Functional dissection of the DORNRÖSCHEN-LIKE enhancer 2 during embryonic and phyllotactic patterning

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Abstract

Main conclusion The Arabidopsis DORNRÖSCHEN-LIKE enhancer 2 comprises a high-occupancy target region in the IM periphery that integrates signals for the spiral phyllotactic pattern and cruciferous arrangement of sepals.

Abstract Transcription of the DORNRÖSCHEN-LIKE (DRNL) gene marks lateral organ founder cells (LOFCs) in the peripheral zone of the inflorescence meristem (IM) and enhancer 2 (En2) in the DRNL promoter upstream region essentially contributes to this phyllotactic transcription pattern. Further analysis focused on the phylogenetically highly conserved 100-bp En2core element, which was sufficient to promote the phyllotactic pattern, but was recalcitrant to further shortening. Here, we show that En2core functions independent of orientation and create a series of mutations to study consequences on the transcription pattern. Their analysis shows that, first, in addition to in the inflorescence apex, En2core acts in the embryo; second, cis-regulatory target sequences are distributed throughout the 100-bp element, although substantial differences exist in their function between embryo and IM. Third, putative core auxin response elements (AuxREs) spatially activate or restrict DRNL expression, and fourth, according to chromatin configuration data, En2core enhancer activity in LOFCs correlates with an open chromatin structure at the DRNL transcription start. In combination, mutational and chromatin analyses imply that En2core comprises a high-occupancy target (HOT) region for transcription factors, which implements phyllotactic information for the spiral LOFC pattern in the IM periphery and coordinates the cruciferous array of floral sepals. Our data disfavor a contribution of activating auxin response factors (ARFs) but do not exclude auxin as a morphogenetic signal.

Keywords Auxin · Chromatin configuration · DORNRÖSCHEN-LIKE · Lateral organ founder cells · Mutational enhancer analysis · Phyllotaxy

Abbreviations

ARF Auxin response factor
AuxRE Auxin response element
DRNL DORNRÖSCHEN-LIKE
FM Floral meristem
IM Inflorescence meristem
LOFC Lateral organ founder cell
SAM Shoot apical meristem
TSS Transcription start site

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Introduction

The DORNRÖSCHEN-LIKE (DRNL) gene is one of the earliest available markers for cellular determination in the peripheral zone of the Arabidopsis thaliana shoot apical meristem (SAM) (Chandler et al. 2011b). DRNL encodes an AP2-type transcription factor (Kirch et al. 2003) and acts redundantly with its close relatives DORNRÖSCHEN (DRN) (Chandler et al. 2007) and PUCHI (Chandler and Werr 2017) to control meristem identity and organ initiation. DRNL transcription has been analysed by imaging DRNL::erGFP transgenic plants and starts in the apical
domain of the early embryo proper (Chandler et al. 2011a). Later in development, DRNL::GFP expression is restricted to founder cells of lateral organ primordia and starts with cotyledons in the embryo or leaves in the vegetative phase and prepatterns floral primordia in the peripheral zone of the inflorescence meristem (IM) or organs in all four floral whorls during the reproductive phase (Chandler et al. 2011b). The DRNL transcription pattern in lateral organ founder cells (LOFCs) is highly dynamic and is controlled by three enhancer elements designated En1, En2 and En3 (see top of Fig. 1a), which are distributed within 5.6 kb of upstream DRNL promoter sequences (Comelli et al. 2016). Enhancer En2 is located approximately 2.5 kb upstream from the DRNL transcription start site (TSS) and in the IM controls the spiral LOFC arrangement that prepatterns floral primordia redundantly with the more distal element En1 (Comelli et al. 2016). According to progressively increasing external and extended internal deletions of the DRNL promoter, enhancer En2 in the absence of En1 is necessary to activate transcription in the spiral LOFC pattern. The central 100-bp En2core sequence that is highly conserved and shares 87 invariant nucleotide positions in six distant Brassicaceae species is sufficient for En2core expression (Comelli et al. 2016).

Imaging of the inflorescence apex has revealed that DRNL::GFP expression initiates in close proximity to the central stem-cell zone (Seeliger et al. 2016) and ongoing cell divisions displace DRNL::GFP-expressing LOFCs to the IM periphery, where the GFP signal bifurcates into two domains of which the outer or basal signal transiently marks the position of the bract; whereas, the inner or apical domain prepatterns the abaxial sepal (Chandler et al. 2011b; Chandler and Werr 2014). Expression in the abaxial sepal is followed by two expression foci that simultaneously prepattern the

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Fig. 1  *DRNL* promoter structure and mutations in the En2core enhancer element. **a** A schematic representation of the microsyntenic *DRNL* promoter upstream region up to the flanking *At1g24600* gene (*DRNLLONG*) and previously established enhancer elements En1, En2 and En3 (Comelli et al. 2016). Depicted below are the external DRNLshort deletion that identified the redundancy between enhancer elements En1 and En2 and the 400-bp deletion SD En2 within the DRNLshort promoter construct that functionally identified the role of En2 in the IM. Reinsertion of the 100-bp En2core fragment into the 400-bp SD En2 deletion restored enhancer activity, whereas smaller overlapping 50-bp-bp fragments of En2core in En2coreD, En2coreE and En2coreF lines did not. Only the central En2coreD fragment that spans AuxRE1 and AuxRE2 showed occasional weak activity in sepalis of a few transgenic lines (Comelli et al. 2016). **b** Molecular structure of new En2core mutations created within the DRNLshort promoter construct, which from top to bottom depict inversion of the 100-bp En2core fragment in SEn2coreinv, the position of nine scanning mutations (Scan1-9) and the 5-bp deletions Δ 5.1, Δ5.2 and Δ5.3 within the spacer region between AuxRE1 and AuxRE2 or the point mutations created within the core AuxRE tetranucleotides. The brown marked regions represent putative PIF protein binding sites (Mironova et al. 2014).
positions of both lateral sepalas before DRNL::GFP expression is activated in LOFCs for the adaxial sepal. At the end of floral stage 1 (Smyth et al. 1990), the specification of LOFCs for the adaxial sepal coincides with the histological appearance of a furrow that separates the floral meristem (FM) from the IM (Chandler et al. 2011b). The four groups of sepal LOFCs are, thus, all specified before activation of the WUSCHEL and CLAVATA3 stem-cell markers indicates FM autonomy from the IM (Goldshmidt et al. 2008; Seeliger et al. 2016). Accordingly, DRNL transcription accompanies cellular fate decisions that begin close to the stem-cell zone (Seeliger et al. 2016), are perpetuated to the outer IM periphery and extend into the stereotypic cruciferous arrangement of sepalas in the outer floral whorl before the floral meristem gains autonomy (Chandler and Werr 2014). En2 and particularly its central, most conserved En2core part in the absence of distal DRNL promoter elements are sufficient to report phyllotactic information (Comelli et al. 2016).

Plant phyllotaxis exemplifies the highly reproducible patterns within biological systems (Wolpert 1969) and the most widely accepted theory assumes the presence of inhibitory fields that originate from developing lateral primordia at the SAM periphery (Reinhardt et al. 2003; reviewed by Traas 2013). In concert, overlapping inhibitory fields that originate from incipient primordia might generate a dynamic spatio-temporal pattern that allows local organ initiation below a threshold inhibitor level. Auxin has been implicated in this network (Reinhardt et al. 2000) and the polarly localised PIN-FORMED 1 (PIN1) efflux carrier directs auxin to the sites of organ initiation and, thus, creates a concentration gradient around newly initiated organs (Reinhardt et al. 2003). Although auxin functioning as a morphogen was initially considered to be sufficient to generate appropriate phyllotaxy (Reinhardt et al. 2000, 2003); an inhibitory field involving non-cell-autonomous cytokinin signalling by the ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6 (AHP6) inhibitor was identified to regulate the position of lateral organ initiation or the timing of successive primordia initiation in crosstalk with auxin (Besnard et al. 2014). The AHP6 promoter is a potential target of the DRNL transcription factor (Ikeda et al. 2006) and the transcription patterns of both genes in the IM peripheral zone are largely congruent (Chandler and Werr 2015). In contrast, DR5-reported auxin response maxima reside at the outer margin of the DRNL and AHP6 expression domains (Besnard et al. 2014; Chandler and Werr 2014), although point mutations in two core auxin response elements (AuxREs) have provided evidence that the DRNL En2 enhancer integrates an auxin response (Comelli et al. 2016). Two core GACA AuxRE motifs (Yamaguchi et al. 2013) in inverted orientation reside centrally within the 100-bp En2core region at a distance of 19 bp from each other, which approximates to two helical turns, suggesting that both face to one side of the DNA double helix. The entire 27-bp region is invariant in six Brassicaceae species and inactivating GCCA point mutations (Yamaguchi et al. 2013) in both core AuxREs abolishes En2 enhancer function (Comelli et al. 2016).

Auxin is essential for lateral organ initiation (Przemeck et al. 1996; Galweiler et al. 1998; Reinhardt et al. 2003), but auxin responses are complex. At the level of transcriptional control, the auxin response factor (ARF) family functions by binding to AuxREs in promoters of auxin-response genes. However, the ARF family contains activating or repressing members, which can recruit members of a second family of transcription factors, the Aux/IAA repressors, which are degraded depending on the intracellular auxin concentration. In addition, ARFs also homo- or heterodimerise (Boer et al. 2014), which involves combinatorial cooperativity and spacing constraints between AuxREs, such as the 19 bp between the putative core AuxREs in En2core (Comelli et al. 2016). Furthermore, canonical AuxRE motifs co-exist with non-canonical variants (Boer et al. 2014; Mironova et al. 2014) and are possibly recognised with different affinities. Additionally, ARFs functionally interact with other transcription factors (Guilfoyle and Hagen 2012) and well-studied examples are BRASSINAZOLE-RESISTANT1 (BZR) and PHYTOCHROME INTERACTING FACTOR 4 (PIF4) (Oh et al. 2014). G-boxes, the cis-regulatory target sites of these transcription factors, are enriched genome wide in the vicinity of AuxREs in Arabidopsis (Mironova et al. 2014) and both are present within En2core (Comelli et al. 2016). Together, the conservation of sequence and attributes suggests that En2core potentially functions as a unit and serves to recruit multiple transcription factors into a functional enhanceosome (Panne 2008). This interpretation is supported by an open chromatin configuration in IM cells and LOFCs (Frerichs et al. 2019).

Here, we describe a detailed mutational analysis of the DRNL En2core element (Comelli et al. 2016) and compare the effects of mutations in the IM and the embryo. The data reveal differences in the cis-regulatory sequences that are active in the embryo or IM and that include the core AuxREs, which spatially restrict DRNL transcription in the embryo. In the IM peripheral zone, expression in LOFCs appears to be closely coupled to DRNL expression in the sepalas. Apparently, En2core combines cis-regulatory elements used to integrate phyllotactic information for LOFCs specification in the IM periphery with elements that contribute to the determination of sepal anlagen in the outer floral whorl.

Materials and methods

DRNL promoter constructs

The DRNL\textsubscript{LONg}::GFP and DRNL\textsubscript{SHORt}::GFP promoter constructs have been described previously (Comelli et al. 2016)
and consist of 5634 bp or 3176 bp upstream of the DRNL translation start codon, respectively. A unique XmaI restriction site in front of the translation start allowed isolation of the DRNL promoter as an XmaI-AscI fragment, which for mutagenesis was subcloned into pBluescript II KS (+). The Phusion® site-directed mutagenesis kit (NEB) was used to delete the 100-bp En2core element and to create a unique FseI site with which to insert the modified En2core fragments. The double AuxRE1+2mut restriction site has already been described in Comelli et al. (2016), the single AuxRE1mut and AuxRE-2mut TGTC to TGCC mutations were created by primers in Table 1. The 5-bp deletion mutations Δ5.1, Δ5.2 and Δ5.3 were also created by the Phusion® site-directed mutagenesis kit (NEB) with primers listed in Table 1.

The Scan1–9 mutations were generated by the In-Fusion HD Cloning Kit (Clontech/Takara); the SΔ En2core promoter construct in pBluescript II KS (+) served as a recipient, which was linearised at the FseI site and the overhanging 3′ termini were trimmed with T4 polymerase. All nine mutated 100-bp En2core fragments were chemically synthesised as 130-bp single-strand oligonucleotides that contained 13-bp extra DRNL promoter sequences at either end plus a residual 2 bp from the FseI site, which served as targets for PCR amplification and recombination. Homologous recombination followed the manufacturer’s protocol; the resulting Scan1–9 mutation constructs were confirmed by DNA sequencing. Promoter fragments carrying En2core mutations were generally converted to a GFP expression cassette with flanking AscI sites for cloning into the binary pGPTV Asc-BAR vector (Überlacker and Werr 1996). Transformation of Agrobacterium tumefaciens (GV3101) and Arabidopsis thaliana L. Col-0 plants was performed as described in Bechtold and Pelletier (1998). Transgenic progeny were selected via BASTA resistance and were grown on soil in the greenhouse in long-day conditions (16 h light: 8 h dark).

**Confocal imaging**

Expression of GFP was monitored using a Zeiss LSM 700 confocal laser scanning microscope. GFP was excited at 488 nm and emission was analysed between 502 and 525 nm. Image Z-stacks were converted into 3D images using Imaris software (Bitplane, Zürich, Switzerland) to visualise DRNL expression at all floral developmental stages. Floral staging was assigned according to Smyth et al. (1990) and images were processed using Photoshop CS2 software (Adobe). At least six independent transgenic lines for each En2 core mutation were compared for expression in the embryo, IM and stage1 FMs.

**Results**

**En2 mutagenesis**

The DRNL promoter constructs used in previous analyses are schematically summarised in Fig. 1a and their characterisation identified three enhancer elements designated En1, En2 and En3 in the DRNL promoter upstream region extending to the upstream At1G24600 gene (Comelli et al. 2016). Both distal enhancers En1 and En2 synergise in the DRNLlong promoter construct to enhance transcription in the IM or early FM. In contrast, when En1 is absent in the DRNLshort promoter version, En2 is necessary and sufficient to

### Table 1 Primers used to create En2core mutations

| PCR primers to create SΔEn2core | TGGCCCGTTCCTGGATCCTCATTTTCC | TGGCCCGTTCCTGGATCCTCATTTTCC |
|----------------------------------|-------------------------------|-------------------------------|
| Del2b-CseR                      | GCGCGGTTGACCTTTTCC           | GCGCGGTTGACCTTTTCC           |
| Del2b-CseF                      | GCGCGGTTGACCTTTTCC           | GCGCGGTTGACCTTTTCC           |

### Oligos and PCR primers to create scanning mutations Scan1–9

| Scan1          | TGGCCCGGTTGACCTTTTCC |
|----------------|----------------------|
| Scan2          | TGGCCCGGTTGACCTTTTCC |
| Scan3          | TGGCCCGGTTGACCTTTTCC |
| Scan4          | TGGCCCGGTTGACCTTTTCC |
| Scan5          | TGGCCCGGTTGACCTTTTCC |
| Scan6          | TGGCCCGGTTGACCTTTTCC |
| Scan7          | TGGCCCGGTTGACCTTTTCC |
| Scan8          | TGGCCCGGTTGACCTTTTCC |
| Scan9          | TGGCCCGGTTGACCTTTTCC |

2b-c15bpoveryFR   | CATTTTGGATGCTACCTTTTCC |
3b-c15bpoveryFR   | GAGATGCTACCTTTTCC |

### PCR primers to create the 5-bp deletions ΔS–1, ΔS–2 and ΔS–3

| AuxRE Del5bp1bF | GACATTTGCTACCTTTTCC |
|-----------------|---------------------|
| AuxRE Del5bp2bR | GCCGCTTTTCCAAAAGCTG |
| AuxRE Del5bp2F  | ACTTTGCTACCTTTTCC  |
| AuxRE Del5bp2R  | GCCGCTTTTCCAAAAGCTG |
| AuxRE Del5bp3bF | GTATCAATTTGCTACCTTTTCC |
| AuxRE Del5bp3bR | CAGATTTGCTACCTTTTCC |
promote *DRNL* transcription in the spiral LOFC pattern at the IM periphery and in the stage 1 floral buttress (Comelli et al. 2016). This En2 function correlated phylogenetically with a high degree of sequence conservation in the 100-bp En2core element, which is sufficient and fully functional when inserted into a larger 400 bp deletion SΔEn2 (Fig. 1a) that removes En2 in addition to flanking sequences (Comelli et al. 2016). Unfortunately, the 100-bp En2core element was recalcitrant to further dissection and three overlapping 50-bp sub-fragments (En2coreD-F in Fig. 1a) tested previously essentially lacked enhancer function.

We, therefore, adopted a less disruptive strategy and firstly created a set of linker-scanning mutations that shifted a 10-bp stretch of alternating AT nucleotides throughout the En2core sequences (Scan1–9). Secondly, we generated 5-bp deletions (Δ5.1, Δ5.2 or Δ5.3) in the evolutionarily invariant 19-bp spacer that separates the putative core AuxRE1 and AuxRE2 motifs. Although these 5-bp deletions overlapped with scanning mutations Scan3, 4 or 5, the deletions differed substantially; whereas the scanning mutations preserved distances, each 5-bp deletion reduced the distance between AuxRE1 and AuxRE2. More importantly, however, each 5-bp deletion reduced half a helical turn, which might affect the relative orientation of AuxREs from both being oriented to one face of the DNA double helix to being on opposite faces, which potentially also affects sequences at the outer flank of AuxRE1 and 2. Lastly, as in the previous study, we had only simultaneously mutated both AuxREs (AuxRE1 + 2mut in Fig. 1b); we now additionally mutated each putative core AuxRE element by converting GACA to GCCA in each (AuxRE1mut, AuxRE2mut) (Ulmasov et al. 1999; Yamaguchi et al. 2013). All the internal En2core mutations are depicted in Fig. 1b, which also includes SEn2inv, in which the En2core sequences have been inverted.

**En2 mutation affects DRNL expression in the embryo**

To directly compare the expression pattern observed with individual En2core mutants to the wild-type pattern, Fig. 2a–f combines stage-specific expression patterns of the *DRNL*short::GFP construct, which provided the basis for the mutations. Although comparison of *DRNL*Long::GFP and *DRNL*short::GFP expression patterns in developing wild-type embryos revealed no obvious differences, we have combined the *DRNL*Long::GFP embryo patterns in Supplementary Fig. S1a, as they refine a previous analysis described in Chandler et al. (2011a). Following expression in the apical domain of the 8- to 16-cell proembryo (Fig. 2a), expression becomes focused to incipient cotyledon primordia at the late globular stage and is repressed in the prospective SAM (Fig. 2b). From the heart to early torpedo stage, *DRNL*short::GFP expression was present throughout the emerging cotyledons (Fig. 2c, d), but became restricted to a small subset of cells in the cotyledon tips by the late torpedo stage (Fig. 2e). Expression in the cotyledons initially included the L1 layer (Fig. 2g), but was restricted to cells in the sub-epidermal layer during the torpedo stage (Fig. 2e), a layering that was less pronounced in later walking stick embryos (Fig. 2f). This expression pattern strictly depended on En2 activity and was completely lost when En2 was absent, in external deletions starting from *DRNL*Long (ΔEXs) or internal deletions of the *DRNL*short promoter (SΔEn2), both described in Comelli et al. (2016). For direct comparison with the expression of *DRNL*short::GFP pattern on top, the second row (Fig. 2g-l) depicts embryos of SΔEn2core::GFP lines, where the 100-bp En2core element was deleted. Supplementary Fig. S1b shows similar results at two representative stages resulting from the larger 400-bp SΔEn2 deletion, where GFP expression is absent but restored in SΔEn2 + En2core::GFP lines, i.e. when the 100-bp En2core element replaced the deleted native 400-bp sequences (compare Fig. S1b and c). Embryonic GFP signals independent of En2 enhancer activity were only observed late in the walking-stick embryo at the flanks of the embryonic SAM in incipient vegetative leaf primordia. This leaf aspect of the expression pattern was robust and was only absent in external deletions when most of the DRNL promoter upstream sequences were removed, i.e. when enhancers En1, En2 and En3 were absent (Comelli et al. 2016).

Changes observed in the embryonic GFP expression pattern with individual En2core mutations are tabulated in Fig. 2m, where the horizontal axis corresponds to the embryo stages shown in Fig. 2a–f. At least 10 and on average 15 embryos per stage and line were analysed for six independent homozygous transgenic lines for each embryonic stage. Accordingly, the upper vertical row (SΔEn2En2core) in Fig. 2m depicts deletion of En2 and corresponds to Fig. 2g–l, where GFP signals are absent, except in incipient leaf primordia. The non-stained tabular fields in row 1 mean that no signal was observed in at least 60 embryos from six independent lines per stage, although a few (<0.5%) exceptions were occasionally observed in single plants, probably due to cross-fertilisation. In contrast, inversion of En2core in SEn2inv::GFP lines generated patterns that were indistinguishable from those of *DRNL*short::GFP lines and are indicated by green tabular fields in row 2. The overlapping 50-bp sub-fragments En2coreD and En2coreF (Comelli et al. 2016) were insufficient to restore expression of SΔEn2 (Fig. 2m); whereas, we observed expression with the En2coreE insert in the SΔEn2 400-bp deletion in two out of six lines in several late torpedo or walking-stick stage embryos with signals at the cotyledon tips (Suppl. Fig. S1e). This partial rescue is indicated by the light green-filled fields in row four. The late cotyledon activity depends on the integrity of both core AuxREs, because GFP expression in the
The AuxRE1 + 2mut construct was absent in the cotyledon tips from the torpedo stage onwards (Suppl. Fig. S1d). By contrast, both AuxREs were not required for expression at the transition from the globular to the heart stage, when DRNL promoter activity was restricted to cotyledon founder cells and was lost in the SAM anlage (Fig. 2n, Suppl. Fig. S1b). However, single or double AuxRE mutations led to reduced expression in the emerging cotyledons at the heart stage (Fig. 2m) and remaining expression was often asymmetric and occasionally confined to a single cotyledon or ectopically extended into the L1 layer (Fig. 2o, p). According to the 5-bp deletions Δ5.1, Δ5.2 or Δ5.3, neither DRNL LONG promoter constructs at successive embryonic stages as indicated above each CLSM picture. The pattern is identical to that of the DRNL LONG promoter version included in Suppl. Fig. S1a. g–l Results obtained following deletion of the 100-bp En2CORE element in SΔEn2CORE lines and representative examples with the longer 400-bp deletion SΔEn2 (Comelli et al. 2016) are shown in Suppl. Fig. S1b. The only remaining expression was observed in incipient leaf primordia initiated after the late torpedo stage, robust transcriptional activity that is redundantly controlled in the DRNL promoter (Comelli et al. 2016). m Tabular summary of changes in expression patterns observed with individual En2CORE mutations; columns correspond to the series of embryonic stages in a–f. Dark green indicates no difference from the wild-type pattern; no colour means the absence of DRNL expression at this embryo stage, whereas light green indicates quantitative changes in expression, which either consisted of reduced or increased (enhanced) GFP signal intensity or a spatially aberrant (ectopic) GFP pattern. The lower panel (n–t) shows altered expression patterns specific for individual En2CORE deletions. In AuxRE1,2mut lines (n, t), Activity was normal in the emerging cotyledons during the late globular/early heart stage (n), but ectopic activity in the L1 layer was detected at the torpedo stage (o); a similar result was observed for AuxRE1mut (p). Scan4 lines exhibited reduced GFP signal intensity during the early torpedo stage (q), whereas Scan6 or Scan9 (data not shown) lines showed an increased GFP signal, which was also spatially expanded at this stage (r). Subtle dynamic changes were observed later in Scan9 lines: the GFP signal was still absent in the L1 layer at the tip of the cotyledons at the torpedo stage (s) but was observed here later (t), together with epidermis-specific expression in the hypocotyl.

Fig. 2 DRNL::GFP expression during embryogenesis and the contribution of the En2CORE enhancer. a–f GFP expression pattern observed with the DRNLshort promoter constructs at successive embryonic stages as indicated above each CLSM picture. The pattern is identical to that of the DRNL Long promoter version included in Suppl. Fig. S1a. g–l Results obtained following deletion of the 100-bp En2CORE element in SΔEn2core lines and representative examples with the longer 400-bp deletion SΔEn2 (Comelli et al. 2016) are shown in Suppl. Fig. S1b. The only remaining expression was observed in incipient leaf primordia initiated after the late torpedo stage, robust transcriptional activity that is redundantly controlled in the DRNL promoter (Comelli et al. 2016). m Tabular summary of changes in expression patterns observed with individual En2CORE mutations; columns correspond to the series of embryonic stages in a–f. Dark green indicates no difference from the wild-type pattern; no colour means the absence of DRNL expression at this embryo stage, whereas light green indicates quantitative changes in expression, which either consisted of reduced or increased (enhanced) GFP signal intensity or a spatially aberrant (ectopic) GFP pattern. The lower panel (n–t) shows altered expression patterns specific for individual En2CORE deletions. In AuxRE1,2mut lines (n, t), Activity was normal in the emerging cotyledons during the late globular/early heart stage (n), but ectopic activity in the L1 layer was detected at the torpedo stage (o); a similar result was observed for AuxRE1mut (p). Scan4 lines exhibited reduced GFP signal intensity during the early torpedo stage (q), whereas Scan6 or Scan9 (data not shown) lines showed an increased GFP signal, which was also spatially expanded at this stage (r). Subtle dynamic changes were observed later in Scan9 lines: the GFP signal was still absent in the L1 layer at the tip of the cotyledons at the torpedo stage (s) but was observed here later (t), together with epidermis-specific expression in the hypocotyl.

S1d). However, single or double AuxRE mutations led to reduced expression in the emerging cotyledons at the heart stage (Fig. 2m) and remaining expression was often asymmetric and occasionally confined to a single cotyledon or ectopically extended into the L1 layer (Fig. 2o, p). According to the 5-bp deletions Δ5.1, Δ5.2 or Δ5.3, neither
the distance nor the relative orientation of AuxRE1 and AuxRE2 affected DRNL expression at these stages.

The early globular embryo stage was most sensitive to En2core mutations, in which expression was abolished (i) by the AuxRE1 + 2mut double point mutation but not by single AuxRE mutations; (ii) by each of the three central core 5-bp deletions (Δ5.1, Δ5.2, Δ5.3) and (iii) by six scanning mutations, Scan1, 3, 4, 5, 7 and 8. Strikingly, although individual point mutations in the AuxRE1mut or AuxRE2mut constructs did not affect early GFP expression, the overlapping Scan3 or Scan5 mutations and Scan4, which affected sequences in the invariant 19-bp spacer that separates AuxRE1 and AuxRE2, abolished En2core activity. This central region of En2core is not only particularly important for enhancer activity in the early Arabidopsis embryo, but also consistent with the unique partial rescue of the late embryonic pattern observed with SΔEn2 + En2core and a similar partial rescue previously detected in the IM (Comelli et al. 2016).

The central Scan3, 4 and 5 mutants also concordantly and quantitatively reduced En2core activity at the late heart and early torpedo stage (Fig. 2q), when other mutations that affected the earliest embryonic DRNLshort::GFP expression, i.e. Scan1, 7 and 8, Δ5.1, Δ5.2 and Δ5.3 did not affect DRNL expression. Notably, DRNL promoter activity in the incipient cotyledons at the late globular stage was not affected by AuxRE1mut, AuxRE2mut and AuxRE1 + 2mut; whereas, these quantitatively reduced or caused ectopic expression in the L1 layer at subsequent stages (compare Fig. 2p–e). After the heart stage, several En2core mutations outside of AuxRE1 or 2 quantitatively affected GFP activity, for example: Scan4 (Fig. 2q) reduced and Scan6 (Fig. 2r) enhanced expression in early torpedo stage embryos. These quantitative effects normalised towards the late torpedo stage, when GFP expression in the cotyledon tips of Scan1, Scan3–8 and Scan9 lines again was similar to that of wild type (compare Fig. 2s, e). Scan9, however, was unique in causing ectopic DRNL expression at the walking stick stage: i.e. in the epidermis at the base of the cotyledons or the hypocotyl (compare Fig. 2s, t). The only En2core mutation without any phenotypic consequences in the early or late embryo was Scan2.

**Effects of En2 mutations on DRNL expression in the inflorescence apex**

Within the reference construct DRNLshort::GFP (Comelli et al. 2016), En2core activity is essential to promote expression in the phyllotactic LOFC pattern in the IM and accordingly, the SΔEn2core deletion construct was not active in the IM peripheral zone (compare Fig. 3a, b); whereas, inversion of the En2core sequences in SEn2core−inv did not affect expression (compare Fig. 3c to a). However, En2core deletion affected not only the LOFC-specific pattern in the IM peripheral zone, but also expression in early sepal primordia (compare Fig. 3a, b). This is similar to previous results with the larger SΔEn2 and also, due to established redundancy between En1 and En2, is only observed in the context of the DRNLshort promoter (Comelli et al. 2016). Expression patterns in the IM and sepals were extremely dynamic and therefore, we have grouped aspects of the pattern that were affected by En2core mutation into four categories that are schematically depicted in the top row of Fig. 3g. The diagrams are simplifications deduced from the scheme in Fig. 3d that illustrates the IM, associated developing flowers and signals therein that prepattern individual organs in the top view on the IM of the DRNLshort::GFP reference pattern in Fig. 3a. The most frequent phenotype was (i) the absence of GFP signals in the IM and sepal primordia, similar to SΔEn2core (Fig. 3e) or (ii) the selective absence of GFP signals in the IM but their presence in early sepals (Fig. 3e); permutations thereof were (iii) reduced GFP signal intensity in the IM peripheral zone and the absence of promoter activity in the abaxial and lateral sepal primordia, but remaining activity at the position of the adaxial sepal until floral stage 2 or (iv) no En2 activity in the IM and in lateral sepals but activity in the abaxial and adaxial sepals (Fig. 3f).

We previously showed that when both core AuxREs are mutated, En2 activity in the IM is lost (Comelli et al. 2016). Here, however, only AuxRE1mut abolished enhancer activity in the IM and in sepals; whereas, AuxRE2mut led to only mild reductions in DRNL promoter expression. This selectivity contrasts with the phenotype of scanning mutations Scan3 and Scan5 that covered AuxRE1 or AuxRE2, respectively, and that both eliminated En2core activity in the IM peripheral zone. Clear differences in the effect of both scanning mutations were observed in sepal primordia: for Scan5, DRNL expression was observed in the adaxial sepal and occasionally somewhat weaker in the abaxial sepal; whereas similar to AuxRE1mut + AuxRE2mut, expression was absent in the sepal of Scan3 lines. The third-strongest phenotype after AuxRE1mut and Scan3 was obtained with Scan7, which eliminated DRNL expression in the IM but retained weak signal in the adaxial sepal. More subtle effects on expression were caused by Scan1, 4, 5 and 8, which led to weaker DRNL promoter activity in the spiral LOFC pattern in the IM but differed with respect to the degree of relative expression in the abaxial and adaxial or lateral sepal primordia. Scan4 is located between core AuxRE1 and AuxRE2 and partly or fully overlaps the three 5-bp deletions (Δ5.1, Δ5.2 and Δ5.3), which all reduced DRNL expression in the IM periphery, but not in sepals, a phenotype that was strongest with Δ5.3 (Fig. 3e). Only Scan2 and Scan9 did not affect DRNLshort::GFP expression in the IM or early FM; however, Scan9 and Scan6 (see asterisks in Fig. 3g) both affected late DRNL promoter activity in the tips of growing sepals after floral stage 3.
In vivo footprinting of the active En2 enhancer

We previously applied fluorescence-activated cell sorting (FACS) with assay for transposase-accessible chromatin with high-throughput sequencing (ATAC-seq) and compared the chromatin state of DRNL::erGFP-expressing LOFC protoplasts in the apl1 cal IM (GFP +) with that of non-fluorescing (GFP-) cells (Frerichs et al. 2019). Peak calling revealed multiple transposase hypersensitive sites (THSs) throughout the microsyntenic region between DRNL and the upstream flanking AtG24600 gene (Fig. 4a). The qualitative difference between DRNL-expressing GFP + LOFCs and GFP- IM cells was greatest upstream of the DRNL TSS, where the chromatin in the proximal promoter region was only accessible in GFP + protoplasts (Fig. 4a). By contrast, peak calling of ATAC-seq reads across the En2 region revealed an open
chromatin configuration in GFP- cells but further increased openness in LOFCs, with highest read frequencies within the 100-bp En2core element (Fig. 4b).

Calculation of mean normalised read frequencies and read termini in three biological replicates in the En2core region on the nucleotide level (Fig. 4c) revealed minimal variability in the frequency and position of read termini between the GFP + or GFP- replicates, but consistently showed substantially higher frequencies of read termini in DRNL-expressing LOFCs. Accordingly, in the ap1 cal IM, the En2core element locates within an open chromatin region that becomes significantly more accessible (P < 0.0001) in LOFCs, when DRNL is actively transcribed. Novel cleavage sites unique to the transcriptionally active enhancer element were rare; a single potential candidate site is marked by an arrow at position 68 (Fig. 4c) and resides within Scan7, which eliminated En2core activity. However, En2core activity was also abolished by Scan3 or Scan5, which span AuxRE1 and AuxRE2, respectively. According to the AuxRE1mut phenotype, the distal element is functionally important, which correlates with few read termini within the AuxRE1 region in GFP + and GFP- cells. Although ATAC-seq read termini exhibited a sequence bias (Calviello et al. 2019), this contrasts to read termini within AuxRE2 in GFP- chromatin and even higher numbers in transcriptionally active chromatin from GFP + LOFCs. Differential in vivo transposase accessibility, thus, supports the phenotypic differences between AuxRE1mut and AuxRE2mut, i.e. accessibility in the latter is consistent with En2core activity that is unaffected in AuxRE2mut lines. By contrast, an adjacent 4-bp sequence towards the DRNL TSS that is devoid of read termini suggests it is protected from transcription and might explain the phenotypic discrepancy between the effects of AuxRE2mut and the overlapping Scan5 mutation.

**Activating ARFs and En2core function**

The phenotype resulting from the point mutation AuxRE1 + 2mut and AuxRE1mut suggests that AuxREs are functionally relevant for DRNL promoter activity, which overlaps with auxin response maxima in the IM. We, therefore, introduced DRNL::GFP into loss-of-function backgrounds of activating ARFs (Guilfoyle and Hagen 2007), i.e. arf5, arf6, arf7, arf8 or arf19 single mutants and due to known redundancy, into arf6 arf8 (Nagpal et al. 2005) and arf7 arf19 (Okushima et al. 2005) double mutants. No difference in the wild-type DRNL::GFP expression pattern was observed in inflorescence apices or embryos in any of these mutant backgrounds. Among arf mutants, arf5/monopteros is unique in exhibiting a lowly penetrant basal embryo domain phenotype followed by early seedling arrest (Hardtke and Berleth 1998). We, therefore, analysed DRNL::GFP expression in three arf5 alleles: the strong arf5-1 or mp-U55 alleles (Mayer et al. 1991) and the hypomorphic mp-S319/SALK_021319/arf5-2 allele (Alonso et al. 2003; Cole et al. 2009; Donner et al. 2009). Among a population segregating for the strong arf5-1 and mp-U55 alleles, wild-type DRNL::GFP expression was observed in embryos until the early globular stage. Homozygous mutant embryos only become morphologically distinguishable later and DRNL::GFP expression was detectable in the apical domain and in discrete foci in the presumptive cotyledons during the heart stage. Expression remained in the sub-epidermal layer of the cotyledon tips, although often in a broader expression domain than in wild-type embryos (Fig. 5a–d). By contrast, DRNL::GFP expression in the weak mp-S319 background differed spatially from that in both strong alleles and wild type; from the late heart stage, ectopic DRNL::GFP expression was observed in a central embryo domain and subsequently in the apical hypocotyl, where it weakly extended into the SAM and the cotyledon tips (Fig. 5e–h). By contrast
to in strong mp alleles, DRNL\textsubscript{SHORT}:GFP expression in mp-S319 cotyledon tips remained weak.

Because arf5-1 and mpU55 alleles show the developmental arrest of early seedlings, post-embryonic DRNL promoter activity can only be studied in the weak mp-S319 allele (Fig. 5i), for which we compared expression of DRNL\textsubscript{LONG}:GFP and DRNL\textsubscript{SHORT}:GFP. Within DRNL\textsubscript{LONG}, En2 synergises with the distal enhancer element En1 that is absent in DRNL\textsubscript{SHORT} (Comelli et al. 2016). In the occasional flowers that develop in the mp-S319 inflorescence, both promoter versions showed a patchy, discontinuous ring of DRNL::GFP expression (Fig. 5j, l). This contrasts with that in the IM, where DRNL\textsubscript{LONG}:GFP showed a similar ring-shaped expression (Fig. 5j, k), whereas expression of DRNL\textsubscript{SHORT}:GFP was substantially weaker and was frequently restricted to isolated cells (Fig. 5i, m). This reduction in signal intensity with DRNL\textsubscript{SHORT} in the IM relates to the synergy between En1 and En2 (Comelli et al. 2016), but in the mp-S319 background, neither the DRNL\textsubscript{LONG}:GFP nor the DRNL\textsubscript{SHORT}:GFP transgenes showed a similar phyllotactic expression pattern to wild type (compare Fig. 5 j–m with Fig. 3a).

To date, the mp-S319 allele has been described to contain a left-border T-DNA insertion at position 6,890,755 of chromosome 1, which has been estimated from the MP 3′ terminus and is located within the penultimate exon 12 of the MP
transcription unit (Alonso et al. 2003). Given the ectopic embryonic DRNL::GFP expression in the weak mp-S319 allele compared to the strong arf-1 and mp-U55 alleles, we queried whether a truncated transcript was present. Analysis of the mp-S319 right border revealed a large truncated T-DNA insertion exceeding the potential of long-range PCR reactions and joining two left T-DNA borders. This truncation removes 31 bp (6,890,746–6,890,774) of the MP transcription unit that include the 5′ splice-acceptor site of exon 12. RT-PCR amplifications with several MP-forward primers located in different exons and left-border T-DNA primers confirmed the presence of a spliced mp-S319 transcript that extends into the T-DNA sequence. This truncated mp-S319 mRNA is translatable into a shorter 815 amino-acid (aa) protein that deviates from the native MP gene product after aa 788, which is encoded close to the 3′ splice-donor site of exon 11. Read-through into non-spliced intron 11 sequences adds a 17-aa peptide (KRLSLIHSTNFLCHNLT), which terminates at a stop codon prior to the T-DNA insertion site.

When translated, this truncation causes deletion of the conserved ARF domains III and IV (Ulmasov et al. 1999) and results in a mp-S319 protein similar to the semi-dominant MP Δ transgene (Krogan et al. 2012), especially MPΔ-2 (1–794 aa). Therefore, we analysed expression of
DRNL::GFP in MP Δ transgenic plants, but we observed no difference from the wild-type DRNL expression pattern in embryos or inflorescences either in heterozygous or in homozygous MP Δ progeny, which can be discriminated according to leaf shape, vascular density or floral phenotypes (Krogan et al. 2012). Even when we reduced competing endogenous MP activity by crossing the MP Δ transgene into the arf5-1 mutant background we observed no alteration in the spiral DRNL::GFP expression pattern in LOFCs at the IM periphery. However, the MP Δ transgene failed to rescue homozygous arf5-1 mutants and whereas genotyping revealed Mendelian inheritance of the MP Δ transgene, we could not confirm a single homozygous arf5-1 plant among 50 progeny that according to strength of leaf and floral phenotypes, were homozygous for MP Δ. The changes in the DRNL::GFP expression pattern observed in the mp-S319 IM (Fig. 5j–m), thus, can hardly be explained merely by the absence of domains III and IV in the C-terminally truncated MP polypeptide.

Discussion

Deletion analysis of the DRNL promoter with emphasis on the IM peripheral zone previously identified En2 as an enhancer element that synergistically activates transcription in LOFCs (Comelli et al. 2016), together with a more distal element, enhancer En1. The phylogenetically highly conserved central 100-bp En2core sequences were essential for this function, and these sequences were subjected to a detailed molecular analysis here and in addition to acting in the IM, also function in the embryo, where similar to its parologue DRN, DRNL is initially expressed (Chandler et al. 2011a). The comparison of individual En2core mutations in the IM and the embryo showed: first, potential cis-regulatory target sequences are clustered throughout the 100-bp element; second, cis-elements function synergistically in the early embryo and the IM peripheral zone; third, putative core AuxRE sequences can spatially activate or restrict DRNL expression.

The greatest sensitivity to En2core mutations in the embryo was revealed in an early developmental window, when apical–basal polarity is established and 10 mutations (Fig. 2m) interfered with enhancer activity. This high sensitivity to sequence changes within En2core during early embryonic development suggests that the concerted activity of multiple cis-regulatory elements is required to establish a functional enhanceosome (Panne 2008). Subsequent maintenance of expression might involve fewer cis-regulatory sequences, because 7 out of 10 En2core mutations (Scan1, 2, 7 and 8 or Δ5.1, 2 and 3) had few transcriptional consequences at later embryonic stages. Their distribution within En2core agrees with the result of substitution experiments, in which only the central fragment (En2core) out of three overlapping 50-bp fragments (En2coreD,E or F in Fig. 1) weakly restored late DRNL promoter activity in the cotyledon tips, a rescue that according to AuxRE1 + 2neo lines, depends on the integrity of both AuxREs. A feature during late development is that many mutations, including point mutations in the putative core AuxREs, cause ectopic DRNL transcription, suggesting that some cis-elements potentially restrict DRNL promoter activity.

This loss of cell-type specificity in the embryo differs from that in the IM, where the most prominent phenotype is the absence of DRNL transcriptional activity, either in the spiral LOFC pattern within the peripheral zone or in early sepal primordia (Fig. 3b, g). The 5-bp deletions Δ5.1, Δ5.2 and Δ5.3 specifically affect DRNL transcription in LOFCs at the IM periphery (Fig. 3e, g) and suggest that the En2core centre is apparently critical to activate transcription in the phyllotactic LOFC pattern.

The LOFC-specific En2core activity in the IM and in sepal anlagen that encloses the prospective FM is observed with several mutations and relates to the development of the floral phytomer, which consists of a node, an axillary meristem, an internode and a bract. Bract outgrowth is suppressed in Arabidopsis, but occurs in puchi and leafy mutants, where DRNL::GFP expression persists from LOFCs into the incipient bracts (Chandler and Werr 2014). Bract outgrowth is a modulation of proximo–distal development and does not affect synchronised DRNL activity in the lateral sepals, but alters the sequence in which sepals are initiated, from abaxial, lateral then adaxial in wild type, to bract, lateral, abaxial and then adaxial sepal in puchi and leafy mutants (Chandler and Werr 2014). Within En2core, several mutations (Scan1, 4, 5 and 8) specifically affect DRNL expression in the two lateral sepals (Fig. 3f) and the IM and, thus, affect cis-regulatory sequences common to both patterning aspects. The most robust DRNL expression is observed in the adaxial sepal, which is specified last and reflects the bifurcation of the floral primordium from the inflorescence apex, the initiation of which at the end of floral stage 1 correlates with the de novo initiation of a stem-cell population in the FM (Goldshmidt et al. 2008). Dissection of En2core, thus, reveals: (i) cis-regulatory elements that monitor the phyllo-tactic position of new floral primordia within the IM periphery are either identical or in direct proximity to cis-motifs that provide coordinates for the robust crucifer arrangement of sepals; and (ii) such cis-regulatory sequences distinguish between lateral (Scan1, 4) and abaxial sepals (Scan7) before the FM acquires autonomy.

Available ATAC-seq data depicted the chromatin configuration of DRNL::GFP-expressing LOFCs and non-expressing neighbours in the ap1 cal IM (Frerichs et al. 2019) and in seedling stem cells (Sijacic et al. 2018). The comparison of available chromatin configurations allows three conclusions:
(i) En2 and especially \( \text{En2}\text{core} \) acquire an open chromatin configuration in the \( \text{apl cal IM} \), which is not detectable in seedling stem cells (Frerichs et al. 2019), (ii) chromatin is more open in \( \text{DRNL::GFP} \)-positive LOFCs than in \( \text{DRNL::GFP} \)-negative cells. However, increased openness in LOFCs is not due to largely altered transposase cleavage sites, which would indicate changes in transcription factor binding, but reflects local hypersensitivity in LOFCs at several discrete \( \text{En2}\text{core} \) positions. (iii) This chromatin hypersensitivity in \( \text{En2}\text{core} \) contrasts with that in the \( \text{DRNL TSS} \), which acquires an open configuration only in LOFCs, a long-range effect that is often associated with active transcription due to enhancer activity.

Although ATAC-seq data provide little evidence that transcription factors are recruited de novo to \( \text{En2}\text{core} \) for its expression within LOFCs, they support mutational analyses and indicate a compact genomic region targeted by multiple transcription factors. Such elements either guide the assembly of a multifactorial enhanceosome (Panne 2008), as is suggested by the orientation-independent function of the \( \text{En2}\text{core} \) element, or comprise a high-occupancy target (HOT) region (Foley and Sidow 2013). HOT regions in animals typically integrate signals from diverse regulatory pathways to quantitatively fine-tune immediate upstream promoter regions for RNA polymerase II recruitment. The cell-type-specific opening of chromatin in LOFCs at the \( \text{DRNL TSS} \) is compatible with RNA polymerase II entry (Frerichs et al. 2019); whereas, mutational analysis of \( \text{En2}\text{core} \) shows that phyllotactic signals in the IM peripheral zone integrate with those that pattern sepal anlagen in the flower.

The integration of auxin signalling into \( \text{En2}\text{core} \) function

Current models of phyllotactic signalling mainly focus on auxin as a morphogen (Reinhardt et al. 2003), although cytokinin signalling has also been acknowledged as an additional component (Besnard et al. 2014). The presence of two conserved putative core AuxREs in \( \text{En2}\text{core} \) and the essential integrity of AuxRE1, which also resides within a poorly accessible chromatin region, suggest that auxin contributes to \( \text{En2}\text{core} \) expression. By contrast, the \( \text{AuxRE2}\text{mut} \) mutation only subtly quantitatively affected \( \text{DRNL} \) expression and ATAC-seq reads terminate within the TGTC motif; which differs from directly flanking sequences that are inaccessible and are mutated in the phenotypic \( \text{Scan5} \) mutant that overlaps AuxRE2. Although the detrimental effect of \( \text{AuxRE1}\text{mut} \) and its inaccessible chromatin configuration suggest that auxin contributes to \( \text{En2}\text{core} \) activity in LOFCs, the spiral phyllotactic pattern of \( \text{DRNL} \) expression in the IM is selectively affected only by the deletions \( \Delta 5.1, \Delta 5.2 \) and \( \Delta 5.3 \). Each deletion removes 5 bp from the 19-bp spacer region, which either fully or partially overlaps with scanning mutation \( \text{Scan4} \) (Fig. 1b), which in addition to the spiral LOFCs in the IM, also affects \( \text{En2}\text{core} \) activity in lateral sepal anlagen. It is, therefore, unclear whether \( \text{En2}\text{core} \) transcription in LOFCs is directly due to sequence alterations in the AuxRE spacer region or to the 180° twist that results from deletion of half a helical turn from the centre of the \( \text{En2}\text{core} \) element. Concerted ATAC-seq hypersensitivity at the outer flanks of both AuxRE motifs suggests synergistic interactions between transcription factors bound to motifs at the outer flanks.

The effects of AuxRE point mutations differed in the early embryo, where \( \text{En2}\text{core} \) activity in the apical domain is abolished by the double mutation \( \text{AuxRE1}\text{1mut} + 2\text{mut} \), but single mutations in either \( \text{AuxRE1}\text{mut} \) or \( \text{AuxRE2}\text{mut} \) had no detectable consequences. Based on the hypothesis that changes in \( \text{DRNL::GFP} \) expression are dependent on activating ARFs, we analysed \( \text{DRNL::GFP} \) expression in loss-of-function alleles of \( \text{MP} \) (\( \text{ARF5} \)), \( \text{ARF6} \), \( \text{ARF7} \), \( \text{ARF8} \) and \( \text{ARF19} \). However, the \( \text{DRNL} \) expression pattern and level was similar to that of wild type in the embryos or IMs of single mutant \( \text{arf6} \), \( \text{arf8} \), \( \text{arf7} \) alleles or \( \text{arf6} \), \( \text{arf8} \) and \( \text{arf7} \) double mutants. MONOPTEROS is particularly relevant, as it is essential for transcription of the \( \text{DRNL} \) parologue DRN in the cotyledon tips and physically binds to canonical AuxREs in the DRN promoter upstream region (Cole et al. 2009). By contrast to \( \text{DRN} \), we observed \( \text{DRNL::GFP} \) expression in homozygous \( \text{arf5}\text{-}1 \) and \( \text{mp}\text{-}U55 \) phenotypic embryos (Fig. 5a–d); whereas, \( \text{DRN} \) is expressed in the L1 layer of the cotyledon tips, \( \text{DRNL} \) is present in subtending procambial cells (Chandler et al. 2011a). The differential dependence of \( \text{DRN} \) and \( \text{DRNL} \) transcription on MP, thus, relates to discrete cell-type specificity and is also consistent with distinct genetic interactions between \( \text{drn} \) or \( \text{drnl} \) loss-of-function alleles and \( \text{pinformed1} \) or \( \text{pinoid} \) mutants, which are hampered in auxin efflux or signal transduction, respectively (Chandler et al. 2011a).

Although \( \text{AuxRE1}\text{mut} \) links an AuxRE core motif with enhancer activity in LOFCs at the IM periphery, except for the pattern obtained in the \( \text{mp}\text{-}S319 \) background, we have obtained little evidence for a role of activating ARFs within the \( \text{En2}\text{core} \) element. Our data do not exclude that \( \text{En2}\text{core} \) responds to auxin, but mutational analyses and the chromatin configuration suggest that such a response would potentially have to integrate into a multifactorial protein–DNA complex. Two imperfect G-boxes (Figs. 1, 4) near to the putative core AuxREs within \( \text{En2}\text{core} \) are potential binding sites for PIF transcription factors, which interact with ARFs (Oh et al. 2014), and binding motifs for both transcription factor families co-occur genome wide (Mironova et al. 2014). In this context, the availability or local degradation of Aux/IAA proteins might be functionally relevant and be indirectly affected by the absence of domains III and IV in the \( \text{mp}\text{-}S319 \) background. The \( \text{MP} \) gene is transcriptionally up-regulated
in LOFCs and in the apical IM and belongs to the class of highly expressed ARFs (Frerichs et al. 2016); thus, the absence of domains III and IV might affect the interaction with cognate AUX/IAA inhibitory proteins (Guilfoyle and Hagen 2007). AUX/IAA proteins are locally degraded when auxin concentrations are high, which has been visualised in the IM via the DII-VENUS degron reporter (Brunoud et al. 2012). The DII-VENUS expression pattern spatially coincides with DR5::GFP-marked auxin response maxima in the IM, and both expression domains partially overlap with DRNL::GFP expression in LOFCs. The increased accessibility of the En2 chromatin to transposase cleavage in LOFCs could indicate the degradation of an inhibitory AUX/IAA interaction and gain of enhancer activity without recruitment of an extra transcription factor.

In conclusion, a detailed analysis of the En2\textsuperscript{core} enhancer element reveals that its evolutionary sequence conservation relates to two major functions: embryonic patterning and LOFC specification in the peripheral zone of the IM. The effect of En2\textsuperscript{core} mutations on DRNL expression shows that: first, potential cis-regulatory target sequences are distributed throughout the evolutionarily conserved 100-bp element; second, major differences exist in En2\textsuperscript{core} function between the embryo and IM; third, cis-elements function synergistically in the early embryo and the IM peripheral zone and fourth, putative core AuxRE sequences can spatially activate or restrict DRNL expression. The En2\textsuperscript{core} mutational data are consistent with available chromatin configuration data and suggest that En2\textsuperscript{core} comprises a HOT region that is potentially occupied by multiple transcription factors. The essential integrity of a single putative core AuxRE element within En2\textsuperscript{core} for IM activity suggests that a potential regulation of En2\textsuperscript{core} by auxin integrates into a pre-assembled multi-factorial enhanceosome, although chromatin data for the active enhancer element in LOFCs provide little evidence for massive chromatin remodelling due to the entry of novel transcription factors.

**Author contribution statement** DG, AF, JWC and WW conceived and designed the research. PC and DG conducted the experiments. AF and JE contributed new methods and analysed data. JWC and WW wrote the manuscript. All authors read and approved the manuscript.

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