifications were performed using the LAS X Leica Software. For studying the RAM length, seedlings were mounted in PI (0.02 mg/mL). We measured the distance from quiescent centre till the uppermost first cortical cell which was twice as long as wide as described by (Feraru et al., 2019). For epidermal cell patterning, lengths of four consecutive epidermal cells from neighbouring (tricho/atrichoblast) files in the late meristem were measured (Löfke et al., 2015). For analysing the patterning frequency in GL2::4xYFP, we checked for its expression in cell division and transition zones. We defined the occurrence of trichoblast cells in an atrichoblast cell file and vice versa as a patterning defect and counted the number of such patterning events in each seedling. For root length measurements, ½ MS plates with seedlings were scanned using Epson Perfection V700 scanner and the root lengths were measured using ImageJ.

Data Analysis

We used GraphPad Prism 8 for generating graphs. Error bars in graphs indicate standard error. One-way ANOVA and Tukey’s Multiple comparison test and students t-test were performed for statistical analysis of the data. Sample sizes (n) for all experiments are given in the respective figure legends.

Plasmid construction and generation of transgenic lines

To generate a GA insensitive, stabilized version of RGA in the Col-0 background, RGA::ΔRGA was amplified from genomic DNA of Col-0 using Q5 high fidelity DNA polymerase (NEB). Two overlapping fragments lacking 17 amino acids covering the DELLA domain as described previously by (Feng et al., 2008) were generated using the following primer pairs: #270 (5'-tacaaaaaagcaggctcactagtaatttcttgctgtc-3') and #272 (5'-gttcgagtttcaaagcaacctcgtccatgttaacctccacgtc-3'), #273 (5'-gacggtggaggtaactggaggt tctttgaactcgaac-3') and #271 (5'-gtcgggtctatctcgactcgccggagagctgca-3'); The resulting overlapping fragments were then cloned into a Gateway™ pENTR4™ vector backbone linearized with Ncol/Xhol via Gibson Assembly (NEB). The assembled plasmid was
transformed into electrocompetent DH10b E.coli cells, positive clones were selected on LB medium using kanamycin (50µg/mL) and confirmed by sequencing. Confirmed entry clones were digested with Asil to destroy the kanamycin resistance of the pENTR4-backbone, and recombined with pEarleyGate303 (Earley et al., 2006) using Gateway LR Clonase II enzyme mix to generate a plant expression vector. Positive colonies were selected for kanamycin (50µg/mL) resistance, confirmed plasmids were electro-transformed into Agrobacterium tumefaciens GV3101 and used for transforming Arabidopsis thaliana ecotype Col-0 by floral dipping (Clough and Bent, 1998). Stable transformants with a strong GA-deficient phenotype were selected before crossing with spy-22 and sec-5.

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Author contributions

KVM and DL planned experiments, IZ provided substantial technical support, KVM wrote the manuscript with support from DL.

Competing interests

The authors declare no competing interests.

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**Figures**

Fig. 1 *spy-22* mutants display longer root apical meristems and reduced difference between atrichoblast and trichoblast cells. 

A- Longitudinal cross section images of 7-day old seedlings mounted in PI. Meristem size was defined as the distance from the quiescent center to first uppermost cortical cell which was twice as long as wide, as indicated by white arrows, scale bar – 100 μm. 

B- *spy-22* roots display significantly longer meristems compared to Col-0 and *sec-5*. n = 16–23. 

C- The epidermal layer in the late meristematic region of 7-day old seedlings mounted in PI. Lengths of 4 consecutive cells in neighbouring (tricho/atrichoblast) files in the late meristem were measured, scale bar – 20 μm. 

D- Atricho- and trichoblast cell length in Col-0, *sec-5* and *spy-22*, n = 47-64. 

E- The ratio of the epidermal cell lengths of atrichoblasts/trichoblasts is lower in *spy-22* compared to *sec-5* and Col-0. For statistical analysis, One-way ANOVA with Tukey's multiple comparison was performed for B, D and students t-test was done for E (*** P ≤ 0.001, * P ≤ 0.05), data from three independent biological repeats is shown.
Fig. 2 spy-22 forms ectopic root hairs. A - Maximum projection of Z stacks to visualize root hair patterning of 7-day old seedlings, scale bar – 100 μm. B - WER::4xYFP expression in the epidermal cells in the meristem region. YFP signal in spy-22 is unevenly distributed within the same cell file, scale bar – 50 μm C - Expression of GL2::4xYFP visualized in atrichoblasts. Expression in spy-22 indicates the presence of trichoblast cells in the atrichoblast cell file and vice versa, scale bar – 100 μm. D - EXP7 is exclusively expressed in root hair cells. YFP signal indicates EXP7 promoter activity is not uniform within cell files in spy-22, suggesting the presence of non-hair cells in a hair cell file and vice versa, scale bar – 100 μm. Representative pictures of three biological repeats are shown.
Fig. 3 *wer-1* phenotype is epistatic in *spy-22 wer-1*. Maximum projection of Z stacks to visualize root hair patterning of Col-0, *wer-1*, *spy-22* and *spy-22 wer-1*. In loss of function *wer-1* mutants, all epidermal cells achieve hair cell identity, while root hair patterning in *spy-22* is patchy. *spy-22 wer-1* displays a phenotype similar to *wer-1*, where all epidermal cells take the hair cell identity, scale bar – 100 µm.
Fig. 4 Epidermal patterning in *spy-22* is independent of GA. **A**- Epidermal cell length of 7-day old Col-0, *sec-5* and *spy-22* seedlings grown on ½ MS supplemented with 10 μM GA₃, n = 48-60. **B**- Presence of 10 μM GA₃ does not influence the epidermal patterning, the ratio of the epidermal cell lengths of atrichoblasts/trichoblasts is lower in *spy-22* compared to *sec-5* and Col-0. **C**- GL2::4xYFP expression pattern remains largely unchanged in presence of 10 μM GA₃, scale bar – 100 μm. **D**- Patterning defects per seedling defined as the number of times an atrichoblast appears in a trichoblast cell file and vice versa. The average number of patterning events per seedling is higher in *spy-22*, but remained unaffected in the presence of 10 μM GA₃ in all the lines compared to untreated controls, n = 16-30. For statistical analysis, One-way ANOVA with Tukey’s multiple comparison was done (*** P ≤ 0.001, * P ≤ 0.05), data from three biological repeats is shown.
Fig. 5 Ethylene signaling does not regulate GL2 patterning in the root meristem. A- GL2::4xYFP expression pattern of 7-day old seedlings in Col-0, sec-5 and spy-22 background in the presence of 1 µM ACC and 100 nM AVG, scale bar – 100 μm. B- Patterning defects per seedling defined as the number of times an atrichoblast appears in trichoblast cell file and vice versa. The average number of patterning events per seedling remained unaffected in the presence of 1 µM ACC or 100 nM AVG in all the lines compared to untreated controls, n = 16-20. For statistical analysis, One-way ANOVA with Tukey’s multiple comparison was done data from three biological repeats is shown.
Fig. 6 RGA::ΔRGA mutants display reduced root length. **A** 7-day old seedlings grown on ½ MS plates. **B** RGA::ΔRGA Col-0, RGA::ΔRGA sec-5 and RGA::ΔRGA spy-22 are significantly shorter than Col-0, sec-5 and spy-22, respectively, n = 44-79. **C** Longitudinal cross section images of 7-day old RGA::ΔRGA seedlings mounted in PI. Meristem size was defined as the distance from the quiescent center to the first uppermost cortical cell which was twice as long as wide, as indicated by white arrows, scale bar – 100 μm. **D** RAM lengths of 7-day old seedlings were unaffected by reduced GA signaling in RGA::ΔRGA lines, n = 11-18. For statistical analysis, One-way ANOVA with Tukey’s multiple comparison was done (*** P ≤ 0.001, ** P ≤ 0.01, * P ≤ 0.05), data from three biological repeats is shown.
Fig. 7 Reduced GA signaling does not affect epidermal cell patterning or induce ectopic root hairs. A- The epidermal layer in the late meristematic region of 7-day old RGA::ΔRGA seedlings mounted in PI. Lengths of 4 consecutive cells in neighbouring (tricho/atrichoblast) files in the late meristem were measured, scale bar – 20 μm. B- Atricho- and trichoblast cell lengths from the late meristem of 7 day-old seedlings of RGA::ΔRGA Col-0, RGA::ΔRGA sec-5, RGA::ΔRGA spy-22, Col-0, sec-5 and spy-22, n = 46-68. C- 7-day old RGA::ΔRGA Col-0, RGA::ΔRGA sec-5 and RGA::ΔRGA spy-22 seedlings grown on ½ MS agar mounted in PI. RGA::ΔRGA Col-0 and RGA::ΔRGA sec-5 did not show ectopic root hairs, while in RGA::ΔRGA spy-22 ectopic root hair formation was comparable to spy-22, scale bar – 100 μm. For statistical analysis, One-way ANOVA with Tukey’s multiple comparison was done (*** P ≤ 0.001, * P ≤ 0.05), data from three biological repeats is shown.
Fig. 8 SPY regulates epidermal cell patterning upstream of WER. Various fate determination factors are responsible for imparting epidermal patterning and tissue organisation in the *Arabidopsis thaliana* roots. Non-hair cell fate is largely modulated by WER and GL2. *spy-22* mutants display defects in epidermis and subsequent root hair patterning by regulating cell fate determination upstream of WER.
Fig. S1. *spy-22* mutants have an increased number of cells in the RAM. Number of epidermal cells in the meristem of 7-day old O-glycosylation mutants. Meristems of *spy-22* have a higher number of epidermal cells (39.10 +/- 4.599) compared to Col-0 (29.05 +/- 3.965) and sec-5 (28.92 +/- 5.008). For statistical analysis, One-way ANOVA with Tukey’s multiple comparison was done (**P ≤ 0.001**), data from three independent biological repeats is shown.
Fig. S2. *spy-22* shows abnormal hair cell positioning with respect to underlying cortex. A- Transverse section of the fully elongated zone in 7-day old *spy-22* seedlings. Ectopic root hair cells are adhered to only a single underlying cortex cell in *spy-22*. B- 7-day old *spy-22* seedlings, arrows indicate middle cortex formation. This extra layer of cortex is formed between cortex and endodermis and has been previously described by Cui et al. 2014 in other *spy* alleles. scale bar – 100 μm. C- SCR::4xYFP expression in Col-0, sec-5 and *spy-22* is restricted to the endodermis. The middle cortex proliferation in *spy-22* is unique and independent of SCR expression in the endodermis.
**Fig. S3. RAM length is unaffected by exogenous GA treatment.**

**A**- Longitudinal cross section images of 7-day old seedlings grown in ½ MS plates supplemented with 10 μM GA₃. Meristem size was defined as the distance from the quiescent center to first uppermost cortical cell which was twice as long as wide, as indicated by white arrows, scale bar – 100 μm.

**B**- Addition of 2 μM and 10 μM GA₃ did not alter RAM length. n = 16-25.

**C**- Length of 7-day old seedlings grown on ½ MS plates with 2 μM and 10 μM GA₃. n = 45-60. For statistical analysis, One-way ANOVA with Tukey’s multiple comparison was done (* P ≤ 0.05), data from three independent biological repeats is shown.
Fig. S4. Ethylene signaling does not regulate ectopic hair formation in spy-22. A- 7-day old EXP7:4xYFP seedlings in Col-0, sec-5 and spy-22 background grown on ½ MS plates supplemented with 1 μM ACC and 100 nM AVG. ACC treatment induced formation longer root hairs in Col-0 and sec-5. Additionally, ACC led to occurrence of hair cells in non-hair cell files in Col-0 and sec-5 background in a very low frequency, and treatment with AVG very slightly induced the formation of non-hair cells in a hair cell file in Col-0 and sec-5 background. However, the ectopic root hair formation and root hair length were evidently unaffected by ACC and AVG treatment in spy-22. Scale bar – 100 μM. Representative pictures of three biological repeats are shown.
Fig. S5. Rosette and shoot phenotype of $\text{RGA::ΔRGA}$ mutants. Col-0, spy-22, sec-5 and their crosses with $\text{RGA::ΔRGA}$ Col-0, a line expressing a stabilized version of the GA-signaling repressing DELLA protein RGA. All $\text{RGA::ΔRGA}$ lines show phenotypes characteristic for low GA signaling, like smaller rosette size and shorter inflorescences.
Fig. S6. Atrichoblast/trichoblast cell length ratio of RGA::ΔRGA mutants. The ratio of atrichoblast/trichoblast cell lengths in RGA::ΔRGA Col-0 (1.40) was similar to Col-0 (1.44). Compared to all lines, the ratio of RGA::ΔRGA spy-22 (1.23) was lower, similar to spy-22 (1.21).
Fig. S7. GA deficient ga1-4 and della mutants with high GA signaling do not display ectopic root hairs. A- Longitudinal cross section of 7-day old Ler, ga1-4 and gai rga rgl1 rgl2 rgl3 seedlings, meristem size was defined as the distance from the quiescent center to first uppermost cortical cell which was twice as long as wide, as indicated by white arrows, scale bar – 100 μm. B- 7 day old seedling of ga1-4 display significantly shorter RAM compared to Ler and gai rga rgl1 rgl2 rgl3. n = 16-24. C- The overall root length of 7-day old ga1-4 seedlings is significantly shorter than Ler and gai rga rgl1 rgl2 rgl3. n = 21-44. D- The epidermal layer in the late meristematic region of 7-day old Ler, ga1-4 and gai rga rgl1 rgl2 rgl3 seedlings. Lengths of 4 consecutive cells in neighbouring (tricho/atrichoblast) files in the late meristem were measured, scale bar – 20 μm. E- The atrichoblast cells are significantly longer than the trichoblast cells in all the lines. n = 51-60. F- 7-day old seedlings of Ler, ga1-4 and gai rga rgl1 rgl2 rgl3 do not display ectopic root hair formation in the elongated zone. For statistical analysis, One-way ANOVA with Tukey’s multiple comparison was done (** P ≤ 0.001), data from three independent biological repeats is shown.