

Short communication

Spatial and temporal expression patterns of auxin response transcription factors in the syncytium induced by the beet cyst nematode *Heterodera schachtii* in *Arabidopsis*

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SUMMARY

Plant-parasitic cyst nematodes induce the formation of a multinucleated feeding site in the infected root, termed the syncytium. Recent studies point to key roles of the phytohormone auxin in the regulation of gene expression and establishment of the syncytium. Nevertheless, information about the spatio-temporal expression patterns of the transcription factors that mediate auxin transcriptional responses during syncytium formation is limited. Here, we provide a gene expression map of 22 auxin response factors (ARFs) during the initiation, formation and maintenance stages of the syncytium induced by the cyst nematode *Heterodera schachtii* in *Arabidopsis*. We observed distinct and overlapping expression patterns of ARFs throughout syncytium development phases. We identified a set of ARFs whose expression is predominantly located inside the developing syncytium, whereas others are expressed in the neighbouring cells, presumably to initiate specific transcriptional programmes required for their incorporation within the developing syncytium. Our analyses also point to a role of certain ARFs in determining the maximum size of the syncytium. In addition, several ARFs were found to be highly expressed in fully developed syncytia, suggesting a role in maintaining the functional phenotype of mature syncytia. The dynamic distribution and overlapping expression patterns of various ARFs seem to be essential characteristics of ARF activity during syncytium development.

Keywords: *Arabidopsis*, auxin response factors, cyst nematodes, gene expression, *Heterodera schachtii*, syncytium.

The phytohormone auxin is a key signalling molecule regulating a wide range of growth and developmental processes. These crucial

processes include, for example, apical dominance, root development, vascular differentiation, shoot elongation and embryo patterning (Benjamins and Scheres, 2008; Zhao, 2010). In addition, auxin regulates various cellular processes that are associated with the plant response to biotic stresses (Kazan and Manners, 2009). Consistent with its crucial role in cell differentiation and morphogenesis, it is widely believed that auxin plays key roles in the initiation and formation of the syncytium induced by plant-parasitic cyst nematodes (for reviews, see Gheysen and Mitchum, 2011 and Grunewald *et al.*, 2009b). Syncytium formation is a very sophisticated process that involves proteins from both nematode (effectors) and host cells that co-operatively transform normal root cells into the syncytium cell fate type (Hewezi and Baum, 2013). The nematode first initiates contact with a single competent cell, known as an initial feeding cell (IFC). This contact is translated into dramatic cellular modifications, including endoreduplication, cell wall modification and dissolution, disappearance of large vacuoles, and an increased number of organelles and metabolic activity (Golinowski *et al.*, 1996). Remarkably, these modifications are also stimulated in neighbouring cells, which gradually fuse with each other and with the IFC. The successive cell-to-cell fusion of several hundred cells surrounding the IFC results in the formation of one large multinucleated syncytium as a novel plant structure. Several lines of evidence point to fundamental roles of auxin in syncytium induction and formation. For example, the synthetic auxin response promoter DR5 has been found to be activated in the developing syncytia on cyst nematode infection (Absmanner *et al.*, 2013; Grunewald *et al.*, 2009a; Karczmarek *et al.*, 2004), demonstrating a rapid increase in auxin concentration. In addition, several auxin response or auxin transport mutants show aberrant feeding cell morphogenesis and structure (Goverse *et al.*, 2000; Grunewald *et al.*, 2009a). Furthermore, a role of auxin in the reprogramming of root cells adjacent to the IFC prior to their merging with the developing syncytium has also been suggested (Goverse *et al.*, 2000; Grunewald *et al.*, 2009a; Karczmarek *et al.*, 2004). Although a local increase in auxin concentration in the developing syncytium and neighbouring cells is expected to activate auxin-dependent transcriptional programmes, little is known about the auxin signalling that mediates auxin transcriptional responses during syncytium induction

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Accession numbers: ARF1 (At1g59750), ARF2 (AT5G62000), ARF3 (At2g33860), ARF4 (At5g60450), ARF5 (At1g19850), ARF6 (AT1G30330), ARF7 (AT5G20730), ARF8 (AT5G37020), ARF9 (At4g23980), ARF10 (At2g28350), ARF11 (AT2G46530), ARF12 (T1G34310), ARF13 (AT1G34170), ARF14 (AT1G35540), ARF15 (AT1G35520), ARF16 (AT4G30080), ARF17 (At1g77850), ARF18 (At3g61830), ARF19 (AT1G19220), ARF20 (AT1G35240), ARF21 (AT1G34410) and ARF22 (AT1G34390).

and development. Three main components of the auxin signalling pathway have been identified through forward and reverse genetics approaches. These include transport inhibitor response1/auxin-binding F-box proteins (TIR1/AFB) auxin receptors, auxin/indole-3-acetic acid protein (Aux/IAA) inhibitors and auxin response factors (ARFs) *cis*-acting transcription factors (reviewed by Chapman and Estelle, 2009). In *Arabidopsis*, ARFs are encoded by a large gene family containing 22 members and one truncated gene (*ARF23*), which contains a stop codon in its DNA-binding domain and is most probably a pseudogene (Guilfoyle and Hagen, 2007; Remington *et al.*, 2005). These transcription factors bind specifically to auxin-responsive *cis*-acting elements that are frequently found in the promoters of early auxin-responsive genes, thereby regulating their expression. Although a set of ARFs has been identified as differentially expressed in the syncytium induced by the cyst nematode *Heterodera schachtii* in *Arabidopsis* roots (Szakasits *et al.*, 2009), information about the spatio-temporal expression patterns of ARFs during syncytium formation is limited. Here, we describe the spatial expression patterns of all functional ARFs during the initiation, formation and maintenance stages of the syncytium induced by the sugar beet cyst nematode *H. schachtii* in *Arabidopsis thaliana*.

Seeds from 44 independent homozygous T3 *Arabidopsis* lines expressing promoter–green fluorescent protein (GFP) constructs of 22 ARFs (*ARF1–22*) in the Col-0 background (Rademacher *et al.*, 2011) were planted, and 10-day-old seedlings were inoculated with approximately 200 surface-sterilized, second-stage juvenile (J2) nematodes per plant, as described previously by Hwezi *et al.* (2012). The specific promoter activity of these 44 transgenic lines (two lines per ARF construct) was visualized by GFP fluorescence at different time points after *H. schachtii* infection. All microscopic observations were carried out with a 10× objective on an AxioObserver Z1 (Zeiss, Carl Zeiss Imaging Solutions GmbH, Gottingen, Germany) with a GFP filter set for fluorescent observation (filter set 38 HE, Zeiss). Images were captured with a digital CCD camera (ORCA-ER, Hamamatsu, Japan) controlled by Openlab software (Improvision, Perkin-Elmer, Waltham, MA, USA). The time points were carefully chosen to reflect different phases of syncytium development (i.e. initiation, formation and maintenance) (Hwezi *et al.*, 2012). At 2–3 days post-infection (dpi), the developing syncytia induced by the parasitic J2 stage were structurally distinguishable, wherein several cells surrounding the nematode head were interconnected through cell-to-cell fusion. Mapping the expression of the 22 ARFs during the early stage of syncytium formation revealed a high sensitivity of this gene family of transcription factors to nematode infection. With the exception of *ARF8*, which showed little response to nematode infection, all other ARFs exhibited distinct and overlapping expression patterns. One interesting expression pattern observed was the strong expression of 10 ARFs (*ARF3, 6, 10–12, 14, 15* and *20–22*) inside the young syncytium, but not in the

adjacent cells that would be incorporated into the syncytium at a later stage (Fig. 1). The enhanced activity of these ARFs inside the young syncytia apparently indicates a significant increase in the local accumulation of auxin on nematode infection. These results are consistent with previous studies, in which an increase in auxin accumulation in young syncytia was demonstrated using the synthetic auxin responsive promoter DR5 (Absmanner *et al.*, 2013; Grunewald *et al.*, 2009a; Karczmarek *et al.*, 2004). Because the expression of these ARFs is limited to syncytium, their activities may be involved in the transcriptional regulation of genes involved in specific developmental programmes that are restricted to the syncytial cells. Local accumulation of auxin in developing syncytium that mediates the activation of these ARFs could be a result of the simultaneous activation of the auxin import machinery and inhibition of the auxin export machinery. In support of this suggestion, *AUX1*-mediated auxin import was found to be highly up-regulated in young syncytia (Mazarei *et al.*, 2003), whereas *PIN1*-mediated auxin export was down-regulated (Grunewald *et al.*, 2009a).

It has been suggested frequently that cells surrounding the expanding syncytium also experience a gradual and transient increase in auxin concentration (Goverse *et al.*, 2000; Grunewald *et al.*, 2009b). However, ARFs mediating gene expression changes in response to the gradual increase in auxin concentration in neighbouring cells are completely unknown. Here, we found that *ARF1, 2, 4, 5, 9, 18* and *19* were strongly activated in both syncytial cells and adjacent cells, which are about to be incorporated into the developing syncytium at a later stage. Notably, the expression of some of these ARFs was mainly localized towards the cell edges, as in the case of *ARF4, 5* and *19* (Fig. 1). Interestingly, *ARF7* and *17* showed a very similar expression pattern in which the GFP signals were restricted to the edge of the syncytial and neighbouring cells (Fig. 1). These spatial expression patterns may point to the functional localization of ARF-regulated genes, and are consistent with the finding that several nematode-regulated genes that function in cell wall modification contain auxin response elements (reviewed by Goverse and Bird, 2011). In addition, the auxin influx transporter *LAX3*, which activates several cell wall-modifying enzymes (Swarup *et al.*, 2008), was also found to be induced in the cells surrounding the growing feeding sites (Lee *et al.*, 2011). Therefore, it is tempting to speculate that *LAX3*-dependent auxin signalling, which functions in remodelling the cell walls of neighbouring cells, could involve the activity of *ARF1, 2, 4, 5, 9, 18* and *19* in general, and *ARF7* and *17* in particular. Two lines of evidence support this speculation. First, mutations in two of the above-mentioned ARFs (*ARF7* and *19*) completely abolished the induction of *LAX3* mRNA after auxin treatment (Swarup *et al.*, 2008). Second, of the six cell wall-remodelling genes that have been reported to be regulated by *LAX3* (Swarup *et al.*, 2008), we were able to identify *AuxRE* in the promoters of three genes, including pectate lyase (*PLA2*),

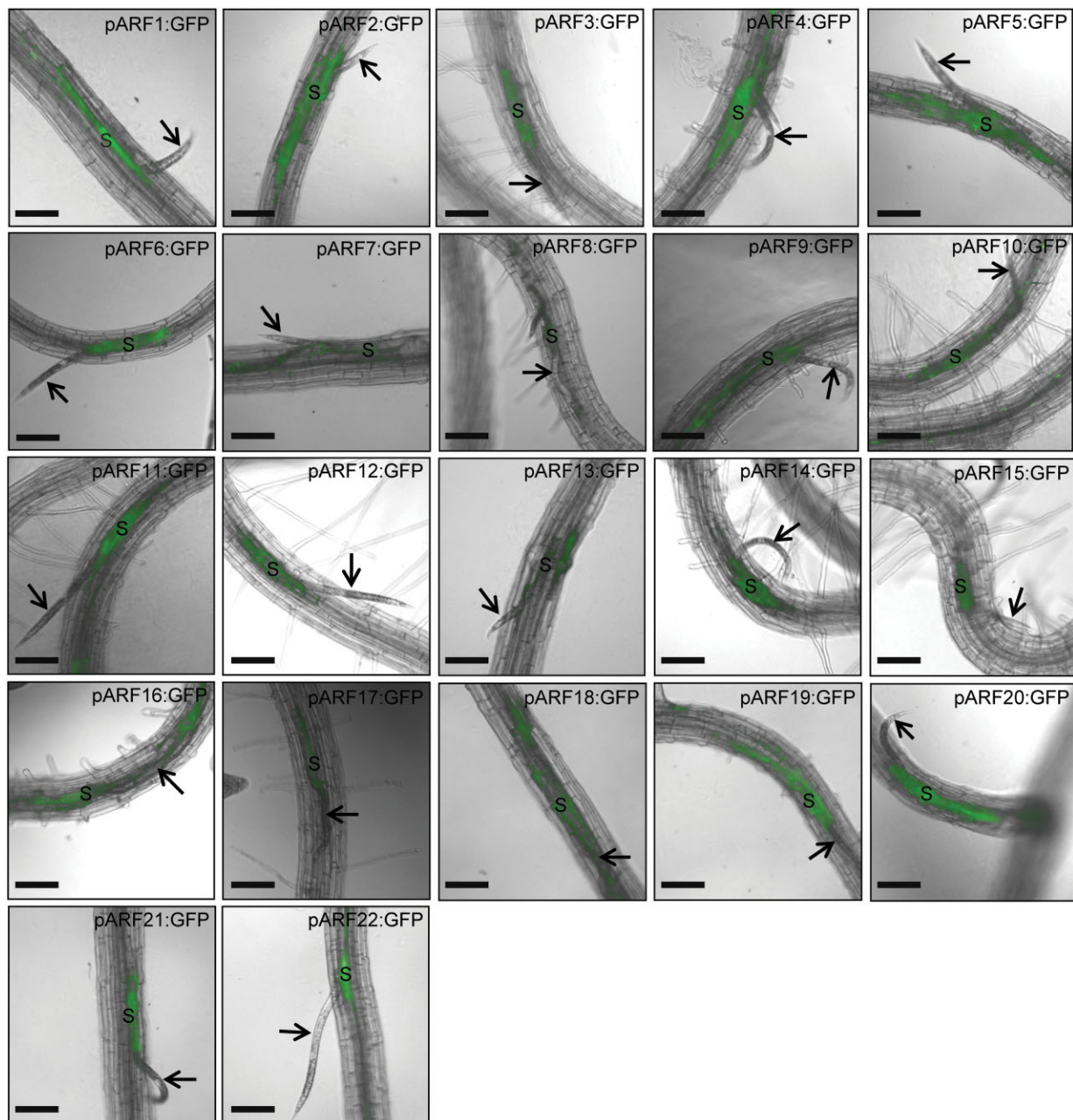


Fig. 1 Promoter activity of auxin response factor (*ARF*) genes visualized by green fluorescent protein (GFP) fluorescence in transgenic *Arabidopsis* roots at the sedentary second juvenile life stage, 2–3 days after *Heterodera schachtii* infection. The spatial expression patterns of 22 *ARFs* were analysed and compared during the syncytium initiation phase. *ARF3*, 6, 10–12, 14, 15 and 20–22 are expressed inside the developing syncytium. *ARF1*, 2, 4, 5, 9, 18 and 19 are active in both the syncytial cells and neighbouring cells. *ARF7* and 17 are mainly expressed at the edge of the syncytial and neighbouring cells. *ARF8* and 16 show a weak response to *H. schachtii* infection. Arrows point to nematode, and 'S' indicates syncytium. Bars, 130 μ m.

xyloglucan:xyloglucosyl transferase (*XTR6*) and glycosyl hydrolase (*GLH17*). Careful examination of the expression profiles of *ARFs* at the 2–3-dpi time points revealed redundant and non-redundant expression patterns of *ARFs* (Table S1, see Supporting Information). For example, *ARF5* and 7 are both expressed on the edge of neighbouring cells, but only *ARF5* is expressed inside the IFCs

(Fig. 1). Interestingly, *ARF5* and 7 were found to function redundantly in oriented cell differentiation and jointly in the control of auxin-responsive cell expansion (Hardtke *et al.*, 2004). We tentatively conclude that specific and overlapping responses of *ARFs* to local increases in auxin concentration at the early stage of syncytium formation correlate with the role of auxin in shaping

the identity of syncytial cells as a new cell type by directing auxin-responsive genes towards new developmental signalling pathways. In addition, the activation of several *ARFs* in the cells adjacent to the initial feeding site as early as 2 dpi (Fig. S1, see Supporting Information) suggests that auxin-dependent gene expression changes take place early on in these cells before they are recruited as part of the syncytium.

We next profiled the spatial activity of *ARFs* during the early third-stage juvenile (J3) stage of infection (5–6 dpi) to determine whether the spatial distribution and activity patterns of *ARFs* are extended to include new cells or merely to maintain the expression patterns observed during the syncytium initiation phase (2–3 dpi). We found that *ARF3*, 11 and 20–22 maintained their spatial expression pattern observed in the syncytium at 2–3 dpi, which is restricted to the IFCs. One remarkable difference was that GFP signals became stronger relative to the earlier time point. This suggests that these *ARFs* are associated with transcriptional programmes that are selectively related to the central syncytial cells. Interestingly, five *ARFs* (*ARF6*, 10, 12, 14 and 15), which primarily showed activity only in the IFCs, displayed an extended expression pattern to include both initial and recently incorporated syncytium cells (Fig. 2). These five *ARFs* seem to be required to mediate auxin-dependent gene expression changes only in the cells that are part of the syncytium.

Because the developing syncytia at 5–6 days after *H. schachtii* infection (i.e. the early J3 stage) undergo extensive cell-to-cell fusion with the distantly reprogrammed neighbouring cells, it was of interest to investigate whether the *ARF* genes, which displayed activity in the neighbouring cells (*ARF1*, 2, 4, 5, 9, 18 and 19), preserve this spatial expression pattern. Notably, *ARF1*, 2, 5 and 18 not only maintained this expression pattern, but there was a gradual increase in the GFP signals in the cells adjacent to the developing syncytia (Fig. 2). In contrast, the expression level of the three remaining *ARFs* (*ARF4*, 9 and 19) was strongly diminished in the neighbouring cells, and intense GFP fluorescence was observed only in the developing syncytium, suggesting that the activity of these *ARFs* became more specific to the syncytium (Fig. 2). *ARF7* and 17 maintained their expression patterns observed at the 2–3-dpi time point, but with a substantial increase in their activities (Fig. 2). *ARF8*, 13 and 16 showed very limited expression, primarily around the nematode head (Fig. 2). It may be important to mention that the neighbouring cells, which showed GFP expression at the 2–3-dpi time point, were incorporated into the expanding feeding sites, supporting a role for *ARFs* in reprogramming neighbouring cells before fusion with the expanding syncytium. The expression of several *ARFs* in neighbouring cells suggests that, once auxin is accumulated in the IFCs, an auxin gradient is established along the syncytium axis, in which a role for the active auxin transport machinery in regulating these *ARFs* can be postulated. It has been suggested recently that PIN3 facilitates local auxin transport from the feeding sites towards sur-

rounding cells to bring about syncytium expansion (Grunewald *et al.*, 2009a). In addition, when the auxin transport machinery was chemically or genetically repressed, both feeding site formation and nematode development were largely affected (Goverse *et al.*, 2000; Grunewald *et al.*, 2009a). In this perspective, mutant combinations between *PIN3* and the above-mentioned *ARFs* will elucidate how PIN3-mediated *ARF* regulation influences syncytium development and size.

The 9–10-dpi time point corresponds to nematode development at the late J3 stage when syncytia reach their maximum size and no additional cells are incorporated. Thus, profiling of the spatial activity of *ARFs* revealed to what extent auxin distribution patterns were associated with the syncytium maintenance phase. It was not surprising to find that the majority of *ARFs* show low expression at this time, with only a detectable expression in the syncytium around the nematode head (Fig. 3). Interestingly, eight *ARFs* (*ARF1–3*, 7, 17 and 20–22) showed noticeable expression levels in the mature syncytium (Fig. 3), suggesting a role of these transcription factors in the transcriptional control of gene expression that maintains the functional phenotype of the mature syncytium. Similarly, other auxin-responsive genes, such as *LAX1* (Lee *et al.*, 2011) and *AUX1* (Mazarei *et al.*, 2003), showed detectable expression levels at and after the J3 stage. However, these results are inconsistent with previous studies in which fully differentiated syncytium did not exhibit an auxin response (Absmanner *et al.*, 2013; Karczmarek *et al.*, 2004). This discrepancy is mainly a result of the fact that these studies used the auxin-responsive DR5 promoter to visualize the auxin response, and that the DR5 promoter activity reflects only the auxin response, but not the auxin concentration. Therefore, the DR5 promoter does not seem to be sufficiently sensitive to determine the auxin gradient in the fully developed syncytium. It has been suggested that an auxin-responsive promoter that is about ten times more sensitive than DR5 is needed to detect an auxin gradient that could be produced under specific circumstances (Yamamoto, 2003).

The expression profiles of the *ARFs* during syncytium formation and development are very specific and, in non-infected plants, we did not observe an expression similar to that associated with nematode infection. The spatiotemporal expression patterns of the *ARFs* are simplified in Fig. 4 and summarized in Table S1. During the initiation phase of the syncytium, auxin accumulates at a high level in the IFCs, which undergo extensive signalling events mediated by *ARFs* to guide these cells into a cell type-specific transcriptional programme. A set of *ARFs* (*ARF3*, 6, 10–12, 14, 15 and 20–22) seems to have overlapping and cooperative transcriptional functions that contribute to the initiation phase of the syncytium. Once the syncytium is initiated, the neighbouring cells should receive auxin, as an instructive signal, from the IFCs in order to precondition these cells to a new cell fate. The commitment of the neighbouring cells to a syncytial fate may require the transcriptional activity of *ARF1*, 2, 4, 5, 9, 18 and 19 initially, and *ARF1*, 2,

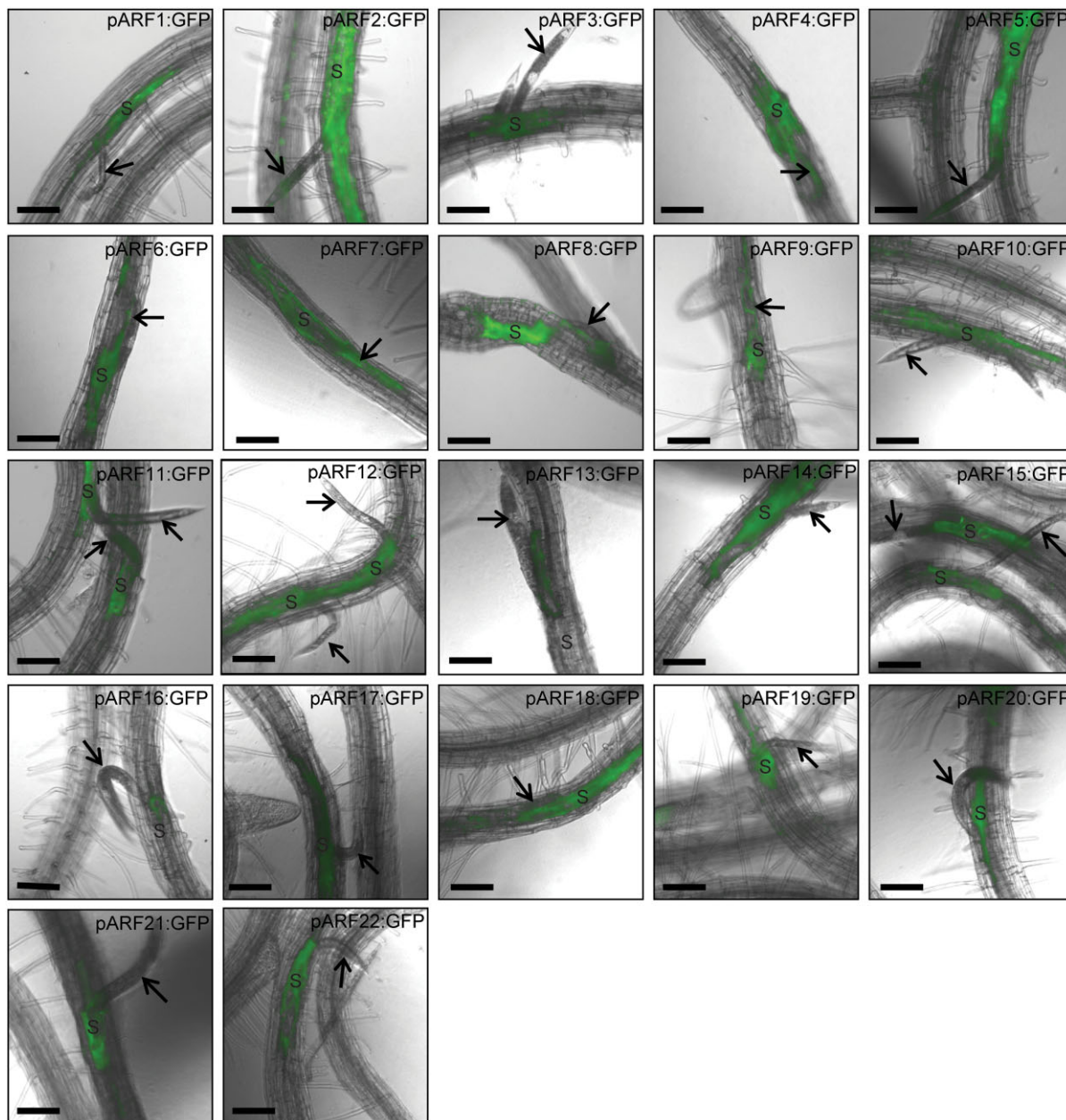


Fig. 2 Promoter activity of auxin response factor (*ARF*) genes visualized by green fluorescent protein (GFP) fluorescence in transgenic *Arabidopsis* roots at the early third juvenile life stage, 5–6 days after *Heterodera schachtii* infection. The spatial expression patterns of 22 *ARFs* were analysed and compared during the syncytium formation phase. The expression of *ARF3*, 11 and 20–22 is restricted to the initial feeding cells and not extended to include recently incorporated syncytium cells. *ARF4*, 6, 9, 10, 12, 14, 15 and 19 are expressed in the developing syncytium. *ARF1*, 2, 5 and 18 are continually expressed in the neighbouring cells. *ARF7* and 17 show a remarkable increase in their activity and are mainly expressed at the edge of the syncytial and neighbouring cells. *ARF8*, 13 and 16 are expressed mostly in the syncytial cells around the nematode head. Arrows point to nematode, and 'S' indicates syncytium. Bars, 130 μ m.

5 and 18 until syncytium formation is complete. The maximum size of the expanding syncytium presumably needs to be marked before major transcriptional alterations occur. *ARF2*, 5 and 18 could be involved in the labelling of syncytial cell boundaries because of their expanding expression patterns at the early stage of infection (i.e. 2–3 dpi). After the syncytia have reached their

maximum size, eight *ARFs* (*ARF1–3*, 7, 17 and 20–22), which are expressed in fully developed feeding sites, could have a role in maintaining the functional phenotype of mature syncytia. The active distribution and overlapping expression patterns of various sets of *ARFs* seem to be essential characteristics of *ARF* activity during various phases of syncytium development. Finally, the tran-

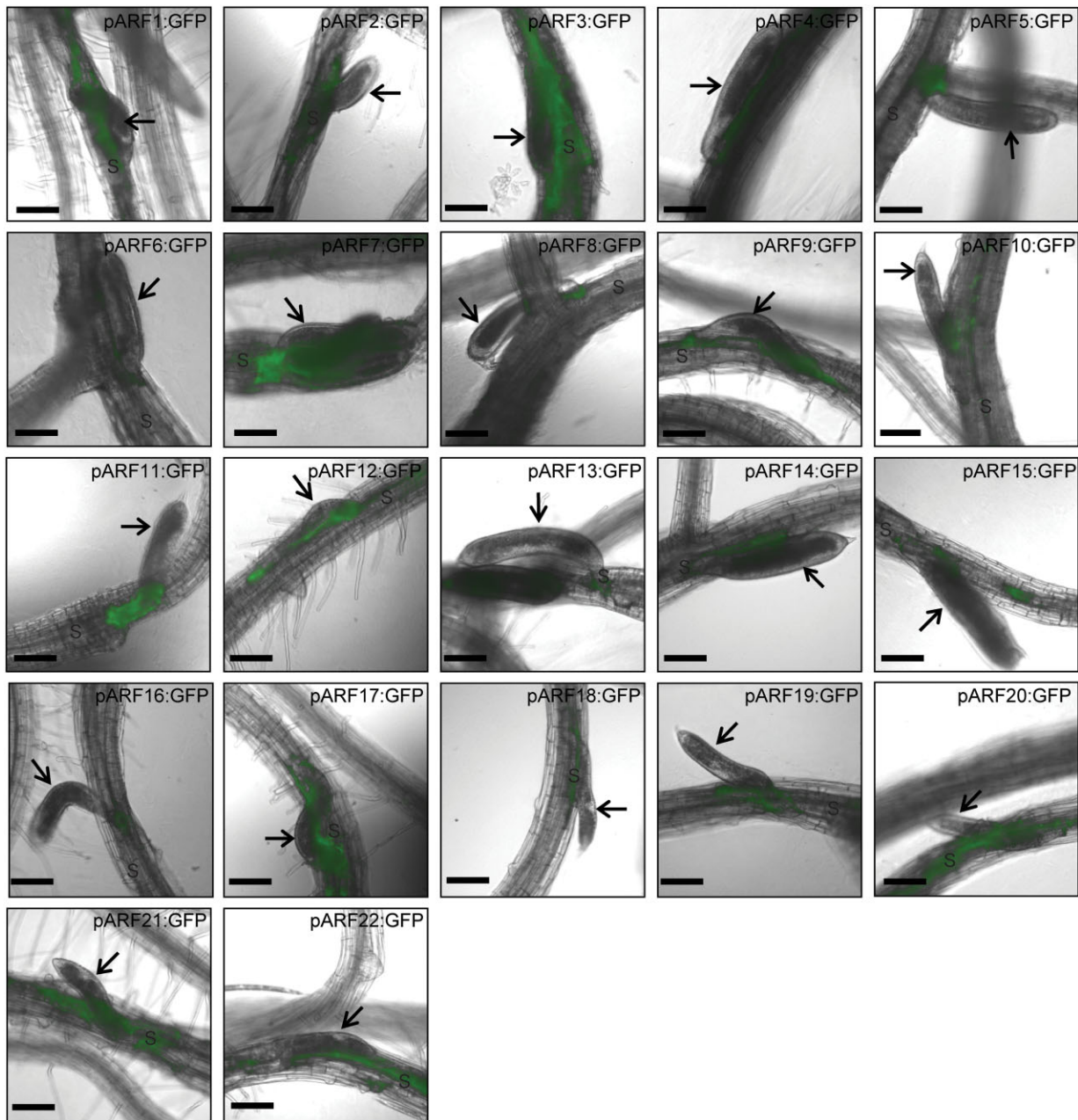


Fig. 3 Promoter activity of auxin response factor (*ARF*) genes visualized by green fluorescent protein (GFP) fluorescence in transgenic *Arabidopsis* roots at the late third juvenile life stage, 9–10 days after *Heterodera schachtii* infection. The spatial expression patterns of 22 *ARFs* were analysed and compared during the syncytium maintenance phase. *ARF1–3*, 7, 17 and 20–22 are expressed in fully developed syncytium, whereas the expression of the other *ARFs* is restricted to the syncytial cells around the nematode head. Arrows point to nematode, and 'S' indicates syncytium. Bars, 130 μ m.

scriptional activity of these *ARFs* might not reflect the exact patterns of mRNA accumulation, as some *ARFs* are known to be post-transcriptionally regulated by microRNAs (miRNAs). *ARF 10*, 16 and 17 are targeted by miR160 (Mallory *et al.*, 2005; Wang *et al.*, 2005), whereas *ARF6* and 8 are targeted by miR167 (Wu *et al.*, 2006), and both miRNAs are regulated by *H. schachtii* infection (Hewezi *et al.*, 2008).

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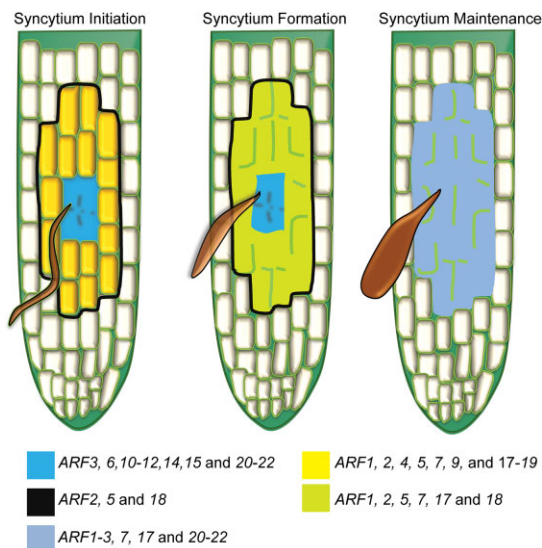


Fig. 4 Model illustrating the spatiotemporal expression patterns of the auxin response factors (*ARFs*) during various stages of syncytium development. During the syncytium initiation phase, *ARF3*, 6, 10–12, 14, 15 and 20–22 are predominantly expressed in the initial feeding cells, whereas *ARF1*, 2, 4, 5, 7, 9 and 17–19 are strongly activated in both the syncytial cells and in neighbouring cells. *ARF2*, 5 and 18 have an expanded expression pattern, which most probably marks the axis of expanding syncytium. During the syncytium formation phase, *ARF1*, 2, 5, 7, 17 and 18 continue to exhibit high expression in the developing syncytium. During the syncytium maintenance phase, only *ARF1*–3, 7, 17 and 20–22 are expressed in fully developed syncytium.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Comparison of gene expression patterns between auxin response factors (*ARFs*) that are exclusively expressed inside the developing syncytium and those expressed in the neighbouring cells before their fusion with the expanding syncytium. Certain *ARFs*, such as *ARF14*, are predominantly expressed inside the developing syncytium (a), whereas others, such as *ARF2* and 5, are expressed in the neighbouring cells before their fusion with the expanding syncytium (b, c). Arrows point to nematode, and developing syncytium is outlined. Bars, 130 μ m.

Table S1 Summarized expression patterns of auxin response factors (*ARFs*) during syncytium formation and development.