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Review

Wounding and the establishment of leaf polarity: From historical perspectives to present controversies

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ABSTRACT

Leaves originate as primordia on the shoot apical meristem and undergo differentiation to become flat structures with distinct upper (adaxial) and lower (abaxial) tissue types. The establishment of adaxial and abaxial tissue types in leaves was investigated by Ian Sussex in 1955 through wounding experiments. Sussex proposed that a signal coming from the shoot apical meristem promotes the differentiation of adaxial leaf tissues and leaf flattening. Since then, many studies have followed up on Sussex's work to understand better how leaf polarity is first established using anatomical approaches, molecular genetics and live-imaging. While some studies have supported Sussex's findings, others have presented conflicting results. In this review, we focus on how recent evidence has shifted the debate.

KEYWORDS: Sussex signal, wounding, leaf polarity, dorsoventrality, adaxial, abaxial.

INTRODUCTION

During leaf development, three patterning axes can be distinguished: the adaxial-abaxial axis (also called dorsoventral), the proximal-distal axis, and the medial-lateral axis (Figure 1). Differential growth along these axes typically results in the formation of a flat or lamina-shaped organ that provides a large surface area to maximise photosynthesis [1]. Tissues of the leaf nearest, or adjacent, to the shoot are termed adaxial, while those farther away are referred to as abaxial. In terms of histology, adaxial and abaxial leaf tissues differ in a number of ways, reflecting their distinct roles in photosynthesis and gas exchange [2]. In *Arabidopsis*, the number of trichomes, or leaf hairs is higher on the early adaxial juvenile leaf surface compared to the respective abaxial side. The adaxial palisade mesophyll cells are elongated in shape and tightly packed to maximize light absorption. Conversely, the abaxial spongy mesophyll cells vary in shape and are loosely packed to better enable gas exchange. The central vasculature tissue is also different; the xylem is positioned towards the adaxial side, while the phloem is located towards the abaxial side.

How is the leaf adaxial-abaxial axis first established? The field has largely been split into two camps. In 1955, Sussex proposed, based on wounding experiments, that an inductive signal from the shoot apical meristem to the leaf primordium was required to specify adaxial identity and leaf flattening [3]. In contrast, others have proposed that adaxial and abaxial leaf tissues derive their character directly from the corresponding tissues of the shoot from which they originate [4]. More recently, due to the central role of auxin in organ initiation [5, 6], studies have investigated auxin in relation to leaf polarity, including the hypothesis that auxin corresponds to the inductive signal proposed by Sussex [7-9]. In this review, we discuss hypotheses on the origin of leaf adaxial-abaxial patterning with reference to recent data on the topic (see Table 1).

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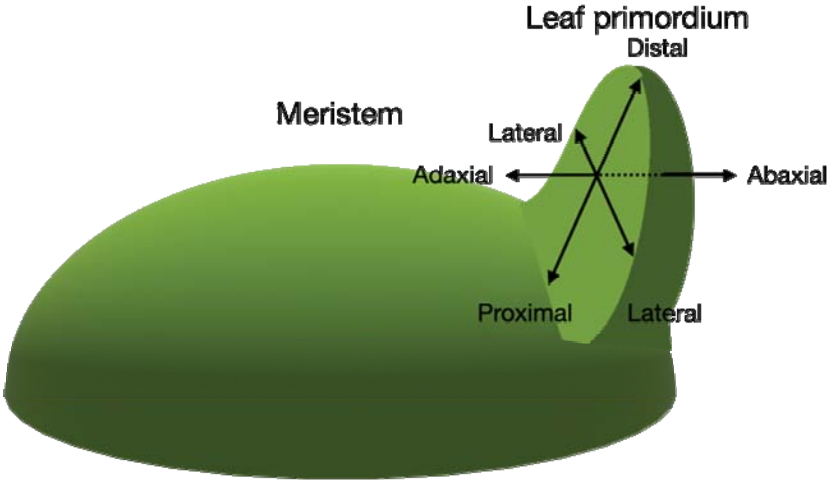

Figure 1. Major axes of a developing leaf with respect to the shoot meristem.

Table 1. Studies discussed in this review.

Research	**Reference**
Morphogenesis in Solanum tuberosum I: experimental investigation of leaf dorsiventrality and orientation in the juvenile shoot	[3]
Morphogenesis in plants; a contemporary study	[11]
Organogenetic capacity of leaves: The significance of marginal blastozones in angiosperms	[4]
Phantastica: a gene required for dorsoventrality of leaves in *Antirrhinum majus*	[14]
Microsurgical and laser ablation analysis of leaf positioning and dorsoventral patterning in tomato	[10]
Auxin depletion from leaf primordia contributes to organ patterning	[8]
Spatial Auxin Signalling Controls Leaf Flattening in *Arabidopsis*	[9]
Cell type boundaries organize plant development	[7]
Stable establishment of organ polarity occurs several plastochrons before primordium outgrowth in *Arabidopsis*	[33]
Specification of leaf dorsiventrality via a prepatterned binary readout of a uniform auxin input	[28]
Coactivation of antagonistic genes stabilizes polarity patterning during shoot organogenesis	[26]
Polar auxin transport modulates early leaf flattening	[29]

Pre-patterning or dependence on a 'Sussex' signal?

In 1955 Sussex reported that in potato, incisions separating the shoot apical meristem from young leaf primordia resulted in the formation of needle-like leaves without a blade. Further histological studies of these radially symmetric lateral organs demonstrated that they consisted entirely of abaxial cell types [3]. Sussex, therefore, concluded that a wound-sensitive inductive signal travelling from the shoot apical meristem to the primordium was necessary for specifying adaxial identity (Figure 2A) on what would otherwise be a radially patterned, abaxialized primordium (Figure 2B). Fifty years later, the same experiment was repeated on tomato shoot tips. Instead of using a sharpened blade to make incisions, an infrared laser was used to ablate tissues. Experiments in which the laser was focused on regions separating young leaf primordia from the shoot apical meristem again resulted in abaxialization and organ radialization, recapitulating the early findings of Sussex based on potato [10]. Despite these results, other scientists in the field from the 1960's onwards, have argued against Sussex's interpretation. Alternatively, they have pointed out that leaf primordia must necessarily be patterned along their adaxial-abaxial axis from inception since the shoot tissue from which the organ is derived is already patterned along its radial axis, which at inception, corresponds to the leaf adaxial-abaxial axis (Figure 3).

For instance, citing the different growth and differentiation status of cells along the surface of the shoot between the centre and periphery, Wardlaw states that 'the growth relationships and rates of differentiation on the adaxial and abaxial sides of the primordium are different from the outset' [11]. Part of the confusion, it is argued, comes from the fact that in some species, including potato and tomato, leaf primordia start as roughly rod-shaped structures where lateral

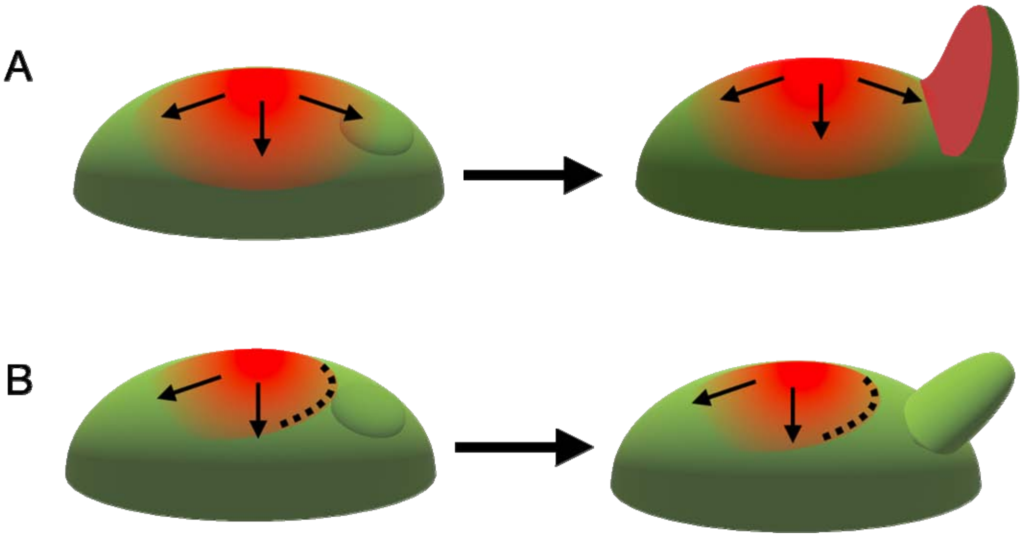

Figure 2. Sussex signal hypothesis for specification of adaxial identity **A.** Induction *via* mobile signal (red) leading to normal leaf development. **B.** Interruption of induction due to wound (dashed line) leading do abaxialized and radialized organ.

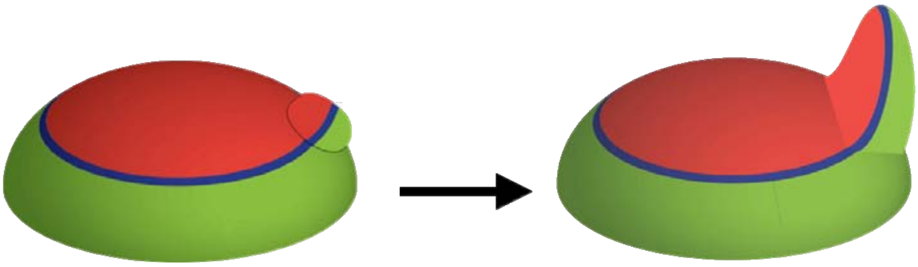

Figure 3. Leaf adaxial-abaxial patterning is derived directly from the radial pattern of the shoot.

growth occurs later, suggesting a radial rather than a flattened, adaxial/abaxial patterning at inception [4]. Weight given to Zimmerman's telome hypothesis, that leaves evolved from axial structures [12], may also promote such an interpretation [4]. In most other species however, leaves initiate as linear structures that extend around the circumference of the shoot. This is most obvious in grasses but also many other dicot species. Debate between these different views has been ongoing, until very recently.

A molecular understanding of adaxial-abaxial patterning

The first clues to understanding adaxial-abaxial patterning at a molecular level were gained by examining *Phantastica* (*phan)* mutants in *Antirrhinum* plants [13, 14] where it was reported that these mutants developed similar abaxialized needle-like leaves to those described by Sussex from wounding experiments, reinforcing the association between loss of adaxial/abaxial patterning and a radially symmetrical organ shape. Weaker phenotypes were also observed in which leaves appeared to develop patches of abaxial cells on the adaxial leaf surface. Along the boundaries of these patches, blade-like protuberances formed. To explain this phenomenon, the authors drew inspiration from wing development in Drosophila [15], suggesting that boundary cells act as 'organizers' to signal to neighbouring regions to promote the growth of laminal tissues. In their discussion, the authors suggest this process occurs downstream of adaxial-abaxial establishment, for which they invoke the Sussex signal hypothesis [14].

After discovering the role of *PHAN* in *Antirrhinum*, several genes involved in leaf morphogenesis and positioning were cloned and characterized in *Arabidopsis*. Members of the Class III HD-Zip gene family act redundantly and are expressed in the adaxial domain of *Arabidopsis* leaves. They include five members named *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *REVOLUTA* (*REV*), *ATHB8* and *ATHB15* [16, 17]. Plants mutant for multiple members of this gene family exhibit a more dramatic phenotype compared to *phan* mutants – they lack an embryonic shoot meristem and in the most extreme cases, form a single radialized and abaxialized cotyledon in its place [17]. Gain of function mutants, in which suppression of HD-ZIPIII expression by miRNAs 165/166 is disrupted, develop leaf phenotypes similar to *phan* mutants in terms of radialized leaf shapes, but in this case, they consist only of adaxial cell types [16]. Plants mutant for multiple members of the abaxially expressed *KANADI (KAN)* genes on the other hand, develop leaves ectopically from the hypocotyl and abaxial side of the leaves [18]. Like gain of function HD-ZIPIII mutants, when KAN1 is expressed ectopically, leaves develop as rod-shaped structures [19]. To summarize, while many of the phenotypes associated with disruptions to adaxial-abaxial patterning result in a radially symmetrical leaf shapes, similar to wound-induced phenotypes, defects in leaf positioning are also apparent in loss of function mutants, hinting at a new connection between organ positioning and adaxial/abaxial patterning.

Is auxin Sussex's inductive signal?

Intriguingly, a phenotype similar to that caused by wounding has been reported to occur after applying auxin to the adaxial side of the leaf primordia in tomato [8] (Figure 4A). Using the DII auxin signalling sensor to monitor auxin levels [20], this study also reported that in *Arabidopsis*, adaxial leaf tissues exhibit lower levels of auxin compared to abaxial parts of the leaf. Linking these observations, the authors propose that low auxin levels in adaxial leaf tissues are critical for adaxial cell type specification (Figure 4B) and that wounding prevents depletion, thereby causing an abaxialized phenotype [8] (Figure 4C). The authors provide evidence that low auxin levels result from auxin movement from developing leaf primordia to the shoot apical meristem, as earlier reported for flowers [21]. Thus, they suggest a different model for the original Sussex signal; instead of a signal originating from the meristem and moving to the incipient leaf, they suggest a signalling molecule, in this case, auxin, is antagonistic to adaxial cell fate and moves from the adaxial side of the leaf primordium towards the shoot apical meristem. Further supporting the auxin depletion model, a subsequent study from the same research group

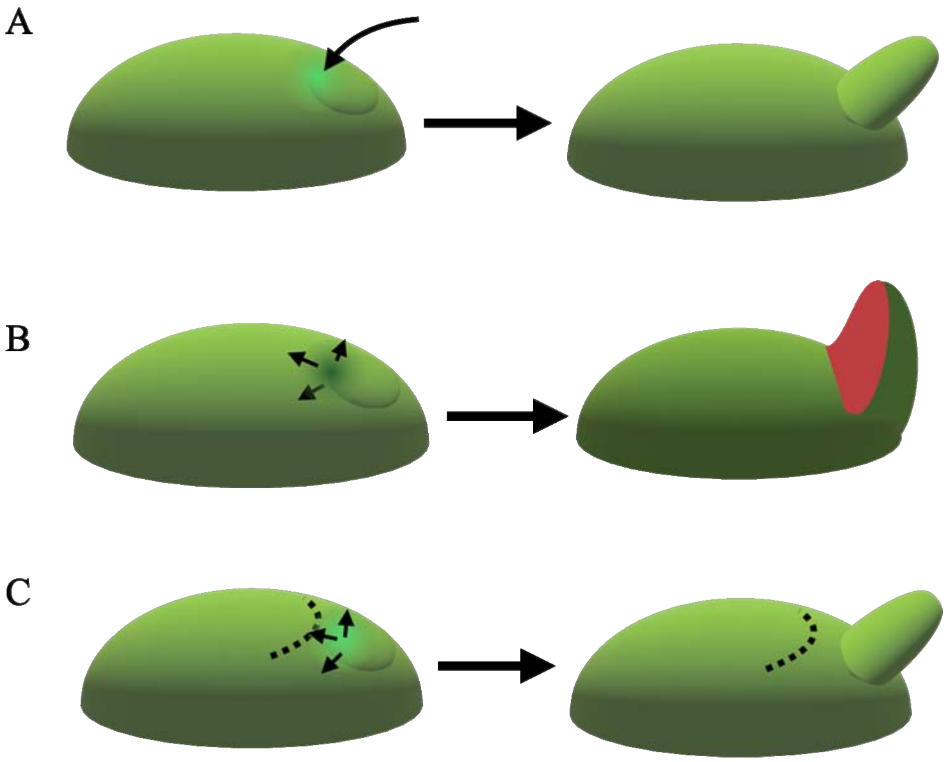

Figure 4. Reverse Sussex signal hypothesis [8]. A. Auxin application to the adaxial side of tomato leaf primordia causes an abaxialized phenotype. **B.** After an initial build-up of auxin that initiates organ formation, auxin is subsequently transported away from the primordium during normal development [21, 8]. **C.** Wounding is thought to prevent transport of auxin away, leading to abnormally high auxin levels in adaxial leaf tissues and subsequent abaxialization, as in (A).

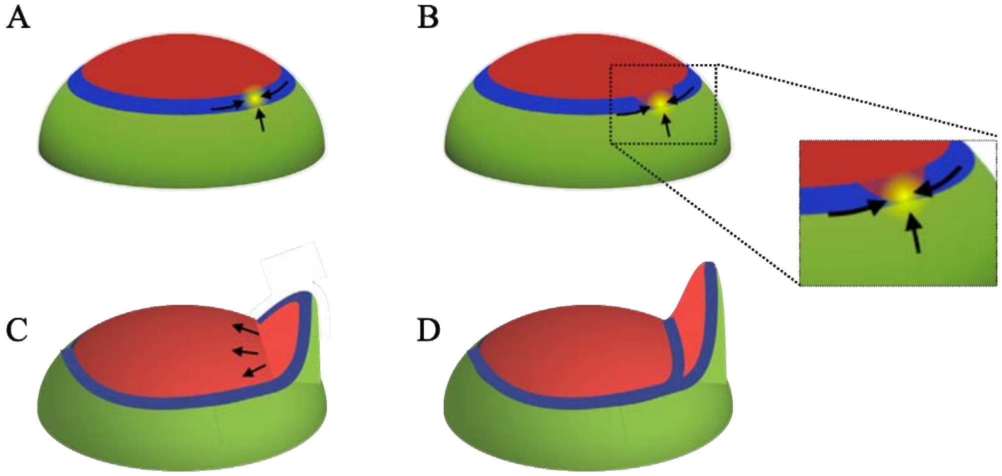

Figure 5. Patterns of REV (red), AS2 (blue) and KAN1 (green) as well as auxin (yellow) during organ inception [7]. Directions of auxin transport shown by black arrows. **A.** Initially, auxin is concentrated between the REV and KAN domains overlapping with AS2. **B.** REV expression extends peripherally towards the primordium. **C.** Auxin transport later directs auxin away from the primordium [21, 8]. **D.** REV expression reduces adjacent to the primordium.

concluded that maintaining low auxin levels in the adaxial leaf primordium restricts the expression of middle domain genes WOX1 and PRS, from adaxial leaf tissues while Auxin response Factors (ARFs) abaxially suppress WOX1 and PRS abaxially [9]. In contrast, it has separately been reported that auxin application to developing *Arabidopsis* leaves does not cause ectopic WOX1 or PRS expression [7]. Furthermore, the reported difference in auxin concentration between the two sides of the leaf has been disputed [22, 23] (see below for further discussion).

Pre-pattern or Sussex signal?

In addition to being expressed in leaves, KANADI and the Class III HD-ZIP transcription factors are expressed in the shoot meristem [16, 24]. Detailed examinations of their shoot expression domains reveal they form non-overlapping concentric patterns [7, 25] and that leaf initiation, as marked by high PIN1 expression, is centred on the boundary region between these domains (Figure 5A) [7]. During organ initiation, the pattern of KAN1 expression remains relatively stable with respect to the primordium progenitor cells while REV expression extended into these progenitors after inception (Figure 5B-D) [7, 25]. These findings reveal that KAN1 already marks the radial axis of the shoot in a way that closely corresponds to the adaxial-abaxial patterning of emerging leaves, supporting prior proposals that leaf tissue polarity is derived from radial patterning of the shoot [4]. By perturbing the expression of KAN1 specifically in shoot tissues, Caggiano *et al*. found that leaf polarity was subsequently changed accordingly, demonstrating a functional link between the two [7].

Given that loss of either KAN or HD-ZIPIII function leads to ectopic leaf formation [18], Caggiano *et al*. [7] also proposed that in the shoot apical meristem, the HD-ZIPIII and KAN genes act where they are expressed to suppress auxin-induced organ initiation. Thus, only at the boundary, where both sets of gene's expression levels are lower (although not completely absent [26]), can organs form (prior to the extension of HD-ZIPIII expression into the primordium). Supporting this proposal, when either REV and KAN1 are expressed ectopically throughout the shoot epidermis, organs fail to initiate [7]. According to this view then, adaxial-abaxial patterning arises in part, as a result of the broader role the KAN and HD-ZIPIII transcription factors have in helping position auxin-dependent cell proliferation, including the formation of new growth axes [7, 27].

More recently, another study also investigated adaxial-abaxial patterning in large vegetative *Arabidopsis* meristems [28]. Three conclusions from this study stand out. Firstly, by examining an auxin transcriptional reporter, the authors find that auxin response at primordium initiation sites is restricted to cells adaxial to the KAN1 expression domain and that in *kan1kan2* mutants, auxin transcriptional response expands abaxially, supporting the earlier proposal for KAN function in restricting auxin activity [7]. Secondly, AS2 was found to be expressed adaxially at the leaf initiation site prior to organ inception in a complementary pattern to KAN1. It was therefore found that AS2 and KAN1 pre-pattern leaf polarity [28]. Finally, after examining the DII auxin marker, the authors conclude that, consistent with Qi *et al*., 2014 [8], an auxin minimum does exist specifically within the adaxial tissues of leaf primordia - but only transiently, before low auxin levels mark both adaxial and abaxial leaf tissues equally [28]. This observation helps to resolve earlier controversy over the auxin distribution in young leaf primordia [22, 23] but leaves open the question of whether low levels of auxin are necessary to restrict WOX and PRS expression to the middle domain of leaves in *Arabidopsis* [7, 9], although the data for tomato unambiguously support such a role [29] (see below).

Pre-pattern and Sussex signal?

If leaf adaxial-abaxial patterning is pre-patterned, what about the effects of wounding as revealed by Sussex? It turns out that one of the consequences of wounding is a change in auxin levels, due to the transport of auxin away from the wound [30, 31]. In addition, auxin regulates the expression of the HD-ZIPIII and KAN organ polarity genes. Caggiano *et al*., 2017 for instance find that when auxin is applied exogenously to the inflorescence meristem, it leads to a slight expansion of the REV expression domain out to the pre-existing boundary of KAN1 expression (mirror the dynamic changes in expression of these genes during organ initiation) [7]. In contrast, lower auxin levels lead to an expansion of KAN1 expression towards the meristem centre at the expense of REV expression [7]. As might be expected from these results, wounding also causes KAN1 expression (i.e. abaxial identity) to expand at the expense of REV expression (adaxial identity) around the wound and this response can be completely blocked by applying exogenous auxin [7]. Thus, auxin appears to be a signalling molecule that promotes adaxial identity, which wounding depletes – corresponding closely to what might be expected for a 'Sussex signal' [32]. Surprisingly however, while wounding was found to alter the expression of adaxial and abaxial genes in the shoot periphery, changes to the polarity of *Arabidopsis* leaf primordia were not detected and no radial leaf phenotype was observed [7], unlike the situation in tomato and potato [3, 10]. One possible reason for this was thought to be that the vegetative meristems used for the *Arabidopsis* experiments were very small. To address this issue, a separate study repeated the wounding experiments in *Arabidopsis* using older plants with larger vegetative meristems - but still the abaxialized leaf phenotype observed in the Solanaceae could not be reproduced [33]. Thus, while wounding's influence on auxin may still account for associated disruptions to leaf polarity, *Arabidopsis* leaves are somehow wound-insensitive.

Tomato revisited

As mentioned above, significant differences have been reported between *Arabidopsis* and tomato in the sensitivity of adaxial-abaxial patterning to both wounding and auxin. In both *Arabidopsis* and tomato, wounding promotes abaxial cell identity over adaxial. However, while this applies to developing leaves in tomato [10], in *Arabidopsis,* wounding only promotes abaxial tissue identity in meristem tissues [7, 33]. Regarding the role of auxin, different studies have reported conflicting results. For instance, Qi *et al*., 2014 [8] report that exogenously applied auxin *antagonises* adaxial identity and promotes a radialized leaf shape in tomato and *Arabidopsis.* Caggiano *et al*., 2017 [7] on the other hand report that exogenous auxin *promotes* adaxial identity (REV expression) in the *Arabidopsis* meristem but does not cause a change to leaf polarity. Indeed, more recent work provides evidence that REV is a direct target of the auxin responsive transcriptional regulator MONOPTEROS [26]. A recent study now investigates wounding and auxin in tomato in more detail [29]. Consistent with the situation in *Arabidopsis*, the authors find that auxin is depleted around tomato wounds and that wounds also lead to a loss of SlREV expression and an expansion of SlKAN2C expression (protein and mRNA) around the wound after 12 hrs and 2 days respectively. In contrast to wounding and consistent with auxin promoting **adaxial** identity, application of auxin and NPA to the tomato apex led to a small increase in REV expression and reduction in KAN expression 24 hrs after treatment. Seven days later however, the leaf developed a radialized shape and SlREV expression was not visible (SlKAN2C expression was not reported). Thus, in response to auxin, there is an initial slight strengthening of adaxial SlREV expression followed by a slower loss of expression - which is different to what is observed for wounding, indicating that the two perturbations are not as related as initially assumed [8]. In agreement, the authors suggest that in *Arabidopsis*, leaves are robust to wounding because they may synthesize their own auxin, while tomato leaves may not [29] - implying that auxin depletion [7] rather than accumulation [26] may be the root cause of wound-induced loss of leaf polarity. Finally, the authors show that by inhibiting the 26s Proteosome pathway, wound-induced reductions in the SlREV protein could be prevented. The authors conclude from this latter experiment that wound-induced SlREV depletion is independent of canonical auxin signalling. However, what happens at the protein level does not explain wound-induced reductions of SlREV mRNA [29]; hence the role of canonical auxin signalling in the transcriptional response of SlREV to wounding remains to be investigated. Overall then, what seems most striking from this recent tomato study is how similar, rather than different, the *Arabidopsis* and tomato wound responses are in terms of auxin, HD-ZIPIII and KAN expression and that the radialization of tomato leaves in response to exogenous auxin, may be a distinct phenomenon.

CONCLUSION

The establishment of leaf adaxial-abaxial patterning has received significant attention from plant developmental biologists and several conflicting models have been proposed to explain this process. In this review, we have discussed and compared them in the context of the inductive signal original proposed by Sussex. While wounding experiments have suggested the existence of a shoot-derived signal necessary to promote adaxial leaf cell fate [3], it has also been argued that leaf primordia must be pre-patterned along their adaxial-abaxial axis at inception, due to the radial patterning of the shoot from which they develop [4]. How have more recent molecular studies influenced the debate? On the one hand, the analysis of molecular markers and experimental perturbations of gene expression demonstrate that radial patterning of the shoot does indeed pre-pattern leaf polarity from inception [7, 25, 28]. However, at the same time, the plant hormone auxin has been identified as a signalling molecule that also helps promote adaxial cell identity (via HD-ZIPIII expression) during organ initiation, which is depleted by wounds, not only in *Arabidopsis* but also in tomato, making auxin a very good candidate for the Sussex inductive signal as originally conceived [3]. Overall, the picture that is starting to emerge then is more nuanced. While a radial pre-pattern of transcription factors helps to establish leaf polarity, the wound-sensitive signalling molecule auxin also contributes, at least in part by promoting adaxial HD-ZIPIII expression over abaxial KAN expression, although the sensitivity of this pathway specifically for leaf development varies between species. We look forward to more updates on this fascinating story including on the role of auxin synthesis for the wound response and the characterization of wound responses in additional species including in monocots.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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