Spatial and temporal expression of the response regulators
ARR22 and ARR24 in Arabidopsis thaliana

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Abstract

ARR22 (At3g04280) is a novel Type A response regulator whose function in Arabidopsis is unknown. RT-PCR analysis has shown that expression of the gene takes place in flowers and developing pods with the tissues accumulating different proportions of splice variants. Spatial analysis of expression, using ARR22::GUS plants as a marker, has revealed that the reporter protein accumulates specifically at the junction between the funiculus and the chalazal tissue. Expression can be up-regulated at this location by wounding the developing seed. A detailed analysis has failed to detect ARR22 expression at any other sites and, to support this assertion, the only evidence for tissue ablation in ARR22::Barnase plants is during seed development, with the consequence that embryo growth is attenuated. Ectopic expression of ARR22, driven by either the CaMV 35S or the pea plastocyanin (PPC) promoters, resulted in the generation of plants exhibiting extremely stunted root and shoot growth. No viable progeny could be isolated from the PPC::ARR22 transgenic lines. An RT-PCR analysis of a recently annotated gene (ARR24–At5g26594), that exhibits 66% amino acid similarity to ARR22, has shown that expression is also predominantly in floral and silique tissues. Examination of ARR24::GUS plants has revealed that the activity of the promoter is primarily restricted to pollen grains indicating that this gene is unlikely to display an overlapping function with ARR22. Analyses of individual KO lines of either ARR22 or ARR24 have failed to identify a mutant phenotype under the growth conditions employed and the double knockout ARR22/ARR24 line is also indistinguishable from wild-type plants. These results are discussed in the light of the proposed role of response regulators in plant growth and development.

Key words: Arabidopsis, response regulators, seed development, signalling.

Introduction

It is now well documented that two-component signalling systems play an important role in higher plants for the intracellular transduction of critical signals including assimilates, light, and plant hormones (Hwang et al., 2002; Schaller et al., 2002; Mason et al., 2004; Mizuno, 2005). These systems were first described in prokaryotic organisms as a mechanism by which bacteria were able to respond to a range of environmental stimuli and found to comprise two elements, namely a receptor kinase and a response regulator (Stock et al., 2000). In plants, a modification to the basic system has taken place and the consequence has been the development of a signalling network involving three separate elements: a hybrid receptor kinase, a His-containing phosphotransfer protein, and a response regulator (Hwang et al., 2002).

Analysis of the Arabidopsis genome has revealed proteins that share sequence homology with all three components of the modified two-component signalling system (Hutchinson and Kieber, 2002; Schaller et al., 2002) and...
for each class phosphorylation capacity has been demonstrated. Over 20 putative response regulators (ARRs) have been identified in the Arabidopsis genome and whilst these display highly conserved domains associated with the sites of phosphorylation a considerable amount of divergence has occurred in other areas of the peptide sequences. Basically, they can be classified into two discrete communities: Type-A and Type-B. Type A ARRs comprise primarily a receiver domain attached to a short C-terminal region and the expression of gene family members is up-regulated by cytokinin treatment (Heyl and Schmülling, 2003) and down-regulated in a cytokinin-deficient transgenic line (Brenner et al., 2005). It has been proposed that the function of type-A ARRs is to coordinate signalling events between the cytoplasm and the nucleus in response to cytokinins and possibly other stimuli (D’Agostino et al., 2000; Heyl and Schmülling, 2003; Brenner et al., 2005). Type B ARRs also consist of a receiver domain, but this is linked to a much longer C-terminal output domain and these proteins frequently contain a GARP DNA-binding motif and this sequence, coupled with some cell biology studies, suggests that they function as transcription factors (Mason et al., 2004). Although the expression of Type B ARRs is not influenced by cytokinins, it has been proposed that they may function to modulate the expression of cytokinins primary response genes including Type A ARRs (Mason et al., 2004).

During the course of studies on pod dehiscence in oilseed rape, a cDNA encoding a putative response regulator protein that was up-regulated during siliques development was identified (Whitehat et al., 1999). The transcript for this gene was primarily associated with RNA extracted from dehiscence zone tissues and expression reached a peak at about 40 d after anthesis. The Arabidopsis gene with closest homology to this Brassica napus gene encodes a novel Type A response regulator named ARR22 (At3g04280) (Gattolin, 2003). The role of this gene in planta is unknown, although recent work has demonstrated that the purified protein has the ability to undergo phosphorylation in vitro and that expression is associated with reproductive tissues (Kiba et al., 2004). Another response regulator (ARR24—At5g26594) with close homology to ARR22 has been identified in the genome and it has been hypothesized that the peptides encoded by these genes might act as functional homologues. In this paper, a detailed characterization of the spatial and temporal expression of ARR22 and ARR24 is described and the consequence of down-regulating the expression of these two genes on the growth and development of Arabidopsis plants is reported.

Materials and methods

Plant materials and growth conditions

Seeds of Arabidopsis ecotypes Columbia-0 or Wassilewskija were grown in 3:1 Levington compost (Levington professional F2 fine structure-medium nutrients standard pH without sand:vermiculite mix. Plants were grown under greenhouse conditions with supplementary lighting to generate a photoperiod of 16 h of light at 22 °C. A number of different reproductive stages were studied these were classified as: open flowers, emerging siliques (1–2 Days After Flowers fully open, DAF), small siliques (3–5 DAF), elongating siliques (4–8 DAF), mature siliques (9–12 DAF), and senescing siliques (13–18 DAF).

Plasmid construction and plant transformation

The pARR22-GUS and pARR22-BAR vectors were constructed by cloning a promoter fragment (–1155 to –1 bp of the translational initiation site) of ARR22 amplified by PCR using genomic DNA. A 1155 bp BamHI/SalI PCR fragment was cloned into the pBl101 vector (which contained the GUS gene in the +1 reading frame) generating the pARR22-GUS vector. A 1155 bp SpeI/NcoI PCR fragment was fused to the barnase coding sequence of the Xbal/NcoI–digested WP274 vector (Paul et al., 1992). The final ARR22::Barnase::BarStar cassette was subcloned into pNOS-NPT-SCV (Biogemma UK, Ltd.) generating the pARR22-BAR vector.

The p3SS-ARR22 and pPPC-ARR22 vectors were constructed by cloning the 429 bp intronless open reading frame (cDNA) of ARR22 (+1 to +452 bp downstream of the translational initiation site) amplified by PCR using cDNA. The 3S promoter fused to the Xbal/SacI ARR22 PCR fragment was subcloned into pBl101 (after removing the GUS sequence with HindIII and SacI and leaving the NOS-ter) generating the p3SS-ARR22 vector. A 429 bp intronless NcoI/EcoRI ARR22 PCR fragment was cloned into pLJA (Biogemma UK, Ltd.) and then subcloned into pNOS-NPT-SCV generating the pPPC-ARR22 vector. The integrity of all plasmids generated was confirmed by sequencing.

The binary vectors: pARR22-GUS, pARR22-BAR, p3SS-ARR22, pPPC-ARR22, pARR24-GUS were electroporated into Agrobacterium tumefaciens strain C58 and grown overnight to saturation. Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998) with an Agrobacterium suspension (50 mg l⁻¹ sucrose and 0.05% Silwett L-77). To identify the transgenic plants in subsequent generations, seeds were surface-sterilized and germinated in Murashige and Skoog (MS) salts, 8 mg l⁻¹ agar and 40 mg l⁻¹ kanamycin. T2 seeds were collected from individual lines and screened for kanamycin resistance. Single resistant T2 plants were transferred into soil and used for the isolation of homozygous lines by identifying progeny with 100% resistance on kanamycin plates.

GUS analysis

Different tissues of putative transgenic plants carrying the plasmids: pARR22-GUS and pARR24-GUS were incubated in GUS staining buffer (50 mM phosphate buffer pH 7.2, 0.5 mM K₃Fe(CN)₆.H₂O, 0.5 mM K₄Fe(CN)₆, 0.1% (v/v) Triton X-100, 0.5 mg ml⁻¹ X-Gluc at 37 °C o/n. Flowers, leaves, roots, and seedlings were put directly into GUS substrate after collecting from plants. Siliques from different developmental stages were treated in various ways before testing for GUS activity: some siliques were halved transversally; and in some cases the seeds, still remaining in the siliques, were damaged by piercing the testa with the pointed end of a needle with the aid of a dissecting microscope. The tissues were cleared in ethanol 70% and observed under a Stemi SV6 microscope.

A time-course of ARR22 expression was undertaken by dissecting young seeds at varying development stages and incubating them in GUS substrate as previously described. These tissues were subsequently cleared, as described by Aida et al. (1997) before photographic recording was carried out.
RT-PCR analysis of gene expression

To determine the spatial and temporal expression pattern of ARR22 and ARR24, as well as to confirm the expression of transgenes, total RNA was extracted from different frozen tissues after homogenization (0.25–0.5 g). Total RNA from leaves, stems, buds, and flowers was extracted using RNeasy Plant Kit (Qiagen). Total RNA, from seeds isolated from different stages of silique development, was extracted using the borate isolation method (Modification of Protocol from Cotton Genome Center, UC Davis, http://cottongenomecenter.ucdavis.edu/protocols/RNA). The RNA was digested with RQI RNase-free DNase (Promega), cleaned up with an RNeasy Plant Kit column (Qiagen), quantified with a Nanodrop ND-1000 Spectrophotometer and visualized on a 1% (w/v) agarose gel. Two to three micrograms of total RNA was used to make cDNA using the SuperScript II RNase H RT method (Invitrogen) following the manufacturer’s instructions. One microtitre of the resulting cDNA was used in a 25 μL PCR solution using Red Hot DNA Polymerase (AB gene). The PCR programme used was: 94 °C for 2 min, followed by 35 cycles of ~94 °C for 45 s; 53–57 °C for 40 s (depending upon primers); 72 °C for 1 min; and a final elongation step at 72 °C for 7 min. The sequences of the primers used were: UBQ10For, 5'-TAAAAACCTTCTCT-CAATCTCTCTCT-3' and UBQ10Rev, 5'-TTGCTCAATGGTGTCCAGGCTT-3'; ARR22For, 5'-TGATGCAATGCCTACCTTCT-3' and ARR22Rev, 5'-ATTAAGGACCTCCTCAACTGAC-3'; ARR24For, 5'-AAGTAGCTGAGAAGACTCTCC-3' and ARR24Rev 5'-TAAAGTTTGACGATGAAATAGA-3'. All PCR products were separated by electrophoresis.

Isolation of ARR22, ARR24, and ARR22/24 knockout lines

Putative knockout lines of ARR22 and ARR24 were obtained from two different sources: ARR22 Knockout Facility at the University of Wisconsin-Madison (Sussmann, 2000) and ARR24 (SALK:124785, Nottingham Arabidopsis Stock Centre (NASC)). The T-DNA insertions were identified as being localized 75 bp downstream of the start codon of ARR22 and 860 bp downstream of the start codon of ARR24. Homozygous single KO plants for ARR22 and ARR24 were obtained using gene-specific primers flanking the T-DNA insertion for each line together with the appropriate LB primer. These single KO plants were crossed and a double homozygous KO plant for ARR22::ARR24-ARR24 was identified from the F2 generation and used for subsequent analysis. The DNA of all the plants screened was isolated using the REDExtract-N-Amp™ Plant PCR Kit. PCR reactions were performed in 25 μL using 4 μL of DNA template. The PCR programme used was: 94 °C for 2 min, followed by 35 cycles of 94 °C for 45 s; 53–57 °C for 40 s (depending upon primers); 72 °C for 1 min; and a final elongation step at 72 °C for 7 min. The sequences of the primers used were: ARR24.0124.LP, 5'-GCTAAAAAACCGTGCTTGAGGT-3'; ARR24.0124.RP, 5'-TTGCTTCTGTGACCAAAAAC-3' and JMLB1, 5'-GGCAATCGTCTGCGCCATCTCTGTC-3' for ARR24 and AT3JR2F, 5'-GAGAAAAAAGTCGTAGAGTG-3'; AT3JR2R, 5'-TCCATCAGCTGAGGAAGAGGT-3' and SK1JLWIS, 5'-TGACCTGAGATGTTGGCCGACGAT-3' for ARR22. All PCR products were separated by electrophoresis.

**Results**

**Structure of the ARR22 (At3g04280) gene and reverse transcription-PCR analysis of expression**

The ARR22 gene contains two introns. One (183 bp) sited within the 5’ UTR of the gene, 25 bp upstream from the ATG, and the other (123 bp) located within the ORF (Fig. 1). RT-PCR analysis, to determine the spatial presence of the transcript, identified expression largely within open flowers, and small and elongating siliques (Fig. 2). No expression could be detected in leaves, stems, or floral buds. Using suitably designed primers all four potential splice variants, associated with the expression of the gene, could be detected. In young flowers the predominant forms were the fully processed transcript (526 bp) and the message containing the 5’ UTR intron (709 bp). In small pods there was a more equal distribution between the partially (709 bp/649 bp) and completely processed transcripts. While in elongating the majority of the mRNA was completely processed. In both flowers and small siliques some unprocessed transcript could be detected (832 bp). The use of ubiquitin primers demonstrated that this was not the consequence of contamination of the samples with genomic DNA. Cloning and sequencing of the different amplified transcripts was carried out to confirm their identity (data not shown).

**ARR22 expression analysis using reporter genes**

β-glucuronidase and Barnase

In order to identify the precise spatial expression of ARR22 in flower and silique tissues, 1155 bp of the ARR22 promoter region was amplified from genomic DNA, cloned upstream of the β-glucuronidase reporter gene and transformed into Arabidopsis plants. Expression of the reporter construct was assessed visually by histochemical staining. GUS activity was localized within the silique tissues, precisely at the junction between the seed and the funiculus (Fig. 3A). Staining was evident in mature green siliques and remained apparent until pod shatter. A discrete layer of GUS expression was also observed at the hilum of mature seeds that had been shed. To characterize ARR22::GUS expression further, seeds were dissected from siliques at different stages of development and tested for GUS activity (Fig. 3B, C). Expression of the transgene was first detectable in developing seeds isolated from pods that were still emerging from the flower and was restricted to the area where the vascular

![Fig. 1. Genomic structure of the ARR22 (At3g04280) gene identifying the introns located within the 5’ UTR region (183 bp) and the ORF of the mRNA (123 bp). The sites of primer binding to undertake the RT-PCR analysis are identified with broad arrows. Site of T-DNA insertion in ARR22 KO line denoted by the open triangle.](https://academic.oup.com/jxb/article-abstract/57/15/4225/552196)
strands terminate at the chalaza. At later stages, the whole chalazal zone became stained with dye accumulation extending into the distal portion of the funiculus. GUS expression was also strongly evident at these sites during the shedding of mature seeds collected at the time of siliques dehiscence.

As GUS accumulation, in young and developing seeds, was rarely seen in intact pods it is hypothesized that the expression of ARR22 (observed in Fig. 3B, C) might be promoted by wounding. To test this hypothesis one siliques valve was carefully peeled away from an immature pod at a developmental stage when no GUS staining was normally observed. After confirmation that this treatment was not sufficient to induce GUS expression, alternating seeds were punctured using a sharp needle before histochemical analysis of the treated siliques was undertaken. Seeds damaged in this way showed intense GUS expression at the seed-funiculus junction, irrespective of where the damage to the seed was carried out. Adjacent non-wounded seeds did not exhibit this response (Fig. 4). No expression was detected in undamaged seeds stored in the unpeeled side of the siliques.

Although analyses using RT-PCR and ARR22::GUS promoter fusion had failed to detect expression of ARR22 in tissues other than reproductive organs, it was decided to verify this by fusing 1155 bp of the ARR22 promoter region to the open reading frame of the Barnase gene (Paul et al., 1992). This gene encodes a ribonuclease from

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**Fig. 2.** RT PCR analysis of (A) ARR22 or (B) ubiquitin transcripts in leaf (Lf), stem (St), buds (Bd), open flowers (Fl), small (Sml), elongating (Elg), mature (Mat), or senescing (Sen) siliques. Transcript sizes: unprocessed, 832 bp; ORF intron excised, 709 bp; 5′ UTR intron excised, 649 bp; fully processed, 526 bp.

**Fig. 3.** Histochemical localization of ARR22 expression in Arabidopsis seeds. (A) GUS activity detected at the seed-funiculus junction in a mature siliques. (B) Low power and (C) Higher power. GUS activity localized in developing seeds isolated from siliques at different stages of development. Bar=100 μm.
Bacillus amyloliquefaciens. Only small amounts of Barnase expression are necessary to bring about cell ablation and this has previously been found to be a valuable tool in the detection of trace amounts of promoter activity (Jenkins et al., 1999). The vegetative development of ARR22::Barnase Arabidopsis seedlings and mature plants was indistinguishable from wild-type (WT) controls with transgenic plants progressing normally through their life cycle to flowering and siliques development and maturation. However, seeds obtained from mature green siliques of ARR22::Barnase plants were smaller than from WT plants and contained embryos of an attenuated size (Fig. 5). A close inspection of the developing seeds revealed tissue damage at the seed:funiculus junction. ARR22::Barnase seeds that had matured to their final dry stage were more shrivelled than the WT (Fig. 5), exhibited a reduced capacity to germinate, and resultant seedlings were frequently of a stunted nature.

Ectopic expression of ARR22

To express ectopically ARR22 in Arabidopsis plants, both the CaMV 35S and the Pea Plastocyanin (PPC) promoters were used to drive the expression of the cDNA. The PPC promoter was employed as it is particularly effective in up-regulating expression in photosynthetic tissues (Pwee and Gray, 1993) including siliques (S Gattolin, personal observations). All 35S::ARR22 and PPC::ARR22 primary transformants grew as phenotypic dwarfs with small, rounded, dark-green leaves and a greatly reduced number of flowers (Fig. 6A, B).

PPC::ARR22 primary transformants were sterile, while seeds were obtained from approximately 10% of the 35S::ARR22 primary transformants. Phenotypic analysis of this selfed population revealed a 1:3 segregation ratio of WT to ‘mutant’ plants and this was confirmed by selection on kanamycin plates (data not shown).

ARR24 has 66% amino acid sequence similarity to ARR22 and a 553 bp intron located at an equivalent position within the ORF of the gene. No 5’ UTR intron is apparent or has been detected in ARR24. To determine whether ARR24 might encode a functional homologue of ARR22, an analysis
of expression was undertaken using RT-PCR on the same RNA samples tested for ARR22 expression. Figure 7 shows that the transcript for ARR24 also accumulates in floral and silique tissues of Arabidopsis with expression reaching a maximum in buds and open flowers before declining during pod development and maturation. The only transcript that could be detected in all these floral stages was in the form of the fully processed mRNA (400 bp).

A 1.9 kb DNA fragment upstream of the translation start of ARR24 was fused to the reporter gene GUS and used to generate transgenic lines of Arabidopsis. Strong expression of the reporter gene could be detected in pollen grains within the anthers of young and mature flowers (Fig. 8A, B). A low level of expression was occasionally found to be associated with the testa of developing seeds, but this observation was not consistent between all the independent homozygous lines studied. No expression was found at the seed:funiculus junction and GUS accumulation was not promoted by wounding the seeds.

**Down-regulation of ARR22 and ARR24**

An ARR22 KO line was obtained from the KnockOut facility at the University of Wisconsin-Madison (Sussman et al., 2000). The T-DNA insertion was located in the coding sequence, 75 bp downstream of the ATG codon. Lines homozygous for the T-DNA insertion into the ARR22 sequence were identified and expression of the gene was found to be silenced in these plants (Fig. 9A). These KO lines were grown under standard greenhouse conditions and plant growth and development compared with negative segregant (wild-type) material. No impact of silencing the ARR22 gene on vegetative growth, reproductive growth, or seed development could be detected under the growing conditions employed.

A putative KO line of ARR24 was obtained from the SALK collection and plants homozygous for this T-DNA insertion (SALK-124785) were identified. Expression of the ARR24 gene in floral tissues of these plants revealed that silencing had taken place (Fig. 9B). No phenotypic consequence of reducing expression of ARR24 could be detected on vegetative or reproductive growth under the growing conditions employed.

A KO line of both ARR22 and ARR24 was generated by crossing the material described above, allowing the resulting

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Fig. 7. RT PCR analysis of (A) ARR24 or (B) ubiquitin transcripts in leaf (Lf), stem (Sl), buds (Bd), open flowers (Fl), small (Sml), elongating (Elg), mature (Mat), or senescing (Sen) siliques.

Fig. 8. Histochemical localization of ARR24 expression. (A) GUS activity within the anthers of young flowers. (B) GUS activity associated with pollen grains in anthers of open flowers.

Fig. 9. Expression analysis of ARR22 and ARR24 by RT-PCR. RNA was isolated from a mixture of buds and open flowers from knockout lines of (A) ARR22, (B) ARR24, or (C) ARR22-ARR24 and used as a template for PCR amplification. Ubiquitin primers were used as a positive control in the RT-PCR reaction.
material to self-fertilize, and then screening the progeny of these selfed plants for a line homozygous for insertions into both genes. After screening by PCR, a KO plant of both *ARR22* and *ARR24* was identified and expression analysis revealed that transcripts of both genes were absent (Fig. 9C). A detailed analysis of this genotype failed to detect a consistent phenotypic consequence of silencing both *ARR22* and *ARR24*.

**Discussion**

The *ARR22* gene contains two introns, one located in the 5′ UTR region and the other in the ORF. Using primers designed to amplify different variants of the *ARR22* mRNA, it is shown that reproductive tissues in *Arabidopsis* accumulate all four potential transcripts, albeit to different extents. The main transcript identified in the tissues where *ARR22* expression took place was the fully processed version. However, young flowers also accumulate a substantial amount of transcript that retains the 5′ UTR intron, whilst in small siliques both partially processed variants can be detected. It has been proposed that changes in the stability of transcript variants may afford a mechanism for the translational control of gene expression (Ner-Gaon et al., 2004) and, as discussed later, there is some evidence that post-transcriptional regulation of *ARR22* may take place.

Kiba et al. (2004) showed that expression of *ARR22* could be detected in a spectrum of plant tissues including leaves, stems, flowers, and siliques. Detailed RT-PCR analysis in this study has failed to detect any expression of the gene in vegetative tissues, however, it has been confirmed that the *ARR22* transcript is present in reproductive organs of *Arabidopsis* with the greatest accumulation being in mature flowers, small and elongating siliques. By fusing 1155 bp of the promoter of *ARR22* to the reporter gene *GUS* it has been possible to identify the precise spatial and temporal expression of the gene. This has recently been confirmed using a 1600 bp fragment of the promoter (S Gattolin, unpublished results). GUS accumulation can be detected specifically at the junction between the funiculus and the developing seed with expression being restricted to the micropylar region. Intense GUS staining could also be seen in the hilum region of seeds that had been shed. This region of tissue has a number of important functions. It is a site that plays a key role in regulating assimilate partitioning into the developing seed (Thorne, 1985) and also the location where the mature seed is shed from the parent plant. Interestingly, the Type B response regulator *ARR21* has also been shown to be expressed in this region (Tajima et al., 2004). This observation raises the possibility that it might play a role in transcription of *ARR22* as in cytokinin signalling Type B ARRs are known to regulate the expression of Type A (Kakimoto, 2003). Intriguingly, a recent report by Aloni et al. (2006) has indicated that free IAA is also elevated at this site during silique development and, therefore, the expression of *ARR22* might be linked to levels of auxin at the seed: funiculus junction.

Work on *ARR22* was initiated by our discovery that a possible orthologue in *B. napus* was up-regulated during silique development (Whitelaw et al., 1999). Fusion of the promoter of the *B. napus* gene to GUS has also revealed that expression is visualized at the seed-funiculus junction (S Gattolin, unpublished results).

By using *Barnase* as a reporter gene other possible cellular locations where *ARR22* might be expressed have been examined. Expression of the ribonuclease leads to cell ablation and signs of tissue injury. Under the greenhouse conditions that were used here for raising the plants, no evidence could be found of sites of expression other than within the developing seed and this confirms the RT-PCR analysis. It is possible that *ARR22* could be expressed in other tissues under different growing conditions to account for the observations reported by Kiba et al. (2004).

Although *ARR22::GUS* expression could be detected after the isolation of developing seeds from small to mature siliques, accumulation of the reporter protein was only occasionally observed in these tissues when intact pods were incubated in GUS substrate. This was particularly surprising as our RT-PCR studies had shown high levels of *ARR22* transcript accumulate in small siliques. Therefore it was investigated whether *ARR22::GUS* expression might be up-regulated by wounding. If siliques are carefully opened and seeds are punctured with a sharp needle without removing them from the pod expression of *ARR22::GUS* is clearly visible at the seed–funiculus junction. *GUS* expression is not detectable at the site of wounding or in adjacent unpunctured seeds. Although an inability of GUS substrate to penetrate into cells could account for our observations, this hypothesis has been excluded. No evidence has been found that the impermeability of the seed tissues restricts *GUS* expression and we, and others, have characterized additional genes that have been shown to be up-regulated in this region of the seed–funiculus junction, using the same reporter, in the absence of wounding (S Gattolin, unpublished results; see also Tan et al., 2003). It is believed that the most likely explanation for these observations is that wounding promotes the post-transcriptional processing of *ARR22* and, as this takes place some distance from the site of tissue damage, intercellular signalling events must be involved. The transgene used to study expression comprised the 5′ UTR untranslated region of *ARR22* fused to *GUS* and this included the intron. Introns sited in regions upstream of the open reading frame have been shown to influence mRNA stability or translational control of gene expression. It is possible that this is the case for *ARR22* and that this situation may be reflected in the expression of *GUS* as well.

In an effort to determine the role of *ARR22* in seed development, expression of the response regulator was driven ectopically. Two promoters were used, 35S *CaMV* provides strong expression in many tissues although its efficacy
across all cell types, for instance within silique tissues, is variable whilst the pea plastocyanin promoter is particularly effective in photosynthetic tissues. Ectopic expression of ARR22 driven by either of these promoters results in broadly similar phenotype with plants exhibiting extreme stunting and inflorescences producing only a limited number of flowers (Gattolin, 2003). Whilst these observations broadly concur with those reported by Kiba et al. (2004), unlike those workers, we were able to obtain viable seeds from the CaMV 35S::ARR22 plants and isolate homozygous lines. The segregation ratio of these lines indicated that a single insertion was responsible for the phenotype. No viable seed could be obtained from the PPC::ARR22 plants and, in general, these plants showed more extreme stunting than the CaMV35S::ARR22 plants. Whether the failure to isolate seed from the PPC::ARR22 plants is a consequence of the ectopic expression of ARR22 within the developing silique or the impact of expressing this gene on the overall assimilation capacity of the plants is not clear.

Although ARR22 has features in common with other Arabidopsis Type A response regulators (Hwang and Sheen, 2002) only relatively recently has it been possible to identify a sequence in the Arabidopsis genome that shares substantial homology with the protein encoded by this gene (Gattolin, 2003). At5g26594 (ARR24) exhibits 66% amino acid similarity to ARR22 and it is therefore possible that the genes are functional homologues. In an effort to examine this hypothesis, the expression pattern of the gene was studied initially using a RT-PCR approach. The results show that the tissue profile of transcript accumulation is similar to ARR22 in that expression is at its highest level in reproductive tissues. The greatest level of expression of ARR24 was observed in young buds and this then declined in flowers and siliques. Fusion of the ARR24 promoter to GUS revealed that expression was primarily restricted to pollen grains within the anthers. GUS staining could be seen in developing buds and mature flowers. Other ARRs such as ARR1, ARR2, and ARR18 have also been shown to be expressed specifically in the anthers of developing flowers (Lohrmann et al., 2001; Mason et al., 2004). No expression of ARR24 could be detected at the seed–funicle junction even after wounding of the developing seed. As the spatial patterns of ARR22::GUS and ARR24::GUS expression do not overlap it seems unlikely that these response regulators play a similar role during reproductive development and operate as functional homologues.

An alternative strategy to the ectopic expression of a gene to determine its function is to study the phenotype of plants where the gene has been silenced. Although no knock out lines of ARR22 are documented as being available, such a line was isolated using the Wisconsin gene knockout facility. The insertion of the T-DNA is 75 bp downstream of the translation start site and, therefore, it is reasoned that this would provide an effective KO line. Although it has been established that the KO line exhibits an undetectable level of expression of ARR22, analysis of the material has failed to identify a phenotype of this mutant material under the growth conditions used here. KO lines of other response regulators have been generated and these have also failed to result in major phenotypic effects although this has been attributed to functional redundancy between the family members (Sakai et al., 2001). One possible role for ARR22 that is postulated here is that it could contribute, in some way, to either the development or activation of the seed abscission zone. However, it has not been possible to detect any effect on seed shedding. A second possible role for the peptide is to influence assimilate partitioning into the developing seed as the site of expression coincides with the tissues where symplastic unloading takes place and apoplastic uptake occurs. Intriguingly the expression of an amino acid importer (AAP1) has also been localized to the chalazal region of the seed and it has been proposed that this protein might function during the importing of assimilates into the developing seed (Hirner et al., 1998). A third possibility is that ARR22 might play a role in the guidance of the pollen tube as an analysis of the transcriptome of the pop2 mutant, that exhibits misguided pollen tubes (Palanivelu et al., 2003), has shown a 42-fold decrease in the expression of ARR22 (Updegraff et al., 2005). However, detailed phenotypic analyses of this ARR22 KO line has failed to identify any consistent effect of silencing the ARR22 gene on seed or silique development under the growth conditions employed.

A SALK insertion line (124785) has been characterized where the T-DNA is located in the second exon 860 bp downstream from the ATG start codon of ARR24. This insertion results in the absence of transcript accumulation of ARR24. Once again no phenotype was apparent as a consequence of down-regulating the gene. To confirm that ARR22 and ARR24 were not functional homologues, a double KO was generated that results in the silencing of both genes. This plant also fails to exhibit a phenotype, providing further evidence that the genes do not act in concert with one another.

Kiba et al. (2004, 2005) have speculated, on the basis of ectopic expression studies, that ARR22 plays a role in cytokinin signalling circuitry. Although this was looked into carefully, it was not possible to find any evidence that application of a range of concentrations of cytokinins to flowers or developing siliques can influence either ARR22::GUS or ARR24::GUS expression. The fact that these two ARRs exhibit clear sequence distinction from the remainder of the Type A ARRs in Arabidopsis may suggest that they are involved in a signalling chain regulated by another hormone, such as IAA (Aloni et al., 2006), or an environmental factor that remains, as yet, unknown.

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