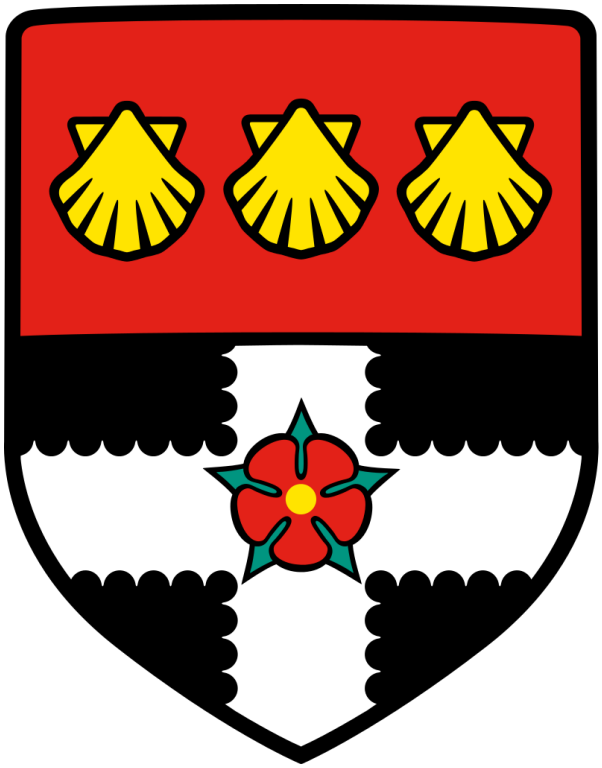

Dissecting the Role of the Response Regulator *SAC29* in *Brassica* Species

Thesis submitted for the degree of Doctor of Philosophy

Department of Food and Nutritional Sciences

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Content

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ABBREVIATIONS

%	Percentage
°C	Degrees centigrade
ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
AHK	Arabidopsis histidine kinase
ANOVA	Analysis of variance
Asp (D)	Aspartate
ATP	Adenosine triphosphate
β	Beta
bp	Base pair
bZIP	Basic Leucine Zipper
cDNA	Complementary deoxynucleic acid
cm	Centimetre
CTAB	Hexadecyl trimethyl-ammonium bromide
d	Days
DAF	Days after flowering
DAP	Days after pollination
ddH_2O	Double distilled water
DEPC	Diethylpyrocarbonate
DEX	Dexamethasone
DNA	Deoxynucleic acid
dNTP	Deoxynucleotide
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
g	Grams
GA	Giberellin

GFP	Green fluorescence protein
GUS	β-glucuronidase
h	Hours
HA	Hemagglutinin
HK	Histidine kinase
HPt	Phosphotransfer protein
His	Histidine
hrs	Hours
IgG	Immunoglobulin G
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JA	Jasmonic acid
Kb	Kilobase
L	Litre
LB	Lysogeny broth
Lys (K)	Lysine
M	Molar
mA	Milliamps
Mb	Megabase
mins	Minutes
ml	millitre
mm	Millimetre
mM	Millimolar
ng	Nanogram
nm	Nanometre
NLS	Nuclear localisation signal
OB	Oil bodies
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis

PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
s	Seconds
SA	Salicylic acid
SAM	Shoot apical meristem
SDS	Sodium dodecyl sulfate
SSP	Seed storage protein
TAG	Triacylglycerol
TCS	Two component system
TE	Tris-EDTA
TBS	Tris-buffered saline
Tris-HCL	Tris-hydrochloride
TTBS	Tris-buffered saline + Tween 20
U	Uracil
µg	Micrograms
µl	Microlitre
µM	Micromolar
UTR	Untranslated region
V	Volts
v/v	Volume to volume
w/v	Weight to volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-gluc	5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid

Abstract

Response regulators (RRs) are crucial signalling components that allow plants to respond to fluctuations in their environment. *ARR22* is a unique type-C RR previously identified in *Arabidopsis* that is hypothesised to be post-transcriptionally up-regulated in response to wounding at the seed:funiculus junction and hence has a predicted role in assimilate partitioning. A putative orthologue known as *SAC29* has been isolated in the economically important allotetraploid crop *Brassica napus* (*B. napus*).

A total of 83 putative RRs in *B. napus* (*BnRR*s) have been identified which can be classified into type-A, -B and –C RRs comparable to *Arabidopsis*. A subset of putative type-A and type-B *BnRR*s were examined further and expression was detected in early seed development stages which may reveal novel functions for these genes in *B. napus*.

In silico and expression analyses have identified and characterised four putative ARR22 orthologues (BnRR76 – BnRR79) that exhibit 81.25% amino acid similarity. Distinct differences in nucleotide and amino acid sequence were observed in *BnRR76* and *BnRR78* that originate from *B. rapa* and *B. oleracea* parental genomes respectively. All genes contain two introns, one located within the 5'UTR and one in the ORF, similar to *ARR22*. RT-PCR analysis revealed differences in spatial and temporal expression of *BnRR76* and *BnRR79* during seed development. Retention of an intron located within the open reading frame in *BnRR77* and *BnRR79* was also observed at different stages of seed maturation.

Mechanical wounding of seeds did not elicit a change in seed storage protein or cysteine protease expression even after 120 mins and hence

does not support the hypothesis that putative *B. napus* orthologues of *ARR22* are necessarily involved in assimilate partitioning. An antibody was designed to recognise an amino acid sequence present in ARR22, and BnRR76 – BnRR79, and was subsequently used in Western blot analysis. Expression of BnRR76 – BnRR79 proteins in seeds was rapidly up-regulated at 60 mins post-wounding while gene expression levels remained at a baseline level until 120 mins when protein level decreased suggesting that a rapid wound response occurs at the protein level rather than at the level of gene expression.

Using a dexamethasone (DEX) inducible system, physiological effects of *ARR22* overexpression were elucidated. DEX-induced overexpression resulted in severe phenotypes comparable to cytokinin receptor mutants such as reduced rosette area and stunted inflorescence. Transgenic lines in which a predicted phosphorylation site, hypothesised to be critical for protein function during stress response, had been mutated exhibited comparable phenotypic effects and hence suggests a possible different mode of mechanism of ARR22 when ectopically expressed.

This project explores and characterises response regulators, with particular focus on their involvement in seed development, for the first time in the economically important oilseed crop *B. napus*. Future work should examine wounding effects at longer time points as well as aim to elucidate downstream components and targets of *ARR22* and its putative *B. napus* orthologues *BnRR76 – BnRR79*.

Chapter 1:

Literature Review

1.1 The *Brassicaceae* family

The Brassicaceae family, also known as the mustard family, comprises over 3,700 flowering species (The Plant List 2013).

1.1.1 *Arabidopsis thaliana*

Arabidopsis thaliana (**Fig. 1.1**; thale cress) is a small weedy dicotyledonous plant belonging to the Brassicaceae family and found widely across Europe and Asia (Meyerowitz and Somerville 1994). It grows to approximately 25 cm in height and produces siliques up to 20 mm in length.

Although of no agronomic or economic importance, it is a popular model system for plant genetics and molecular biology research which is attributable to several advantageous characteristics. It has a rapid life cycle, taking 6-8 weeks from germination to maturity and is prolific in producing seed through self-pollination. Its small diploid 125 Mb genome was the first of any higher plant species to be fully sequenced which allowed the research community to begin large scale projects to determine the roles of its complement of approximately 25 000 genes (Arabidopsis Genome Initiative 2000). Genetic analysis in *Arabidopsis* has become somewhat straightforward with the advent of such molecular techniques as mutagenesis, introducing DNA via *Agrobacterium tumefaciens* as well as the development of mutant

genetic maps (Koornneef et al., 1982; Koornneef et al., 1983; Lloyd et al., 1986).

Figure 1.1. *Arabidopsis thaliana* during the vegetative phase (left); fully grown (middle); flowering (right); and seeds (right). Flower and seed bar 1 mm, other bars 1 cm. Source: http://www-ijpb.versailles.inra.fr/en/arabido/arabido.html.

Thanks to such advances, the fundamental growth and developmental processes common to all plants is relatively well understood. While it is necessary to continue to exploit the advantages of *Arabidopsis* to gain a full understanding of all its genes, it is of course warranted to translate and progress this research base into more complex crop species. Indeed comparative genetic analyses between *Arabidopsis* and crops such as rice and maize have been carried out (Gale and Devos 1998; Keller and Feuillet 2000; Liu et al., 2001) and genome sequencing has allowed for the comparison of genomes and proteomes (*The International Rice*

Genome Sequence Project 2005; Schnable et al., 2009; Schmutz et al., 2010).

1.1.2 Brassica genus

Owing to their wide morphological diversity, *Brassica* species are found in several edible forms within the human diet and hence are of significant economic importance. They also provide many nutritional benefits and are a source of anti-cancer compounds (van Poppel et al., 1999; Finley 2003).

1.1.2.1 *Brassica napus*

Brassica napus (*B. napus*) is an oilseed crop cultivated in several parts of the world including India, China, Europe, Canada and Australia used primarily for animal feed, vegetable oils and biofuel. *B. napus* is the third major source of vegetable oil after soybean and oil palm (USDA-FAS 2016). Global demand and growth for the oil has significantly increased over the past decade due to its nutritional advantages, containing the least amount of saturated fat than other edible oils and a mix of essential omega-3 and omega-6 fatty acids. It has hence become a crop of high interest for genetic improvement. *B. napus* (**Fig. 1.2**) possesses an allotetraploid (AACC) genome derived approximately 7 500 years ago from the natural hybridization of *B. rapa* (AA genome)

and *B. oleracea* (CC genome) forming part of the 'Triangle of U' (**Fig. 1.3**; Nagaharu 1935; Chalhoub et al., 2014).

Figure 1.2. *Brassica napus*. Source: http://www.biopix.com/rape-brassica-napus_photo-43537.aspx.

The Multinational *Brassica* Genome Project (http://brassica.nbi.ac.uk/welcome.htm) was formed in 2002, aiming to develop and bring genomic resources into the public domain. In 2011 the *B. rapa* genome (accession Chiifu-401-42) was published which was

followed 3 years later by the *B. oleracea* genome (Liu et al., 2014; Parkin et al., 2014). Recently a draft genome of *B. napus* was also released, sequenced using whole genome sequencing (line Darmor-*bzh) and mapped to B. rapa and B. oleracea (*Chalhoub et al., 2014) which will significantly aid in crop improvement.

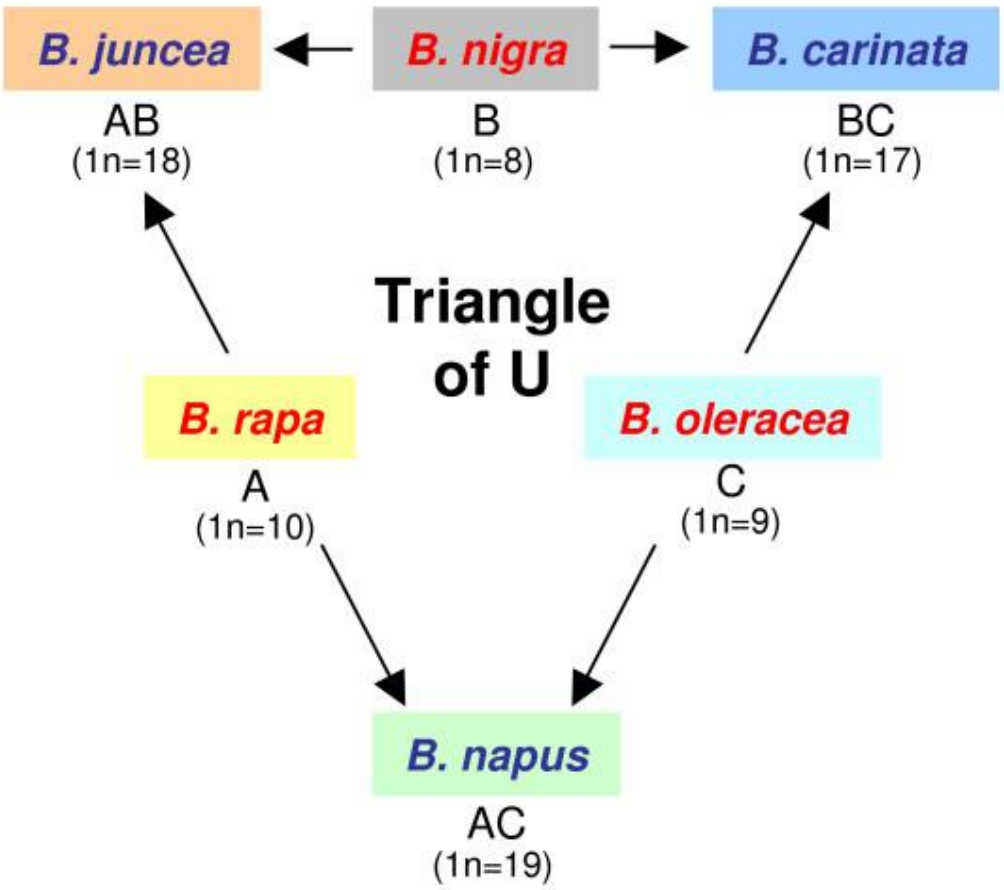


Figure 1.3. 'Triangle of U' theory depicting genetic relationship between *Brassica* species. Taken from Østergaard and King 2008.

1.2 Plant signalling: Two component systems

Two-component systems are sophisticated intracellular signalling mechanisms which allow prokaryotic and eukaryotic organisms to both sense and transduce an environmental signal into the necessary response. Initially identified in bacteria as a chemotaxis apparatus, simple two-component systems comprise a membrane bound receptor histidine kinase (HK) to sense an extracellular signal and a response regulator (RR) to translate the signal (**Fig. 1.4**; Kofoid and Parkinson, 1988; Stewart and Dahlquist, 1988). The activity of the response regulator is altered when an autophosphorylation event occurs on a conserved His residue of the histidine kinase in response to an environmental stimulus. Subsequently a phosphoryl group is transferred to a conserved Asp residue in the receiver domain on the response regulator, activating its output domain (Appleby et al., 1996; Mizuno 1998).

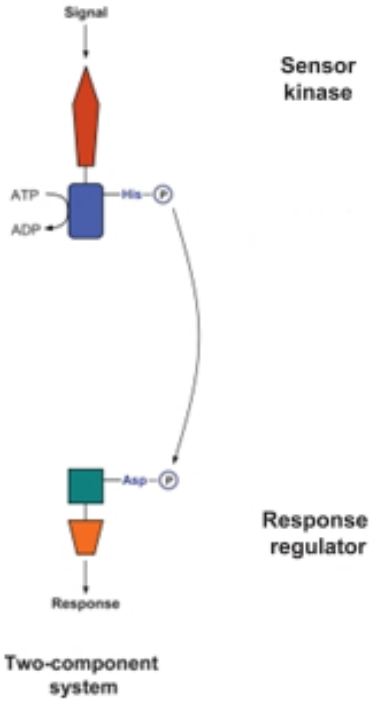


Figure 1.4. Two component systems and the proteins involved. Sensor kinase receives signals resulting in autophosphorylation and the transfer of a phosphoryl (P) group from the sensor kinase to a response regulator. Adapted fromMitrophangy and Groisman (2008).

1.3 The multistep phosphorelay system

The signalling system in plants, however, generally includes a third 'bridge' component known as a histidine phosphotransfer protein (HPt) which is also present in some prokaryotic systems (Burbulys et al., 1991; Appleby et al., 1996; Mizuno 1998). In this evolved system, a phosphoryl group is transferred multiple times (following a His→Asp→His→Asp pattern) and is branded the multi-step His-to-Asp phosphorelay (**Fig. 1.5**; Appleby et al., 1996; D'Agostino and Kieber 1999; Perraud et al., 1999). This modification to the simple system has been hypothesised to provide a number of regulatory checkpoints to

allow for, and co-ordinate, signal cross-talk (Urao et al., 2000; Urao et al., 2001).

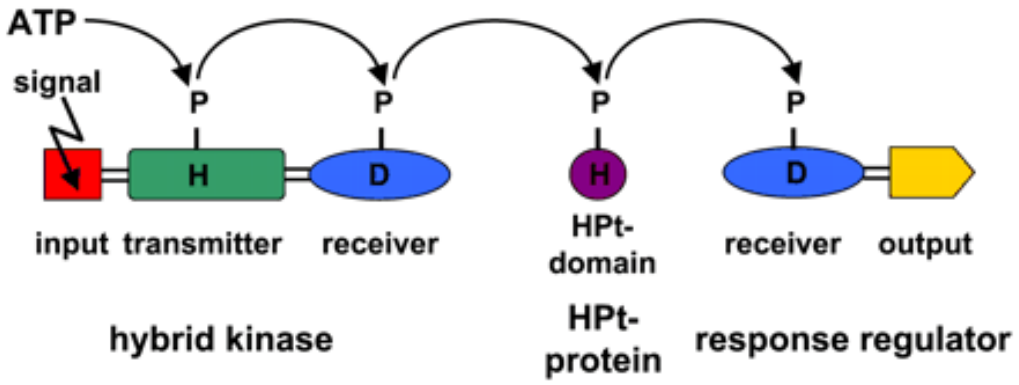


Figure 1.5. Features of the multistep phosphorelay system. A hybrid kinase receives a signal, such as cytokinin binding, resulting in the autophosphorylation of a His residue (H). A phosphate (P) is passed to an attached receiver domain before being relayed to a His-containing phosphotransfer protein (HPt) and subsequently to the receiver domain of a response regulator. Adapted fromLohrmann and Harter (2002).

1.4 Response Regulators

Response regulators are crucial components in plants for the transduction of a signal in response to a variety of stresses, such as heat, salinity and drought, in order to transcriptionally influence growth and development. In *Arabidopsis,* 24 response regulator genes (*ARR1* to *ARR24*) have been identified (Kiba et al., 2004) and each possesses a 120 amino acid receiver domain that contains a conserved DDK motif (Asp, Asp, Lys) (Imamura et al., 1999). This receiver domain is fused to a carboxy terminal output extension. Response regulators were originally classified into two major groups, based on structural composition (**Fig. 1.6**), known as type-A and type-B (Imamura et al., 1999; Hwang et al., 2002).

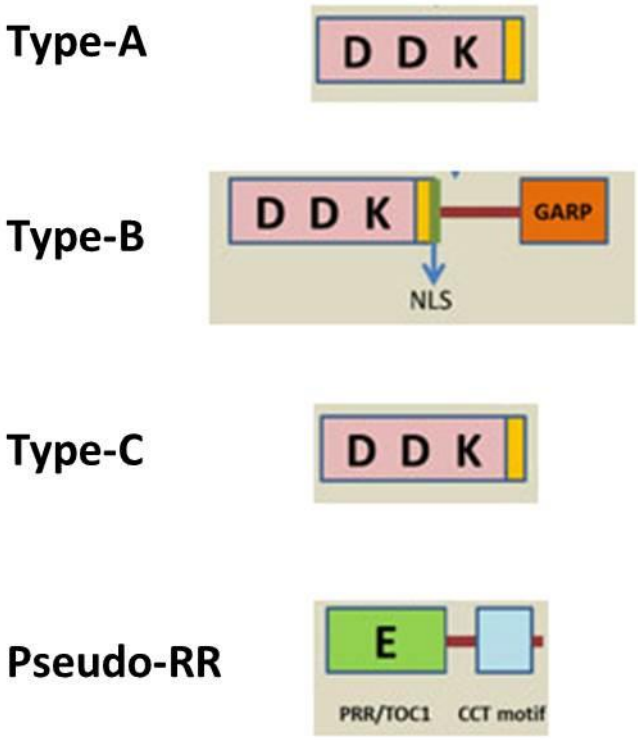


Figure 1.6. Structural composition of type-A, -B, -C and pseudo Response Regulators. Type-A and –C RRs possess only receiver domains (pink) containing the D, D, K motif for phosphorylation. Type-B RRs possess this in addition to a nuclear localisation signal (NLS) and a C-terminal extension (orange) containing the GARP domain. In contrast, the structure of Pseudo-RRs lacks these domains. Adapted from Gupta 2012.

Type-A ARRs are categorized by the possession of a short carboxy terminal extension whereas type-B ARRs possess a much longer carboxy terminal extension (Imamura et al., 1999). However phylogenetic analysis has since extended the classification with two additional groups added referred to as type-C and pseudo response regulators (PRR) (**Fig. 1.7**; Mizuno and Nakamichi 2005; Schaller et al., 2007). The five members of the PRR gene family (*PRR9*, *PRR7*, *PRR5*, *PRR3*, and PRR1/*TOC1*) are not directly considered as players within phosphorelay systems since they lack the necessary aspartate residue for

phosphorylation which is often replaced by glutamate (Makino et al., 2000; Matsushika et al., 2000). Their role lies within maintaining circadian rhythms which is facilitated by the possession of a CCT motif within their C-terminal extension (Mizuno and Nakamichi 2005; Nakamichi et al., 2005).

The multi-step phosphorelay system is not solely confined to *Arabidopsis* and a number of components have been found in a variety of important crop species such as soybean, rice, maize and Brassicas (Sakakibara et al., 1999; Whitelaw et al., 1999; Asakura et al., 2003; Du et al., 20007; Mochida et al., 2010) which will later be discussed.

1.5 Role of type-A ARRs in cytokinin signalling

The type-A response regulator family consists of 10 members (*ARR3*, *ARR4*, *ARR5*, *ARR6*, *ARR7*, *ARR8*, *ARR9*, *ARR15*, *ARR16*, and *ARR17*) and these have been implicated in a number of functions particularly during hormone signalling as well as in response to drought and nutritional status (Coello and Polacco 1999; To et al., 2004; Wang et al., 2011). The sub-cellular localisation of type-A ARR expression has been examined through the use of reporter genes such as green fluorescent protein (GFP) which has shown *ARR5*, *ARR6*, *ARR7* and *ARR15* to be restricted to the nucleus whereas *ARR4* and *ARR16* appear to additionally be expressed in the cytoplasm (Imamura et al., 2001;

Sweere et al., 2001; Kiba et al., 2002). This evidence alludes to their extensive role in plant signalling.

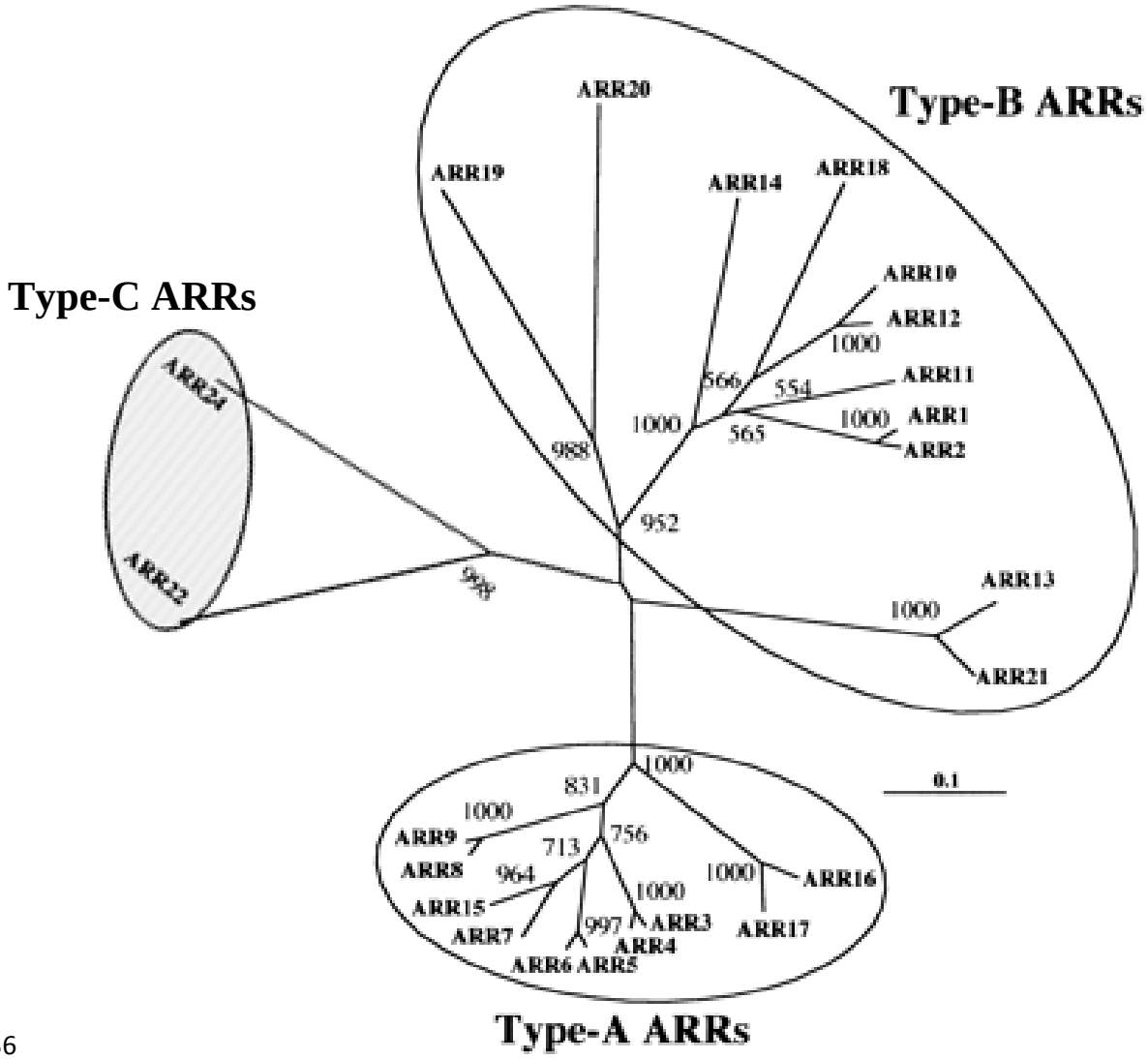


Figure 1.7. Phylogenetic tree constructed from amino acid sequences of ARR receiver domains showing the three main groups. Adapted from Kiba et al., 2004.

The exogenous application of cytokinin, strikingly, leads to the rapid up-regulation of type-A ARRs demonstrating their role in the signalling of this plant hormone (Brandstatter and Kiever 1998; Taniguchi et al.,

1998, Kiba et al., 1999; D'Agostino et al., 2000). Microarray analyses have revealed that each gene appears to accumulate at a different level, for example *ARR5*, *ARR6*, *ARR7* and *ARR15* are rapidly induced to a higher level with evidence suggesting that this is via transcriptional activation without *de novo* protein synthesis, hence they can be denoted "primary response genes". In comparison, *ARR4*, *ARR8* and *ARR9* have a comparatively high basal level (Imamura et al., 1998; Taniguchi et al., 1998; D'Agostino et al., 2000; Che et al., 2002; Rashotte et al., 2003). It has in fact come to light through loss-of-function and gain-of-function mutational studies that type-A ARRs are partially redundant negative regulators of cytokinin and are involved in a negative feedback loop with type-B ARRs (Hwang and Sheen 2001; Kiba et al., 2003; To et al., 2004). Specifically, the suggested cytokinin signalling mechanism involves cytokinin signal perception by histidine kinase cytokinin receptors *AHK2*, *AHK3* and *CRE1/AHK4* which activates the phosphorelay and leads to downstream phosphorylation of type-B ARRs (Sakai et al., 2000; Hwang and Sheen 2011). The type-B ARRs transcriptionally activate type-A ARR genes which subsequently feedback to prevent their transcription (Inoue et al., 2001; To et al., 2004; To and Kieber 2008).

It was hypothesised that type-A ARRs had redundant (or overlapping) functions and this was particularly observed after the application of cytokinin (To et al., 2004). A *GUS* analysis was performed for six type-A

ARRs in seedlings in which reporter expression was observed to expand to tissues surrounding their normal localization after cytokinin treatment (To et al., 2004). However some type-A ARRs exhibit tissue specific expression with some antagonistic interactions among them (Leibfreid et al., 2005; Salome et al., 2006; Ishida et al., 2008a).

Some developmental processes rely on interactions between type-A ARRs, transcription factors and cytokinin. For example during development of the shoot apical meristem (SAM), *STIMPY* (or *STIP*) is transcriptionally regulated by cytokinin and acts upstream of type-A ARRs for meristem establishment in seedlings (**Fig. 1.8**; Skylar et al., 2010). Within shoot development it has been observed that a homeodomain transcription factor that maintains stem cells in an undifferentiated state known as *WUSCHEL (WUS*) represses a number of type-A ARRs to increase cytokinin signalling for normal meristem function (Leibfreid et al., 2005).

Cytokinin has an antagonistic relationship with other hormones throughout plant development. Type-A RRs have a number of roles in cytokinin signalling and, interestingly, appear to mediate hormone communication in order to integrate and transcriptionally synchronize numerous developmental processes. For example the control of meristematic function is linked with cytokinin and other hormone signalling. Within the root apical meristem cytokinin and auxin modulate

size and growth through the regulation of PIN-FORMED proteins and auxin repressor *SHORT HYPOCOTYL 2* (*SHY2/IAA3*) (Dello Ioio et al., 2008). Polar auxin transport, cell division and differentiation were severely disrupted in the octuple mutant *arr3*,*4*,*5,6*,*7*,*8*,*9*,*15* thus implicating type-A ARRs within root development through regulation of PIN proteins, specifically at a post-transcriptional level (Zhang et al., 2011). Auxin also influences *ARR7* and *ARR15* in the determination of the inflorescence apical meristem through *AUXIN RESPONSE FACTOR 5* (*ARF5*)/*MONOPTEROS* (*MP*) (Zhao et al., 2010).

Seed germination is governed by ABA interactions with other hormones, such as auxin and giberellins (GAs), and also relies on regulation by bZIP transcription factors such as *ABI5* (Lopez-Molina et al., 2001; Finkelstein et al., 2002; Lopez-Molina et al., 2003). It has been demonstrated that ABA and cytokinin can also interact during germination and seedling growth through the novel interplay of certain type-A ARRs. In the *arr3*,*4*,*5*,*6* quadruple mutant, *ABI5* expression was noticeably increased when compared to the control and was hypersensitive to ABA (Wang et al., 2011). It has therefore been proposed that *ABI5* is a target for a subset of type-A ARRs in the presence of elevated cytokinin levels.

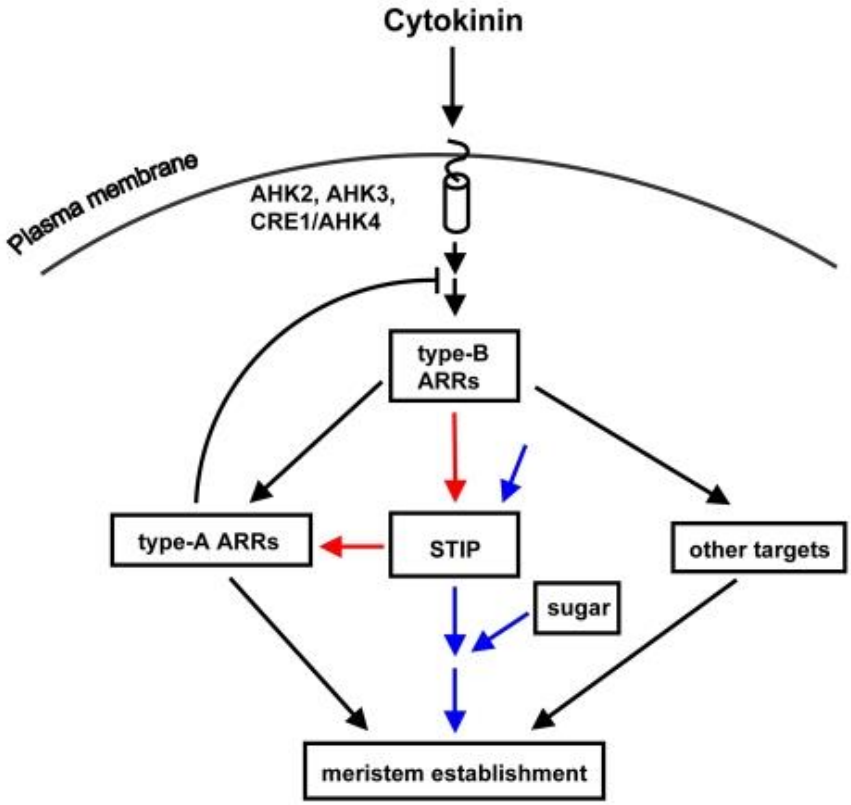


Figure 1.8. Involvement of *STIMPY* in cytokinin signalling for the establishment of the shoot apical meristem. *STIP* acts downstream of type-B RRs after perception of cytokinin. Taken from Skylar et al., 2010.

Type-A ARRs have additional roles in cytokinin signalling such as partly modulating plant immunity. Recently it was demonstrated that there is cross talk between cytokinin and salicylic acid, which is mediated partly by type-A ARRs, for defence against the oomycete *Hyaloperonospora arabidopsidis (Hpa)* isolate *Noco2* (**Fig. 1.9**; Argueso et al., 2012).

Moreover, type-A ARRs may have a role in plant nutrient signalling. For example, *ARR6* expression may be influenced by plant nutritional status. When plants were starved of phosphorous, nitrogen or potassium the protein accumulated in roots and rosette leaves (Coello and Polacco

1999). When phosphorous deficient plants were resupplied, *ARR6* protein levels decreased.

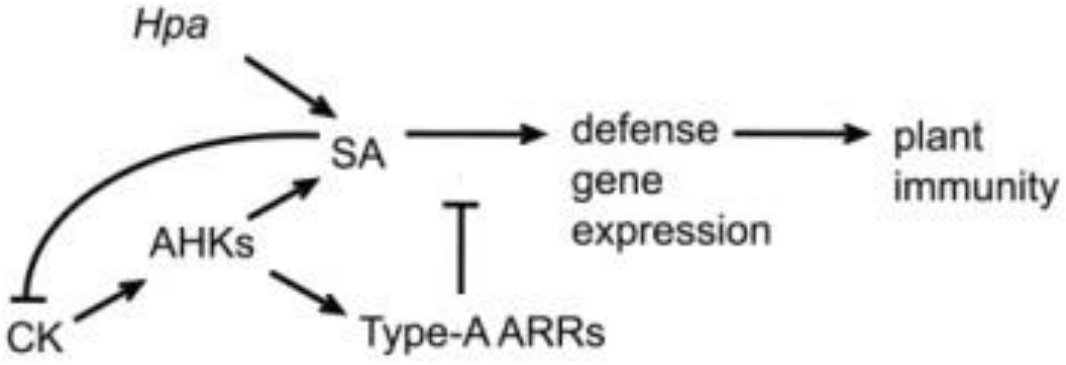


Figure 1.9. Interaction of type-A ARRs with cytokinin signalling for the moderation of plant immunity. Detection of the pathogen *Hpa* results in salicylic acid (SA) responses and the expression of defence genes. High concentrations of cytokinin increases the defense response. Type-A ARRs regulate the process which leads to SA inhibiting cytokinin signalling. Taken from Argueso et al., 2012.

A small number of type-A ARRs (*ARR3*, *ARR4*, *ARR8* and *ARR9*) have been identified as regulators of circadian rhythm (Salome et al., 2006; To and Kieber 2008). Of particular interest is *ARR4* which physically interacts with the NH_2 terminal red light photoreceptor phyB in order to stabilize it in its active light absorbing Pfr form (Sweere et al., 2001; To et al., 2004). Seedlings in which *ARR4* was overexpressed exhibited reduced hypocotyl growth and hence red light hypersensitivity (Sweere et al., 2001). A relationship between *ARR4*, PhyB and cytokinin signalling has been hypothesised potentially requiring phosphorylation of an Asp residue in *ARR4* (Mira-Rodado et al., 2007; Zheng et al., 2006). Moreover an antagonistic interaction seems to exist between *ARR3/ARR4* and *ARR8/ARR9* (Salome et al., 2006; To and Kieber 2008).

It has been suggested that the activity of type-A ARRs could be regulated via proteasomal degradation for cytokinin signalling control (Ren et al., 2009). For example the degradation of the luciferase fusion *ARR7:LUC* was moderately reduced by *MG132*, a proteasome inhibitor (Lee et al., 2008). Similarly Ren et al. (2009) observed that ARR3, ARR5, ARR16 and ARR17 proteins accumulated when treated with *MG132* as well as when treated with cytokinin. The mechanism of proteasomal degradation would thus allow type-B ARRs to accumulate. Other type-A ARRs were unaffected implying that there could be further regulatory mechanisms.

1.6 The function of type-A ARRs in abiotic stress

There is evidence emerging that implies that type-A RRs could be involved in a variety of biotic stress responses. For example, the transcriptome analysis carried out by Wolbach et al. (2008) revealed that *ARR4*, *ARR5*, *ARR6*, *ARR8*, and *ARR9* were all co-expressed with His kinase *ATHK1*, a potential osmosensor (Urao et al., 1999; Tran et al., 2007). Analysis of null mutants during seed germination indeed found a function for these ARRs in osmotic stress. The quadruple mutant *arr3,4,5,6* exhibited increased sensitivity to stress, however sensitivity in the *arr5,6,8,9* mutant was slightly decreased (Wohlbach et al., 2008). Interestingly the *arr3,4,5,6,8,9* mutant was comparable to the wild-type and therefore insinuates that an antagonistic relationship again exists

between *ARR3/ARR4* and *ARR8/ARR9* (To et al., 2004; Wohlbach et al., 2008) as previously mentioned.

Kang et al., (2012) also investigated the effect of drought stress on type-A ARR expression and found that dehydration induced *ARR5, ARR7, ARR15*. The same group have shown that the expression of *ARR5, ARR6, ARR7*, and *ARR15* is induced by cold stress treatment (Jeon et al., 2010). The expression of *ARR7* was particularly induced as confirmed by an *ARR7::GUS* analysis in 17-day old seedlings.

Shi et al., (2012) established a connection between ethylene and cytokinin signalling mediated by type-A ARRs to modify freezing tolerance. Specifically, the transcription factor ETHYLENE INSENSITIVE3 (EIN3) is believed to bind to the promoters of *ARR7* and *ARR15* and the stress responsive transcriptional activators C-repeat Binding Factors (CBFs) in order to repress their activity.

1.7 Type-B *ARR*s

There are 11 type-B ARRs found in *Arabidopsis* (*ARR1, 2, 10, 11, 12, 13, 14, 18, 19, 20* and *21*) that that can be further divided into one major and two minor subfamilies, based on phylogenetic analysis (**Fig. 1.10**; Mason et al., 2004). *ARR1, 2, 10, 11, 12, 14*, and *18* belong to B-1; *ARR13* and ARR*21* to B-II; and *ARR19* and *ARR20* B-III. Subfamily 1 members exhibit a much broader expression profile with RT-PCR and

GUS analyses revealing expression throughout almost the entire plant whereas expression of subfamilies 2 and 3 appear to be confined to reproductive organs (Mason et al., 2004; Tajima et al., 2004). All members of the type-B family contain a conserved nuclear localization signal motif (Imamura et al., 2001; Hosoda 2002). As confirmed by GFP and GUS analyses, type-B RRs are indeed nuclear localised (Lohrmann et al., 1999; Hwang and Sheen 2001; Imamura et al., 2001; Hosoda 2002).

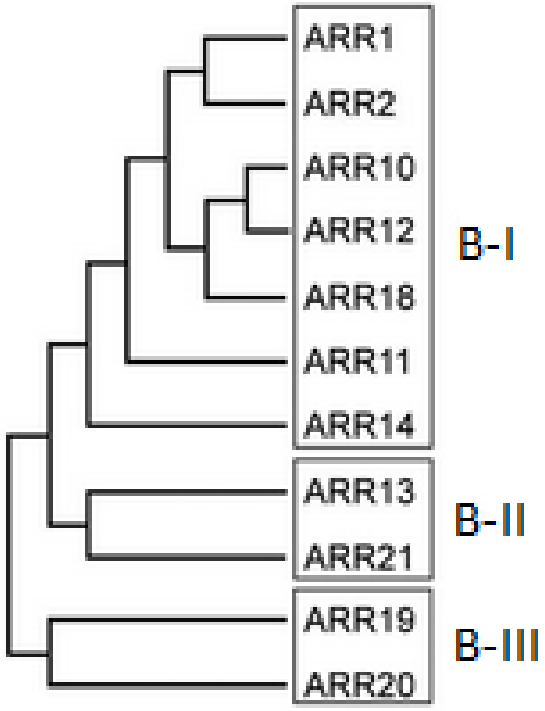


Figure 1.10. Cladogram showing the three subfamilies of *Arabidopsis* type-B RRs. Adapted from Hill et al., 2013.

A distinguishing feature of type-B ARRs is the 60 amino acid GARP motif that enables DNA binding, a characteristic that, to some extent, resembles Myb transcription factors (Sakai et al., 2001). A yeast two-

hybrid analysis uncovered *ARR2* as being a transcription factor specifically expressed in pollen (Lohrmann et al., 2001). Subsequent evidence has confirmed the type-B RRs as transcriptional activators which is consistent with their nuclear localization (Sakai et al., 2000; Sakai et al., 2001; Mason et al., 2004; Rashotte et al., 2006).

Within cytokinin signalling, the cytokinin signal is transmitted to the nucleus from the membrane which results in induction of type-A ARRs by type-B (Hwang and Sheen 2001; Sakai et al., 2001). It has been shown that at least five members (*ARR1*, *ARR2*, *ARR10*, *ARR11* and *ARR12*) of the largest subfamily are principally involved in cytokinin signalling (Mason et al., 2005; Yokoyama et al., 2007; Ishida et al., 2008). Specifically type-B ARRs have a crucial role in the early transcriptional response to cytokinin.

Analysis of loss-of-function mutants has revealed that type-B ARRs act as positive functionally redundant regulators in cytokinin signalling (Sakai et al., 2001; Mason et al., 2005; Argyros et al., 2008). For example, single mutant knockouts are not generally phenotypically altered (Sakai et al., 2001; Horak et al., 2003). However, in the *arr2* mutant, retarded growth and early flowering were noted and in the *arr1* mutant, the size of the root apical meristem was increased (Hass et al., 2004; Dello Ioio et al., 2007). A dominant repressor form of *ARR1* resulted in cytokinin resistance, reduced shoot growth and leaf size, and

enhanced root growth, a strong phenotype comparable to triple loss-of-function cytokinin mutants (Heyl et al., 2008). Conversely, root and shoot phenotypes in the *arr1-3 arr10-5 arr12-1* triple mutant were severely affected and sensitivity to light was increased, effects equivalent to those observed in *ahk* and *ahp* cytokinin receptor mutants hence indicating that these type-B ARRs are crucial for cytokinin action in a variety of processes (Argyros et al., 2008; Ishida et al., 2008). Additionally, analysis of the gain-of-function mutant *arr2* revealed that *ARR2* is also involved in cytokinin mediated regulation of leaf senescence (Putterill et al., 1995) which is consistent with the finding that *ARR2* expression is up-regulated in leaves (Wagstaff et al., 2009).

Mutational analysis has demonstrated that some type-B ARRs also contribute to other signalling networks in order to influence other developmental process. For example overexpression of *ARR1* led to a decrease in root apical meristem size which has been confirmed to be due to an interaction between *ARR1* and *SHY2*, a negative regulator of PIN proteins in auxin signalling (Dello Ioio et al., 2008). *ARR2* has been shown to have a function within ethylene signalling (Hass et al., 2004). Additionally, it has been demonstrated that ARR2 and the salicylic acid response factor *TGA3* can bind thus ultimately resulting in resistance to *Pseudomonas syringae pv. Tomato DC3000* (*Pst*) (Cho et al., 2010). ARR1 and ARR12 regulate shoot sodium accumulation by controlling the

expression of *Arabidopsis* high-affinity K+ transporter 1;1 (AtHKT1;1) in the roots (Mason et al., 2010).

1.8 Pseudo Response Regulators

Pseudo response regulators (PRRs) are nuclear localised proteins that lack the phospho-accepting aspartate residue that is essential for TCS activity (Makino et al., 2000). Within the carboxy extension of PRRs is a CCT motif which is a characteristic feature of the CONSTANS (CO) protein family that are implicated in control of long-day flowering (Putterill et al., 1995; Matsushika et al., 2000). It has been established that PRRs along with the LATE ELONGATED HYPOCOTYL/CIRCA-DIAN-CLOCK ASSOCIATED 1 (LHY/CCA1) protein family regulate circadian rhythm (Makino et al., 2002; Mizuno 2004).

1.9 Type-C ARRs: A novel group

ARR22 and *ARR24* belong to a unique group of *ARR*s. Structurally they are similar to type-A RRs however phylogenetic analysis of their receiver domains places them outside of the type-A and type-B groups (Kiba et al., 2004). Several studies have examined the transcriptional regulation of *ARR22* and *ARR24* by cytokinin and ethylene signalling yet their roles within such networks are unclear (Kiba et al., 2004; Gattolin et al., 2006; Horak et al., 2008). For example, Horak et al. (2008) fused the promoter of *ARR22* to the green fluorescence protein (*GFP*) gene

(*ARR22::GFP*) for GFP analysis. Fluorescence intensity was analysed in the siliques of inflorescences that were excised and placed in solutions of cytokinin (benzyladenine) or the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) however no fluorescence was observed. Despite this, the ability of *ARR22* to act within a phosphorelay system has been confirmed by a yeast two-hybrid and an *in planta* bimolecular fluorescence complementation approach (Horak et al., 2008). Specifically, *ARR22* has been shown to interact with *AHP2*, *AHP3* and *AHP5* (Kiba et al., 2004; Horak et al., 2008).

1.9.1 *ARR22*

Within *the ARR22* gene two introns have been identified; one (183 bp) located within the 5'UTR; and another (123 bp) within the ORF (Gattolin et al., 2006). RT-PCR analysis of *ARR22* expression showed that *ARR22* produces four splice variants (**Fig. 1.11**) and is expressed in flowers and in small (3–5 days after flowering) and elongating siliques (4-8 days after flowering). The fully processed transcript (526 bp) and the transcript containing the 5'UTR (709 bp) are the most prevalent in flowers. In small siliques high levels of transcript were observed with the partially processed transcript (649 bp) detected equally with the 709 bp transcript as well as the 526 bp transcript. In elongating siliques the 526 bp transcript is predominant. The unprocessed transcript (832 bp) can be detected in flowers and small siliques but at a low level.

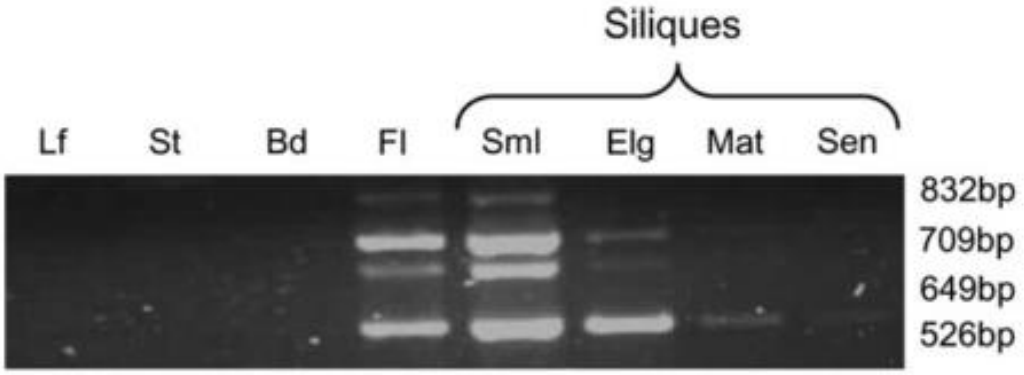


Figure 1.11. RT-PCR analysis of *ARR22* expression, demonstrating splice variants in leaf (Lf), stem (St), bud (B), flowers (Fl), small (Sml), elongating (Elg), mature (Mat) and senescing (Sen) siliques. Transcript sizes: 526 bp (fully processed); 649 bp (retention of ORF intron and 5' UTR intron excised); 709 bp (retention of 5' UTR intron and ORF intron excised); and 832 bp (unprocessed). Adapted from Gattolin et al., 2008.

Two mutant alleles of *ARR22* containing a T-DNA insertion in the intron within the ORF, one of which was located 3 bp upstream from the intron splicing site, have been analysed in order to characterise gene function (Horak et al., 2008). RT-PCR analysis confirmed absence of the *ARR22* transcript in siliques of the mutant lines. No difference in seed development, morphology or metabolic state was observed in the mutant lines compared to wild type. However, overexpression of *ARR22* ectopically, driven by a *CaMV 35S* promoter, results in a dramatic dwarf phenotype with a reduced number of flowers (**Fig. 1.12**; Gattolin et al., 2008).

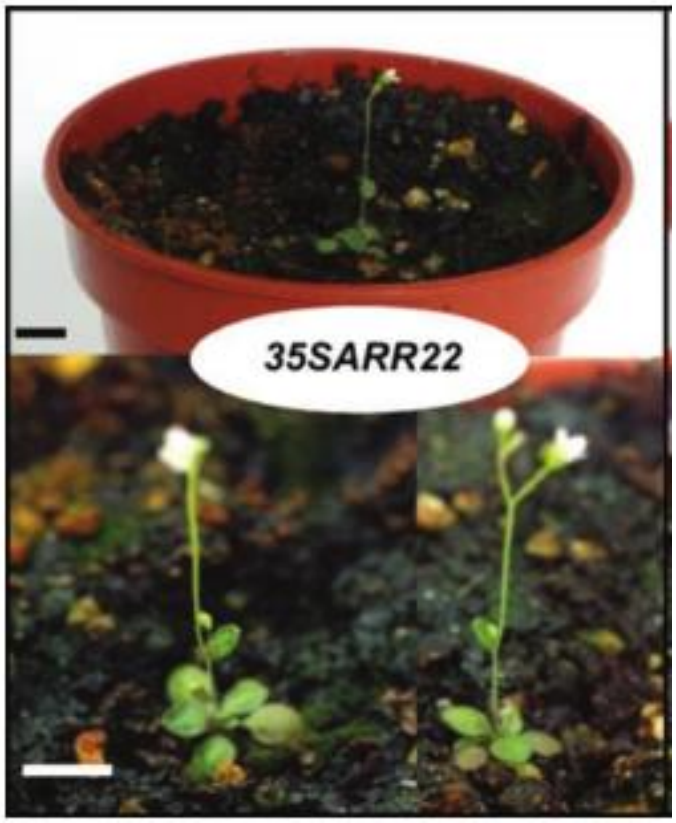


Figure 1.12. Overexpression of *ARR22* under a *CaMV 35S* promoter produces a dwarf phenotype. Taken from Gattolin et al., 2008.

To elucidate the precise location of *ARR22* activity, *ARR22::GUS* *Arabidopsis* lines were created for a β-glucuronidase (GUS) reporter analysis. In seeds isolated from siliques, GUS activity was localised at the seed:funiculus junction (Gattolin et al., 2006). Despite high levels of *ARR22* transcript having been observed in small siliques via RT-PCR analysis, little GUS activity was in fact observed in seeds in intact pods. Therefore it was hypothesised that wounding promoted *ARR22* expression. This was confirmed via an additional GUS analysis in which alternating seeds were mechanically wounded with a needle. The expression of GUS was not identified at the location of wounding nor in adjacent unwounded seeds. Therefore it is believed that *ARR22* is post-

transcriptionally up-regulated and additional intercellular signalling events are implicated (Gattolin et al., 2006). It has, additionally, been shown that at 90 mins after wounding proteolysis genes are up-regulated whilst seed storage protein genes are down-regulated in wildtype plants but not in an *ARR22* T-DNA insertion (knock out) line (Naomab, 2008). Therefore it has been hypothesised that *ARR22* may act as a gate to co-ordinate grain filling in damaged seeds.

As *ARR22* is unusual amongst response regulators in that it does not respond to cytokinin, other hormones or even an environmental signal could be involved in co-ordinating its expression. Recently, Kang et al. (2012) demonstrated that *ARR22* may respond to water deficit and thus could respond to abiotic as well as biotic stresses.

1.10 Response Regulators in Crop Plants

In addition to *Arabidopsis*, a number of RRs have been isolated and characterized in major crop plants. For example, in soybean (*Glycine max*) 18 type-A (*GmRR 1 – 18*), 15 type-B (*GmRR19 – 33*) and 3 type-C RRs (*GmRR 34 - 36*) have been discovered (Mochida et al., 2010). Interestingly, all type-B *GmRR*s were grouped into subfamily 1 however this information was gathered from only ~85% of the sequenced genome and therefore other *GmRR*s could be revealed in the remaining 15% (Mochida et al., 2010). Expression profiles of these *GmRR*s have been analysed in roots and shoots under normal and dehydrated

conditions in order to identify candidate genes for improving drought resistance (Le et al., 2011). Under normal conditions, type-C *GmRR* expression was much lower than that of type-A and type-B *GmRR*s however when dehydrated expression of all 3 type-C *GmRR*s was significantly induced in both roots and shoots whereas a more diverse pattern was seen for type-A and type-B *GmRR*s.

In maize (*Zea mays*), 6 type-A RRs (*ZmRR1, ZmRR2, ZmRR4–ZmRR7*) and 3 type-B RRs (*ZmRR8–ZmRR10*) have been distinguished and their roles in cytokinin signalling analysed (Sakakibara et al., 1998; Asakura et al.,2003; Giulini et al., 2004). GFP analysis showed that three type-A ZmRRs were localized in the cytosol (*ZmRR1, ZmRR2 and ZmRR3*), three in the nucleus (*ZmRR4*, *ZmRR5* and *ZmRR6*) and all type-B ZmRRs in the nucleus (Asakura et al.,2003). Cytokinin treatment results in an increase of type-A ZmRR transcripts (Sakakibara et al., 1998; Sakakibara et al., 1999; Asakura et al., 2003). Cytokinin response was also investigated in the type-A ZmRR homologue mutant *abphyl1* (*ABERRANT PHYLLOTAXY1*) in which phyllotaxy and shoot organ initiation are severely altered (Giulini et al., 2004).

Thirteen type-A and three type-B OsRRs have been identified in rice (*Oryza sativa*) (Ito and Kurata 2006; Jain et al., 2006). Expression of OsRRs was investigated by real-time PCR in seedlings after a number of different treatments: hormone application, salinity, dehydration, and low temperature (Jain et al., 2006). Cytokinin treatment induced

expression of most OsRRs whereas other hormones such as ethylene and auxin had no effect. In response to environmental stresses, the expression of one particular OsRR (*OsRR6*) was significantly induced in all stress conditions indicating its potential role in abiotic stress signalling. In transgenic lines overexpressing *OsRR6*, rice plants were dwarfed with small root systems and the expression of cytokinin responsive genes altered (Hirose et al., 2007).

In *Brassica* species, Liu et al. (2014) have identified 42 RRs (21 type-A, 17 type-B, 4 type-C) in Chinese cabbage (*BrRR*s; *B. rapa*) in a database search. Unsurprisingly, application of cytokinin led to the transcriptional up-regulation of type-A *BrRR*s.

1.11 *SAC29*: an *ARR22* orthologue in *Brassica napus*

During a study of genes expressed throughout silique development in oilseed rape (*Brassica napus*), a cDNA was identified in dehiscence zone tissues that appears to be an orthologue of the type-C *ARR22* in *Arabidopsis* (Whitelaw et al., 1999; Gattolin et al., 2006). Vegetable oils are a major source of calories for human diets and are routinely used within the food industry as well as in non-food products such as biodiesel. *ARR22* is a unique gene that may regulate the response of seeds to stress. Given that it is expressed in a prime location at the seed:funiculus junction it is potentially an ideal candidate for co-ordinating seed storage products into and out of maturing seeds. Since

lipids are major storage products in *Brassica* species, it is of great interest to distinguish whether *SAC29* has a role in determining seed quality, in particular the partitioning of seed resources and composition of the lipid component of the seed. It may perhaps be that the expression of *SAC29* can be influenced in order to manipulate assimilate portioning.

1.12 Seed development, storage proteins, lipids and proteases

Seeds contain proteins that provide a source of nitrogen and amino acids that are required for seed germination. Oilseed development can be divided into approximately four stages: embryo pattern formation, embryo growth, maturation, or seed filling, in which lipids and proteins accumulate, and desiccation (**Fig. 1.13**; Fei et al., 2007; Nietzel et al., 2013). During seed filling, storage reserves are established and there are major gene expression changes particularly associated with lipid biosynthesis and seed storage protein (SSP) accumulation. In *B. napus,* seeds comprise 15% protein and 40% oil (Norton and Harris 1975). The SSPs are classified into groups of which 2S albumins (napin) and 12S globulins (cruciferin) are predominant, representing 20% and 60% of total mature seed protein respectively (Hoglund et al., 1992; Nietzel et al., 2013). Accumulation of these SSPs in protein storage vacuoles in the embryo begins around 20 to 28 DAP and continues until approximately 40 DAP when napin synthesis plateaus but cruciferin synthesis continues for an additional 7 d (Crouch and Sussex 1981).

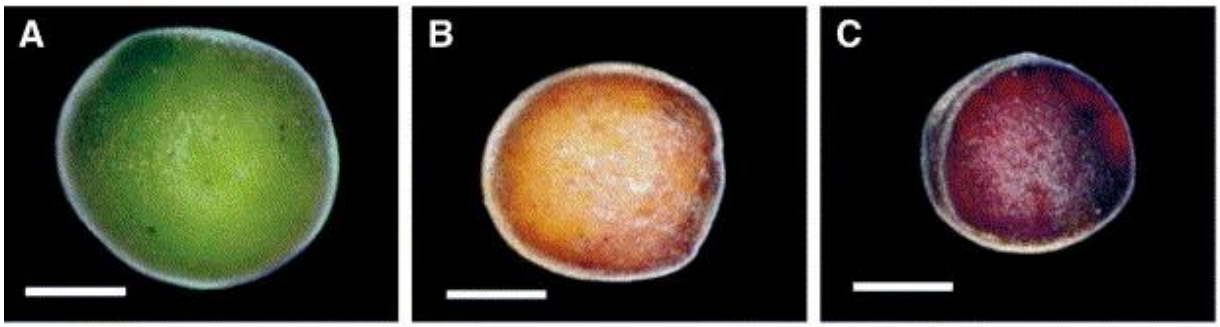


Figure 1.13. Development of *Brassica napus* seeds at selected stages. (A) Full size embryo; (B) Dessicating; (C) Mature dry seed. Bar = 1mm. Adapted from Fei et al., 2007.

Oil bodies (OBs) are lipid particles found primarily in seeds and are comprised of triacylglycerol (TAG), phospholipids and proteins. The most abundant type of protein found in seed OBs are oleosins that represent 75-80% of the total protein content found in OBs (Jolivet et al., 2011).

Cysteine proteases are one of five classes of endoproteases that are implicated in plant proteolysis and act by cleaving internal peptide bonds (Palma et al., 2002; Rawlings et al., 2012). There are approximately 140 cysteine proteases encoded in plant genomes (Rawlings et al., 2006). They play numerous and diverse key roles throughout plant growth and development in response to developmental and environmental signals such as programmed cell death, tissue senescence, breakdown of SSPs and remobilization of amino acids (Ueda et al., 2000; Schaller 2004; van der Hoorn 2008). The most well studied cysteine proteases include calpains, papain-like proteases,

caspase-like proteins (including vacuolar processing enzymes) and deconjugating enzymes (Palma et al., 2002; van der Hoorn 2008).

It has been established that abiotic stress can induce accumulation of cysteine proteases; for example under drought conditions and high and low temperature (Schaffer 1988; Koizumi 1993; Schaffer and Fischer 1990). Studies investigating wounding have also shown that cysteine protease expression is enhanced (Linthorst et al., 1993; Lidgett et al., 1995; Ueda et al., 2000).

1.13 Plant responses to wounding

Plants respond to mechanical wounding, such as insect damage, via signalling systems in order to transcriptionally, post-transcriptionally or post-translationally activate a variety of genes that results in a range of defense mechanisms (Crouch and Sussex 1981). The response can be generated relatively quickly i.e a few minutes after damage or up to several hours and can occur at the site of wounding (local response) or in distal parts (systemic response) of the plant (Crouch and Sussex 1981). Generally, responses are mediated by the increased synthesis, accumulation, perception and crosstalk of hormones such as ethylene, jasmonic acid (JA) and ABA (Norton and Harris 1975; Hoglund et al., 1992). However, other elements such as microRNAs may also be induced in some tissues in response to wounding (Jolivet et al., 2011). As a consequence of wound induced gene expression changes, tissue

repair and metabolism modifications can occur (Crouch and Sussex 1981).

Few studies have addressed or explored the effects of wounding specifically in seeds. It is thus unknown whether mechanical damage is detrimental to yield, particularly in important oilseed crops. However, a recent transcript profiling analysis indicates that wounding in *Arabidopsis* could in fact alter metabolism in seeds (Naomab, 2008). A change in expression in more than 2000 genes was observed with seed storage protein gene expression notably decreased and seed proteolysis genes up-regulated.

1.14 Hypotheses, aims and objectives

It is apparent from the work carried out in *Arabidopsis* that *ARR22* has a likely role in coordinating a response to biotic stress within seeds. Evidence suggests that *ARR22* and its putative orthologue *SAC29* in *B. napus* may act in a unique way as a gate in order to regulate the import or export of crucial seed storage products. This has therefore led to the following hypotheses:

- Wounding of *Brassica* seeds results in post-transcriptional up-regulation of the putative *ARR22* orthologue *SAC29* which leads to the remobilisation of proteins and lipids out of the seeds into non-wounded adjacent tissues.

- *SAC29* has a key role in assimilate partitioning during grain filling.
- Through manipulation of *SAC29* expression, grain filling and nutritional composition can be altered in *Brassica* seeds.

1.14.1 Project aims

The general aims of this project are to elucidate the precise role of *SAC29* in *Brassica* species throughout growth and development and in response to biotic and abiotic stress; to understand it's mechanism of regulation; and to monitor remobilisation of important seed storage proteins and lipids after seed damage has occurred. A better understanding of the basic biological process that lead to movement of seed components out of the seed is sought by studying the regulatory role of *ARR22* and its orthologue *SAC29* in *Arabidopsis* and *Brassica* respectively. This will identify potential targets for future work that will allow us to manipulate the uptake and movement of seed storage lipids and proteins into the seeds of crop plants.

1.14.2 Specific Objectives:

- Identify and characterise type-A, -B and –C response regulator genes in *Brassica* species, specifically *B. rapa*, *B. oleracea* and *B. napus* (**sections 3.2 – 3.4.2; Chapter 3**).
- Establish the temporal expression of two type-A and two type-B RRs in *B. napus* (**section 4.2; Chapter 4**).

- Establish the spatial expression of *SAC29* in *B. napus* (**section 4.3; Chapter 4**).
- Determine the expression of *SAC29* in *B. napus* seeds post-wounding (**section 4.5.1; Chapter 4**).
- Analyse the expression of seed storage protein and proteolysis genes in *B. napus* tissues and in unwounded and post-wounded seeds (**section 4.5.2; Chapter 4**).
- Analyse SAC29 protein expression in unwounded and wounded *B. napus* seeds (**section4.6.1; Chapter 4**).
- Though the use of a dexamethasome inducible system, analyse the effect of overexpressing *ARR22* in *Arabidopsis* on physiology and phenotype while monitoring gene and protein expression (**Chapter 5**).

Chapter 2:

Materials and Methods

2.1 Plant material and growth conditions

Dexamethasone (DEX) inducible transgenic *Arabidopsis* lines 11-7 and 15-5 overexpressing *ARR22*:*HA* and lines 17-3 and 20-3 overexpressing *ARR22*^{D74N}:*HA* were obtained from the Department of Bioenergy Science and Technology and Kumho Life Science Laboratory, Chonnam National University, Korea (Kang et al., 2013).

ARR22:*HA, ARR22*^{D74N}:*HA,* and *Arabidopsis* wild type (ecotype Columbia-0) were sown on Clover Seed and Modular in 9 cm pots and supplemented with intercept at a rate of 0.2 g/L (w/v). Seeds were stratified at 4°C for two days.

Brassica napus RV31 (Westar derivative) seeds were acquired from BRACT (John Innes Centre, Norwich) and sown on Clover Seed and Modular compost in 9 cm pots. Seedlings were then transplanted into 20 cm pots on Clover potting compost. Plants were supplemented with Sinclair Sangral soluble fertiliser 3:1:1 twice a week at a rate of 1:200 (w/v).

Arabidopsis and *B. napus* plants were grown in a controlled growth room with a 16 h photoperiod at a temperature of 20°C and 60% humidity. Flowers on the primary inflorescence were tagged for specific silique stages. Seeds were extracted from *B. napus* siliques after detaching the pods from the plant.

2.1.2 Plant wounding

Wounding of *B. napus* seeds was carried out at 20 and 35 DAF. Siliques attached to the plant were punctured with a pin and left for 5 – 120 mins before the silique was detached, opened and seeds collected for RT-PCR analysis (**section 2.4**).

2.2 Dexamethasone treatment

Dexamethasone (DEX) was dissolved in DMSO to produce a 25 mM stock solution. *DEX* inducible transgenic *Arabidopsis* lines 11-7, 15-5, 17-3 and 20-3 as well as ColWT were sprayed every day from germination or post flowering, depending on experiment, with 25 μM DEX solution (stock solution added to Triton X-100 and ddH2O) or (-) DEX control (DMSO added to Triton X-100 and ddH2O). Plants and soil were sprayed until wet.

2.3 Physiology measurements

The following physiological and morphological characteristics were measured and photographed.

2.3.1 Measurement of leaf number

Leaf number was counted every day until flowering. Plants were photographed at 1, 2 and 3 weeks post spraying. At the end of the analysis rosettes were dissected out for photographs.

2.3.2 Measurement of rosette area

After 3 weeks of spraying with +/- DEX plant rosettes were photographed. Rosette area was calculated using ImageJ (Schneider et al., 2012).

2.3.3 Measurement of primary inflorescence height

At 3 weeks of spraying post floral induction the primary inflorescence of each plant was measured with a ruler.

2.3.4 Measurement of axillary branch number

At 3 weeks of spraying post flowering axillary branch number of each plant was counted. Plants were dissected for photographs.

2.3.5 Measurement of aborted silique number

At 3 weeks of spraying post flowering the number of aborted (had not fully developed) siliques were counted.

2.3.6 Measurement of flower emergence rate

Flower number each day was counted on plants sprayed post floral induction for 3 weeks and emergence rate was calculated.

2.3.7 Statistical analysis

All data collected from DEX treatment experiments were analysed using a two-way ANOVA in SPSS Statistics 21.

2.3.8 GUS histochemical analysis

Leaves, whole rosettes and flowers from plants sprayed with +/- DEX were incubated in GUS staining buffer (50mM phosphate buffer pH 7.2, 0.5% (v/v) Triton X-100, 1mM X-Gluc diluted from a 20mM stock) at 37°C overnight. Staining buffer was removed and tissues were subsequently cleared of chlorophyll pigment in 70% (v/v) ethanol. Ethanol was changed frequently until tissues were cleared of chlorophyll. Tissues were mounted as previously described by Aida *et al*., (1997) after fixation overnight in ethanol-acetic acid (9:1 v/v) solution at room temperature before rehydration through a graded ethanol series (90, 70, 50, and 30% v/v) for 20 min each. Tissues were then cleared with a chloral hydrate: glycerol: water solution (8:2:1 w/v/v) and subsequently photographed under a Nikon SMZ 1500 microscope with a Nikon digital camera 5100 attached. Siliques cut off from plants were stood with pedicel in +/- DEX for 48 hrs. These were subsequently incubated in GUS described.

2.4 Bioinformatic analysis

Amino acid sequences for all 24 *Arabidopsis* response regulators (*ARR*s) were obtained from The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org/). These sequences were input into the Basic Local Alignment Search Tool (BLAST; blastp) on the following databases: National Center for Biotechnology (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the *Brassica napus* Genome Browser (Genoscope; http://www.genoscope.cns.fr/brassicanapus/), the *Brassica oleracea* Genomics Database (Bolbase, http://www.ocri-genomics.org/cgi-bin/bolbase/search_component.cgi), Brassica Database (http://brassicadb.org/brad/) and Ensembl (http://plants.ensembl.org/Brassica_rapa/Info/Index). An expected value (E-value) of 1e-50 was used in BLAST searches. Alignment score was also taken into consideration; sequences that aligned to greater than 200 residues across the whole of the query sequence were identified, which appeared as red bars in the BLAST output display. Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) was used to align the *Brassica* and Arabidopsis amino acid sequences in order to confirm an orthologous sequence. Clustal Omega was also used to align *Brassica* and *Arabidopsis* RR genomic sequences in order to identify presence, number and location of introns within the *Brassica* RRs of interest.

2.5 Reverse transcription PCR analysis of gene expression

2.5.1 Primer design

All primers were designed using Primer3 (v4.0.0; http://bioinfo.ut.ee/primer3-0.4.0/) using sequences obtained from the databases mentioned in section **2.2**. See **Appendix I** for list of primers.

2.5.2 Verification of primer specificity

To verify that primers amplified the correct product/ size, PCR was carried out. Genomic DNA was extracted from *Arabidopsis*, *B. napus*, *B.rapa* (pak choi) and *B.oleracea* (kale) leaves using the CTAB DNA extraction method. DNA was then quantified on NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Hemel Hempstead, UK) and diluted to 150 ng/µl. Genomic transcripts from genes of interest were amplified using PCR with the following programme in an ABI Biosystems GeneAmp PCR system 2700: 94°C for 2 min, followed by 35 cycles of 94°C for 30 s; an annealing °C dependent on primers for 30 s; 72°C for 1 min; and a final elongation step at 72°C for 7 min. Products were visualised on a 1% (w/v) agarose gel.

2.5.3 Total RNA extraction and cDNA synthesis

Plant tissues were collected in 1.5 ml Eppendorf tubes and flash frozen in liquid nitrogen. Total RNA was extracted from a maximum of 100 mg frozen tissue using RNeasy Plant Kit (Qiagen). RNA was run on a 1% (w/v) agarose gel against a 1 Kb molecular weight marker (Bioline, London UK). Contaminating genomic DNA was removed from total RNA using the DNA-free DNase Treatment kit (Invitrogen, Thermo Scientific, Hemel Hempstead, UK) and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Hemel Hempstead, UK). cDNA was synthesised from 3 µg RNA using the Tetro cDNA Synthesis kit (Bioline, London, UK) in a final volume of 20 µl according to the manufacturer's guide (**Table 2.1**).

Table 2.1. cDNA synthesis priming premix.

Total RNA 3 µg	*n* µl
Oligo $(dT)_{18}$ Primer	1 µl
10 mM dNTP mix	1 µl
5x RT Buffer	4 µl
RNase Inhibitor	1 µl
Reverse Transcriptase (200 u/ µl)	1 µl
DEPC-treated H_2O	Upto 20 µl

2.5.4 RT-PCR reaction

Transcripts from genes of interest were amplified via RT-PCR in an ABI Biosystems GeneAmp PCR system 2700: 94°C for 2 min, followed by 35 cycles of 94°C for 30 s; an annealing °C dependent on primers for 30 s; 72°C for 1 min; and a final elongation step at 72°C for 7 min. All products were visualised on a 1% (w/v) agarose gel.

2.6 Sequencing of transcripts of interest

2.6.1 PCR purification

15 µl of RT-PCR and PCR products of interest were extracted from a 1% agarose gel (w/v) and purified using the "freeze 'n squeeze" method of DNA gel extraction. Extracted gel pieces were placed into a 1.5 ml Eppendorf tube, 50 µl TE buffer (1x) was added and tubes flash frozen in liquid nitrogen. Tubes were allowed to thaw before centrifuging at full speed for 20 mins at room temperature. Supernatant was removed from the tube and the process repeated a total of three times. Supernatants were pooled before undergoing ethanol precipitation. DNA samples were measured in volume and 1/10 volume of sodium acetate (0.3 M) pH 5.2 added. Two times volume of cold 100% (v/v/) ethanol was added to samples before incubation at -20°C for 2 hrs. Samples were centrifuged at full speed for 15 mins and supernatant removed before addition of 1ml 70% (v/v) ethanol. Samples were centrifuged at full speed for 5 mins and the supernatant removed. The DNA pellet was allowed to air

dry before addition of 20 µl TE buffer (1x). Purified products were quantified using Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Hemel Hempstead, UK) and visualised on a 1% (w/v) agarose gel.

2.6.2 Cloning

Cloning was carried out using the PCR Cloning Plus kit (Qiagen, Manchester, UK) according to manufacturer's instructions (see **Table 2.2** and **Fig. 2.1**). The ligation-reaction mixture was incubated for 2 hrs at 4°C.

Table 2.2. Qiagen Cloning Plus Kit ligation-reaction mixture preparation.

Component	**Volume (µl)**
pDrive Cloning Vector (50 ng/ µl)	1
PCR Product	1 - 4
Distilled water	Variable
Ligation Master Mix	5
Total Volume	To 10

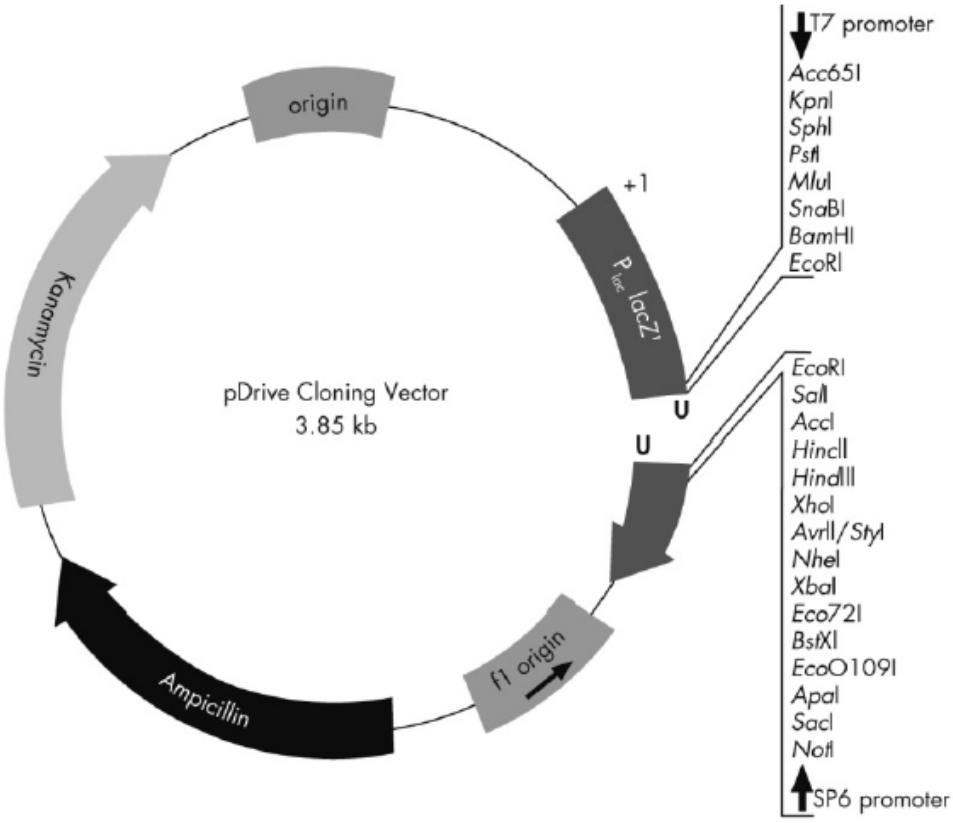


Figure 2.1. pDrive cloning vector showing U overhangs and restriction endonuclease recognition sites. Taken from the QIAGEN PCR Cloning Plus kit handbook.

2.6.3 E. coli transformation

QIAGEN EZ Competent cells (Qiagen, Manchester, UK) were transformed using the heat shock method. Tubes were heated to 42°C for 30 s then incubated on ice for 2 mins. SOC medium was added and cells plated out onto LB agar plates containing kanamycin (30 µg/ml), IPTG (50 µM) and X-gal (80 µg/ml). Plates were incubated at 37°C overnight. Plates were then placed at 4°C for 2 hrs to maximise blue/white screening. Transformed bacterial colonies that appeared white were confirmed by PCR using M13 universal primers using the following programme: 94°C for 5 mins, followed by 35 cycles of 94°C

for 30 s; 50°C for 30 s; 72°C for 30 s; and a final elongation step at 72°C for 7 min. Products were visualised on a 1% (w/v) agarose gel.

2.6.4 Plasmid isolation

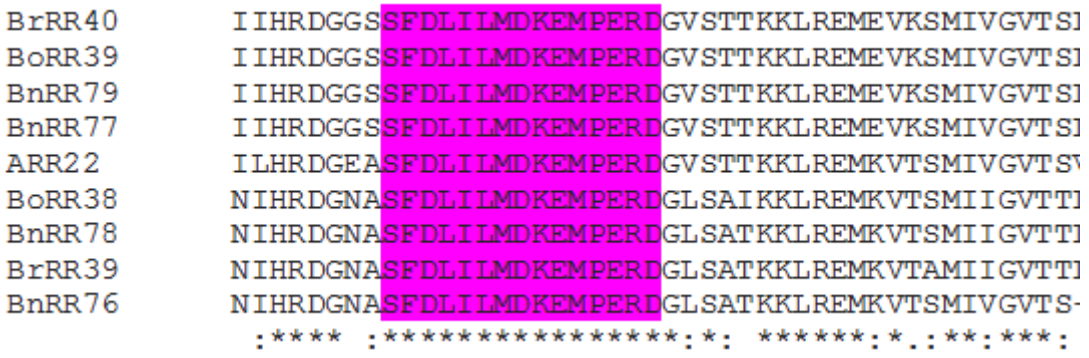
Transformed bacteria were cultured in Luria broth (LB broth) containing kanamycin (30 µg/ml) overnight at 37°C. Plasmid DNA was isolated from bacteria using the GenElute Plasmid Miniprep Kit (Sigma Aldrich, Dorset, UK) and visualised on a 1% (w/v) gel. Plasmid DNA was subsequently sequenced by Macrogen (Amsterdam, The Netherlands).

2.7 Protein expression analyses

Protein expression was analysed in DEX inducible lines 11-7, 15-5, 17-3 and 20-3 treated with +/- DEX. The protein expression of putative *B. napus ARR22* orthologues *BnRR76* – *BnRR79* was analysed in seeds throughout development and in wounded 20 and 35 DAF seed.

2.7.1 Antibody design

A fifteen amino acid sequence was identified in *ARR22* and its putative *B. napus* orthologues (**Fig. 2.2**). This sequence was sent to Agrisera (Vännäs, Sweden) for custom antibody production.



954 **Figure 2.2.** Partial amino acid alignment showing antibody sequence
955 (highlighted in pink).

956

957 ***2.7.2 Protein extraction and quantification***

958 *B. napus* and *Arabidopsis* plant tissues were flash frozen in liquid
959 nitrogen. Protein was extracted using extraction buffer composed of
960 0.5M Tris-HCL; 10% (w/v) SDS; sterile distilled water; and 7x complete
961 Mini EDTA-free protease inhibitor (Roche, Switzerland). Protein extracts
962 were quantified using the Pierce BCA Protein Assay Kit (Thermo
963 Scientific, Hemel Hempstead, UK) using the manufacturer's instructions
964 for the microplate procedure. Samples and standards were measured in
965 triplicate using a Tecan GENios plate reader and Magellan 5 software
966 using a predefined protocol at an absorbance of 540 nm. A standard
967 curve was subsequently drawn (see **Fig. 2.3** for example) and sample
968 protein content determined.

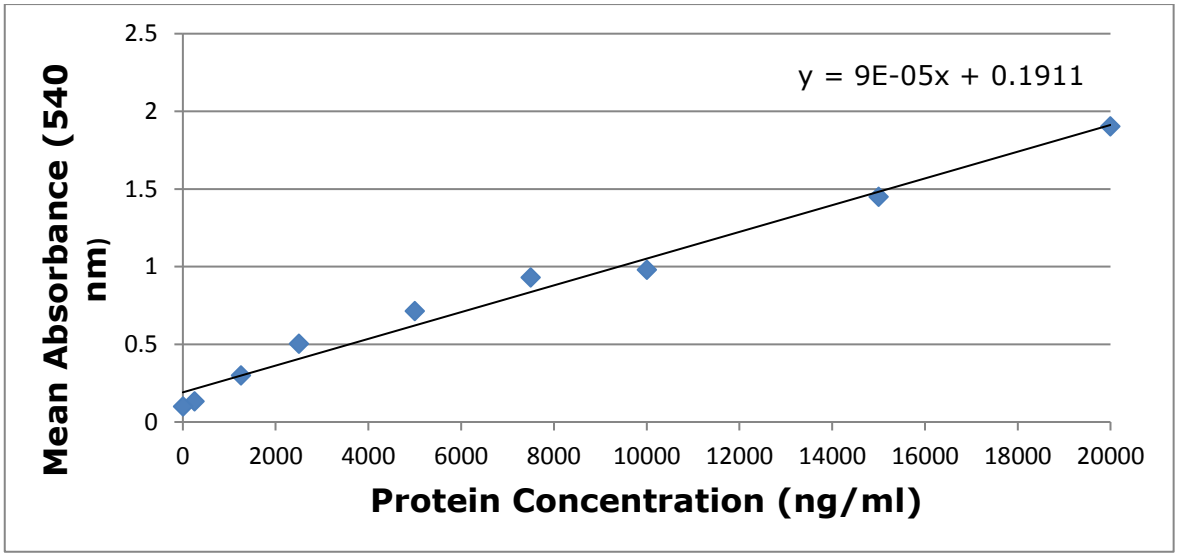


Figure 2.3. Example standard curve produced from BCA assay.

2.7.3 Dot blot detection of proteins with antibodies

To verify the antibody could detect *ARR22* and *BnRR76-BnRR79* proteins a dot blot was carried out. Samples were spotted onto nitrocellulose membrane (GE Healthcare Biosciences, Amersham UK) at a concentration of 20 µg along with 2 µl of the peptide control and allowed to dry. Membrane was blocked in 5% (w/v) non-fat dry milk in TBS (1x) in a 12 cm square petri dish for 0.5 hr on a benchtop rocker at room temperature. Membrane was then washed with TTBS (1x) for 5 mins before incubation with primary antibody (1:10000 v/v) in TTBS (1x) with 1% (w/v) nonfat dry milk for 1 hr at room temperature on a benchtop rocker. Membrane was washed with TTBS (1x; 3 x 5 mins) and incubated with anti-rabbit IgG secondary antibody (1:10000 v/v) conjugated with HRP (Sigma Aldrich, Dorset UK) for 1 hr at room temperature on a benchtop rocker. Membrane was washed (3 x 5 mins)

with TTBS and incubated with ECL reagent (GE Healthcare Biosciences, Amersham UK) for 3 mins. For chemiluminescence imaging the membrane was inserted into a clear plastic pocket and imaged *in a G:BOX (Syngene) using the GeneSys software.*

2.7.4 Western Blotting: SDS-PAGE

Laemmlli buffer (2x) was added to samples at a ratio of 1:1 (v/v) and heated to 95°C for 2 mins. Samples were loaded onto a 15% (v/v) polyacrylamide gel along with a prestained 250 kD ladder (Biorad, *Hemel Hempstead UK*) and run at 200 V for ~1 hr until dye front reached gel line.

2.7.5 Western Blotting: Immunoblotting

Following SDS-PAGE, proteins were transferred to a nitrocellulose membrane (GE Healthcare Biosciences, Amersham UK) using a semi-dry transfer unit (TE77x; Hoefer Inc, *Massachusetts USA).* Power supply was set to 0.8 mA/cm^2 of gel surface. Transfer time was set to 1 hr.

2.7.6 Western Blotting: Coomassie staining

For in-gel protein detection and confirmation of membrane transfer, gels were stained with staining solution (40% (v/v/) methanol, 10% (v/v) glacial acetic acid, 50% (v/v) dd$H_2$0 and 0.1% (w/v) Coomassie Brilliant Blue R-250) for 30 mins on a benchtop rocker. Gels were subsequently

submerged in destaining solution (40% (v/v) methanol, 10% (v/v) glacial acetic acid and 50% (v/v) ddH_2O) which was changed frequently until background was destained.

2.7.7 Western Blotting: Detection of proteins with antibodies

Membrane was blocked in 5% (w/v) non-fat dry milk in TBS (1x) in a 12 cm square petri dish for 0.5 hr on a benchtop rocker at room temperature. Membrane was then washed with TTBS (1x) for 5 mins before incubation with primary antibody (1:10000 v/v) in TTBS (1x) with 1% (w/v) nonfat dry milk o/n in cold room on a benchtop rocker. Membrane was washed with TTBS (1x; 3 x 5 mins) and incubated with anti-rabbit IgG secondary antibody (1:10000 v/v) conjugated with HRP (Sigma Aldrich, Dorset UK) for 1 hr at room temperature on a benchtop rocker. Membrane was washed (3 x 5 mins) with TTBS and incubated with ECL reagent (GE Healthcare Biosciences, Amersham UK) for 3 mins. For chemiluminescence imaging the membrane was inserted into a clear plastic pocket and imaged *in a G:BOX (Syngene) using the GeneSys software.*

Chapter 3:

Characterisation of Response Regulators in *Brassica* species

3.1 Introduction

Response regulators are downstream components of the multistep phosphorelay system in plants that are vital for the conversion of a stress or hormone signal into a transcriptional alteration of growth and development. Previous phylogenetic and structural analyses have shown that in the *Arabidopsis* genome there are 24 putative *ARR* genes based on amino acid homologies. These can be classified into three groups known as type-A, -B and –C (Imamura et al., 1999; Hwang et al., 2002). Type-A ARRs have prominent roles in negatively regulating cytokinin signalling (To et al., 2004) while type-B ARRs are characterised by the possession of a ~60 amino acid region known as the GARP domain that allows them to bind DNA and hence function as transcription factors (Imamura et al., 1999; Hosoda et al., 2002; Schaller et al., 2002; Mason et al., 2004).

Potential orthologues of RRs have been identified in a small number of major crop plants including soybean, rice and maize (Sakakibara et al., 1998; Sakakibara et al., 1999; Asakura et al.,2003; Giulini et al., 2004; Mochida et al., 2010). Little work has examined the presence and/ or precise function of RRs in *Brassica* species. Whitelaw et al. (1999), however, identified a putative *B. napus* orthologue of the type-C *ARR22* during a study of genes expressed during silique development, named *SAC29*.

B. napus possesses an allotetraploid (AACC) genome formed from the hybridisation of the *B. rapa* (AA) and *B. oleracea* (CC) genomes. Here, an *in silico* study was carried out to identify the putative orthologues of all 24 *ARR* genes within the three *Brassica* species and these are referred to as *BrRR*s, *BoRR*s and *BnRR*s. The genomic structures of the type-C *Brassica ARR22* orthologues have been analysed. The putative *B. napus* orthologues of type-A *ARR16* and *ARR17* and type-B *ARR12* and *ARR21* were also chosen to examine. These were selected on the basis of expression data that showed that these *ARR*s are also expressed in seeds although their expression is not confined to seeds.

3.2 Identification of putative Response Regulators in *B. napus*, *B. rapa* and *B. oleracea*

Several databases were used to perform a comprehensive search for response regulator coding and genomic DNA sequences and amino acid sequences in *B. napus*, *B. rapa* and *B. oleracea*. Amino acid sequences for all 24 *Arabidopsis* response regulators (*ARR*s) were obtained from The *Arabidopsis* Information Resource (TAIR; http://www.arabidopsis.org/). These sequences were inputted into the Basic Local Alignment Search Tool (BLAST; blastp) on the following databases: National Center for Biotechnology (NCBI) (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the Brassica napus Genome Browser (Genoscope; http://www.genoscope.cns.fr/brassicanapus/), the *Brassica oleracea* Genomics Database (Bolbase, http://www.ocri-

genomics.org/cgi-bin/bolbase/search_component.cgi), Brassica Database (http://brassicadb.org/brad/) and Ensembl (http://plants.ensembl.org/Brassica_rapa/Info/Index). An expect value (E-value) of 1e-50 was used in BLAST searches for a reliable alignment (Pearson 2013). Alignment score was also taken into consideration; sequences that aligned to greater than 200 residues across the whole of the query sequence were identified, which appeared as red bars in the BLAST output display. Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) was used to align the *Brassica* and *Arabidopsis* amino acid sequences in order to confirm an orthologous sequence. Clustal Omega was also used to align *Brassica* and *Arabidopsis* RR genomic sequences in order to identify presence, number and location of introns within the *Brassica* RRs of interest.

RRs identified were named as *BrRR*, *BoRR* and *BnRR* according to species: *B. rapa*, *B.olearacea* ad *B. napus* respectively. Eighty-three *BnRR*s were identified originating from forty-two *BrRR*s and forty-one *BoRR*s as displayed in **Table 3.1**. For each *ARR* gene between one and three orthologues were found in *B. rapa* and *B. oleracea*. Two orthologues of *ARR16* and *ARR21* and four orthologues of *ARR17* and *ARR12* were identified in *B. napus*. Two orthologues of *ARR22* were identified in both *B. rapa* and *B. oleracea* and subsequently four orthologues were distinguished in *B. napus* (*BnRR80* - *BnRR83*).

3.3 Phylogenetic analysis

Amino acid alignments from Clustal Omega were sent to ClustalW2 Phylogeny (http://www.ebi.ac.uk/Tools/phylogeny/) to create a phylogenetic tree using default parameters. The TreeDyn tool was used to view the tree as a cladogram (Chevenet et al., 2006; Dereeper et al., 2008; Dereeper et al., 2010; http://www.phylogeny.fr/one_task.cgi?task_type=treedyn).

This was generated in order to analyse whether the *Brassica* RRs clustered into the groups type-A, type-B and type-C as seen in *Arabidopsis* or whether a different clustering existed.

Indeed the *Brassica* RRs follow the same phylogenetic pattern as *ARR*s and no additional groups were formed during the divergence from *Arabidopsis* (**Fig. 3.1**). The three type-B subfamilies can also be visibly seen. Interestingly *ARR13* and *ARR21* have evolved together in an almost duplicated manner before the *Arabidopsis* – *Brassica* lineage split.

Table 3.1. Putative *Arabidopsis* response regulator orthologues in *B. napus, B. rapa* and *B. oleracea*. Type-A: Blue; type-B: Pink; type-C: Violet. Genes of interest marked with an asterisks (*). Note (**): BnaA03gXXXXXD is not present in genome databases; this gene was identified through sequencing (Sequence information up-to-date as of December 2015).

B. napus			***B. rapa***		***B. oleracea***		***A.thaliana***
Chromosome locus	**Allocated Gene name**	**Chromosome**	**Chromosome locus**	**Allocated Gene name**	**Chromosome locus**	**Allocated Gene name**	
BnaA09g14370D	*BnRR1*	A09	Bra027829	*BrRR1*			***ARR3*-like**
BnaC09g14930D	*BnRR2*	C09			Bo9g045370	*BoRR1*	
BnaA06g06240D	*BnRR3*	A06	Bra019932	*BrRR2*			***ARR4*-like**
BnaA08g25770D	*BnRR4*	A08	Bra018439	*BrRR3*			
BnaA09g48160D	*BnRR5*	A09	Bra031714	*BrRR4*			
BnaC05g07990D	*BnRR6*	C05			Bo5g010910	*BoRR2*	
BnaC08g14280D	*BnRR7*	C08			Bol022049	*BoRR3*	
BnaA06g16900D	*BnRR8*	A06	Bra033773	*BrRR5*			***ARR5*-like**
BnaA06g20760D	*BnRR9*	A06	Bra019524	*BrRR6*			
BnaAnng26230D	*BnRR10*	A06	Bra018084	*BrRR7*			
BnaC01g42890D	*BnRR11*	C01			Bo1g073610	*BoRR4*	
BnaCnng35610D	*BnRR12*	C03			Bo3g113730	*BoRR5*	
BnaA06g22370D	*BnRR13*	A06	Bra010132	*BrRR8*			***ARR6*-like**
BnaC03g51340D	*BnRR14*	C03			Bol019418	*BoRR6*	
BnaA06g13210D	*BnRR15*	A06	Bra025708	*BrRR9*			***ARR7*-like**
BnaA08g22240D	*BnRR16*	A08	Bra016526	*BrRR10*			
BnaC05g14720D	*BnRR17*	C05			Bol026821	*BoRR7*	
BnaC08g18570D	*BnRR18*	C08			Bo8g068270	*BoRR8*	

BnaA03g19410D	*BnRR19*	A03	Bra000224	*BrRR11*			***ARR8*-like**
BnaA04g23810D	*BnRR20*	A04	Bra016943	*BrRR12*			
BnaA05g02140D	*BnRR21*	A05	Bra004615	*BrRR13*			
BnaC03g23280D	*BnRR22*	C03			Bol020559	*BoRR9*	
BnaC04g01810D	*BnRR23*	C04			Bo4g013160	*BoRR10*	
BnaC04g47580D	*BnRR24*	C04			Bo4g190810	*BoRR11*	
BnaA04g03000D	*BnRR25*	A04	Bra014649	*BrRR14*			***ARR9*-like**
BnaA07g17140D	*BnRR26*	A07	Bra003265	*BrRR15*			
BnaA09g36380D	*BnRR27*	A09	Bra007295	*BrRR16*			
BnaC04g24580D	*BnRR28*	C04			Bol011084	*BoRR12*	
BnaC08g27970D	*BnRR29*	C08			Bol045476	*BoRR13*	
BnaA07g22010D	*BnRR30*	A07	Bra015885	*BrRR17*			***ARR15*-like**
BnaA07g31820D	*BnRR31*	A07	Bra003782	*BrRR18*			
BnaC06g22740D	*BnRR32*	C06			Bol039928	*BoRR14*	
BnaC06g35700D	*BnRR33*	C06			Bol026142	*BoRR15*	
BnaA03g19150D	*BnRR34**	A03	Bra000199	*BrRR19*			***ARR16*-like**
BnaC03g22790D	*BnRR35**	C03			Bol020600	*BoRR16*	
BnaA04g02540D	*BnRR36**	A04	Bra014695	*BrRR20*			***ARR17*-like**
BnaA09g35830D	*BnRR37**	A09	Bra007242	*BrRR21*			
BnaC04g55620D	*BnRR38**	C04			Bol044273	*BoRR17*	
BnaC08g27330D	*BnRR39**	C08			Bo8g090810	*BoRR18*	
BnaA03g34300D	*BnRR40*	A03	Bra001641	*BrRR22*			***ARR1*-like**
BnaA05g23050D	*BnRR41*	A05	Bra022183	*BrRR23*			
BnaC01g44050D	*BnRR42*	C01			Bol034811	*BoRR19*	
BnaC05g36490D	*BnRR43*	C05			Bo5g123620	*BoRR20*	
BnaA01g17750D	*BnRR44*	A01	Bra033527	*BrRR24*			***ARR2*-like**
BnaA03g34320D	*BnRR45*	A03	Bra001643	*BrRR25*			
BnaA03g42350D	*BnRR46*	A03	Bra012743	*BrRR26*			

BnaC01g22100D	*BnRR47*	C01			Bol020274	*BoRR21*	
BnaC03g39750D	*BnRR48*	C03			Bo3g068550	*BoRR22*	
BnaC07g33430D	*BnRR49*	C07			Bo7g104190	*BoRR23*	
BnaA03g51830D	*BnRR50*	A03	Bra023972	*BrRR27*			***ARR10*-like**
BnaC01g06500D	*BnRR51*	C01			Bo1g010830	*BoRR24*	
BnaC07g43590D	*BnRR52*	C07			Bol033755	*BoRR25*	
BnaA07g24890D	*BnRR53*	A07	Bra004076	*BrRR28*			***ARR11*-like**
BnaA07g26610D	*BnRR54*	A07	Bra004245	*BrRR29*			
BnaC06g26570D	*BnRR55*	C06			Bol027853	*BoRR26*	
BnaC06g28780D	*BnRR56*	C06			Bol026109	*BoRR27*	
BnaA04g14760D	*BnRR57**	A04	Bra032035	*BrRR30*			***ARR12*-like**
BnaC04g56320D	*BnRR58**	C04			Bol014767	*BoRR28*	
BnaA03g01960D	*BnRR59*	A03	Bra005928	*BrRR31*			***ARR13*-like**
BnaC03g02950D	*BnRR60*	C03			Bol008869	*BoRR29*	
BnaA02g25910D	*BnRR61*	A02	Bra026635	*BrRR32*			***ARR14*-like**
BnaC02g47700D	*BnRR62*	C02			Bol014787	*BoRR30*	
BnaA02g07870D	*BnRR63*	A02	Bra020390	*BrRR33*			***ARR18*-like**
BnaC02g10960D	*BnRR64*	C02			Bol015562	*BoRR31*	
BnaA05g16250D	*BnRR65*	A08	Bra032275	*BrRR34*			***ARR19*-like**
BnaA08g02850D	*BnRR66*	A08	Bra014172	*BrRR35*			
BnaC05g25970D	*BnRR67*	C05			Bo00904s040	*BoRR32*	
BnaC08g03080D	*BnRR68*	C08			Bol005734	*BoRR33*	
BnaA09g40030D	*BnRR69*	A09	Bra041027	*BrRR36*			***ARR20*-like**
BnaC08g32380D	*BnRR70*	C08			Bol044607	*BoRR34*	
BnaAnng25110D	*BnRR71**	A02	Bra028705	*BrRR37*			***ARR21*-like**
BnaA10g23650D	*BnRR72**	A10	Bra009284	*BrRR38*			
BnaC02g01700D	*BnRR73**	C02			Bol024533	*BoRR35*	

BnaC09g48380D	*BnRR74**	C09			Bol043863	*BoRR36*	
BnaC03g51950D	*BnRR75*	C03			Bol024821	*BoRR37*	***ARR23*-like**
BnaA03gXXXXXD**	*BnRR76**	A03	Bra001099	*BrRR39*			***ARR22*-like**
BnaA05g33120D	*BnRR77**	A05	Bra040204	*BrRR40*			
BnaC03g33640D	*BnRR78**	C03			Bol034163	*BoRR38*	
BnaC05g47370D	*BnRR79**	C05			Bol001327	*BoRR39*	
BnaA02g31620D	*BnRR80*	A02	Bra020537	*BrRR41*			***ARR24*-like**
BnaA09g04220D	*BnRR81*	A09	Bra036579	*BrRR42*			
BnaC07g28850D	*BnRR82*	C07			Bo7g095290	*BoRR40*	
BnaC09g03650D	*BnRR83*	C09			Bol032459	*BoRR41*	

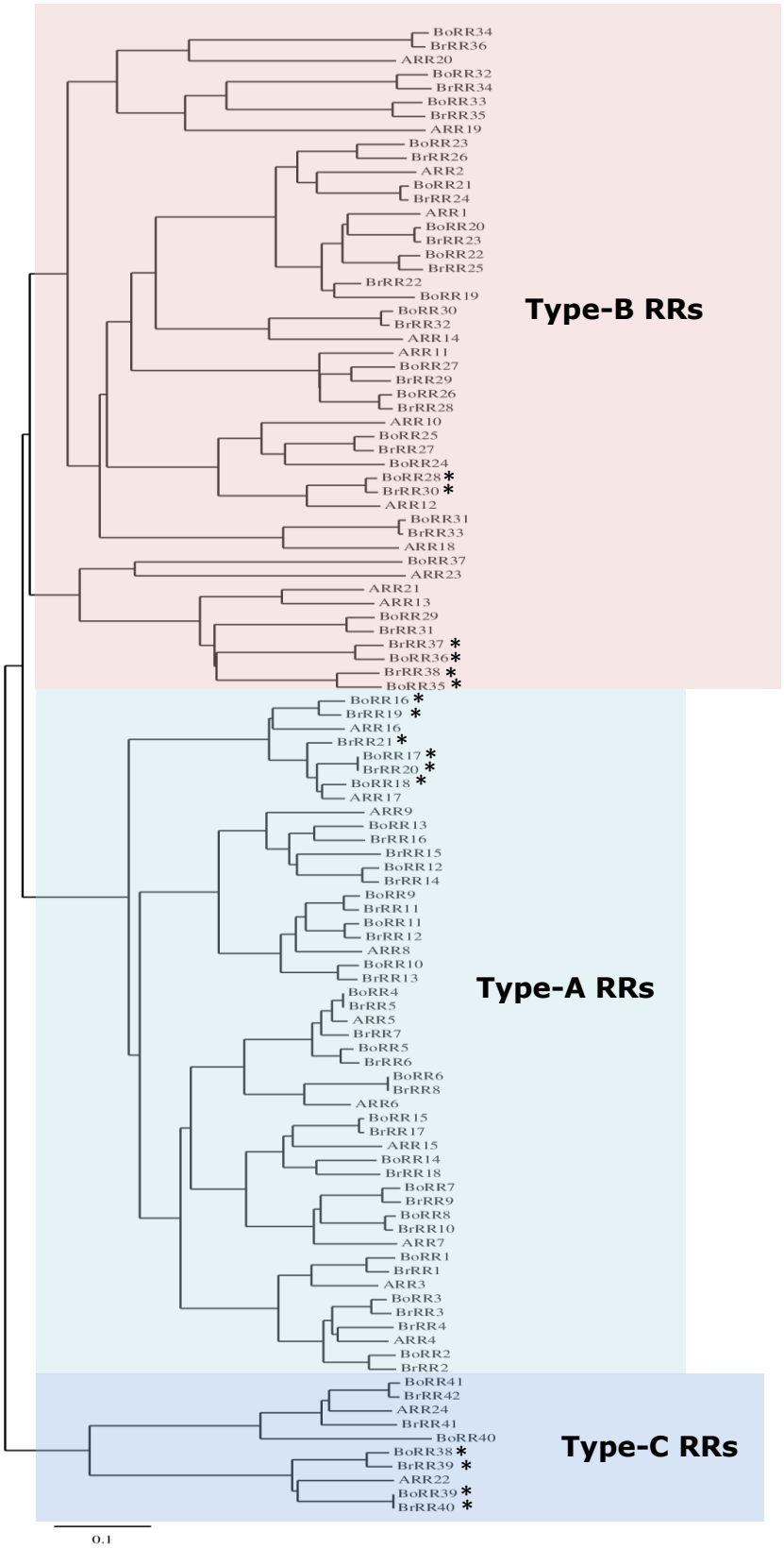


Figure 3.1. Phylogenetic relationship of RR amino acid sequences in *Arabidopsis*, *B. rapa* and *B. oleracea*. Length of bar represents divergence of sequences. Genes chosen for structural and gene expression analysis marked with an asterisks (*).

3.4 Analysis of type-A and type-B *BnRR*s

The gene structure and expression patterns of two type-A (*ARR16* and *ARR17*) and two type-B (*ARR12* and *ARR21*) putative *ARR* orthologues were additionally studied in *B. napus*. These were selected on the basis of expression data gathered from the *Arabidopsis* ePlant Browser tool on the Bio-Analytic Resource for Plant Biology (http://bar.utoronto.ca/; Schmid et al., 2005; Winter et al., 2007). As the contents of *B. napus* seeds determine the ultimate value of the crop, elucidating expression patterns of genes expressed within seeds is of particular interest.

*ARR*s were searched and expression specifically within seeds visualised (**Fig. 3.2**). Genes that exhibited expression in seeds were hence chosen and their putative *B. napus* orthologues identified for gene structure, amino acid and expression analyses.

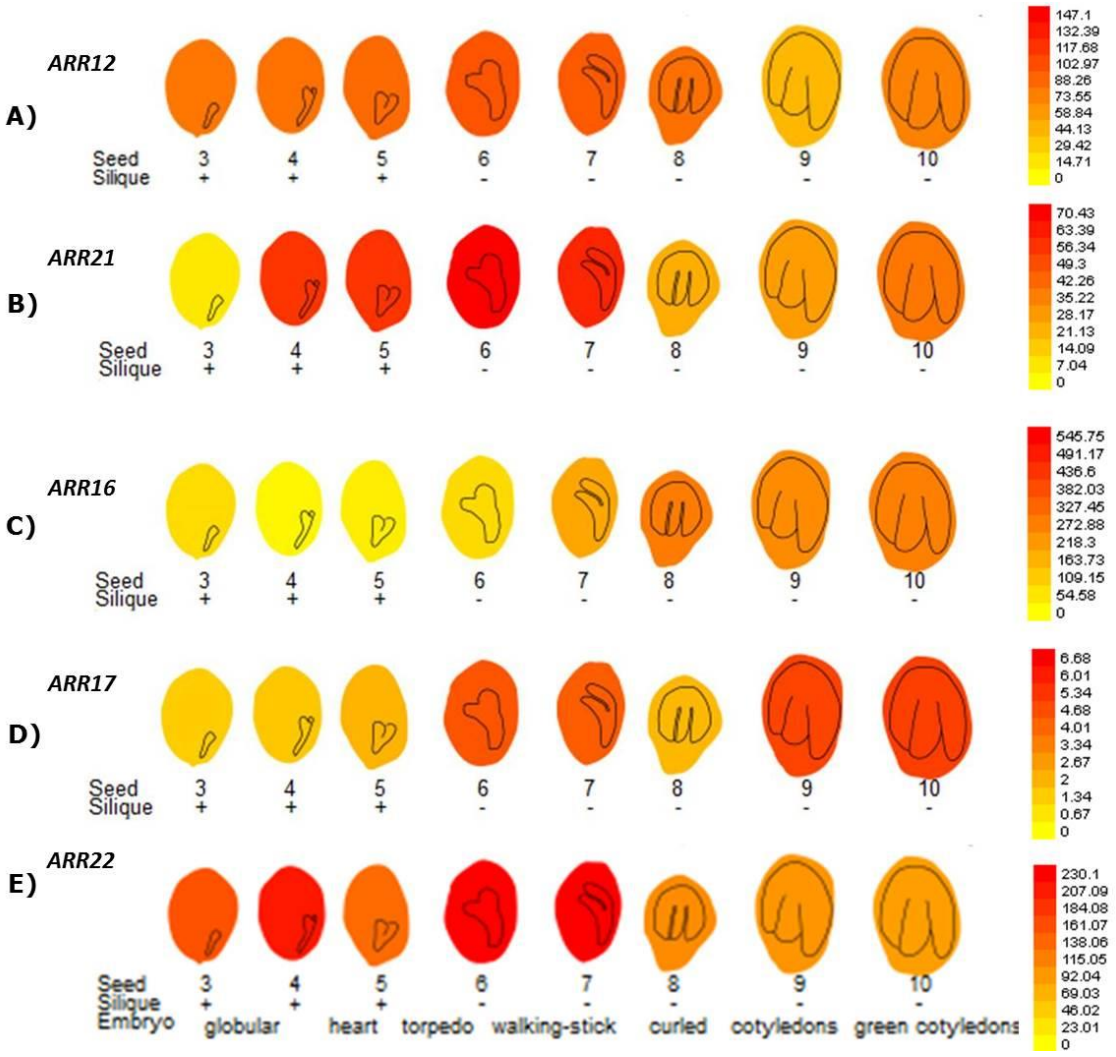


Figure 3.2. Relative gene expression values for **A**) *ARR12* **B**) *ARR21* **C**) *ARR16* **D**) *ARR21* and **E**) *ARR22* in seeds. Information taken from the *Arabidopsis* ePlant Browser tool on the Bio-Analytic Resource for Plant Biology (http://bar.utoronto.ca/; Schmid et al., 2005; Winter et al., 2007). High levels of genes expression in red; low levels in yellow.

3.4.1 Amino acid analysis of type-B BnRRs

A prominent feature of type-B RRs is the possession of the Myb-like DNA binding domain known as the GARP motif, permitting them to function as transcription factors (Imamura et al., 1999; Hosoda et al., 2002; Schaller et al., 2002; Mason et al., 2004). An amino acid alignment of *ARR12* and *ARR21* and their putative *B. napus* orthologues

was performed in order to identify the possession of this domain within the *BnRR*s (**Fig. 3.3**).

The ~60 amino acid region that forms the GARP domain was present in all *B. napus* orthologues of *ARR12* and *ARR21*. It is hence possible to predict that they too may function as transcription factors.

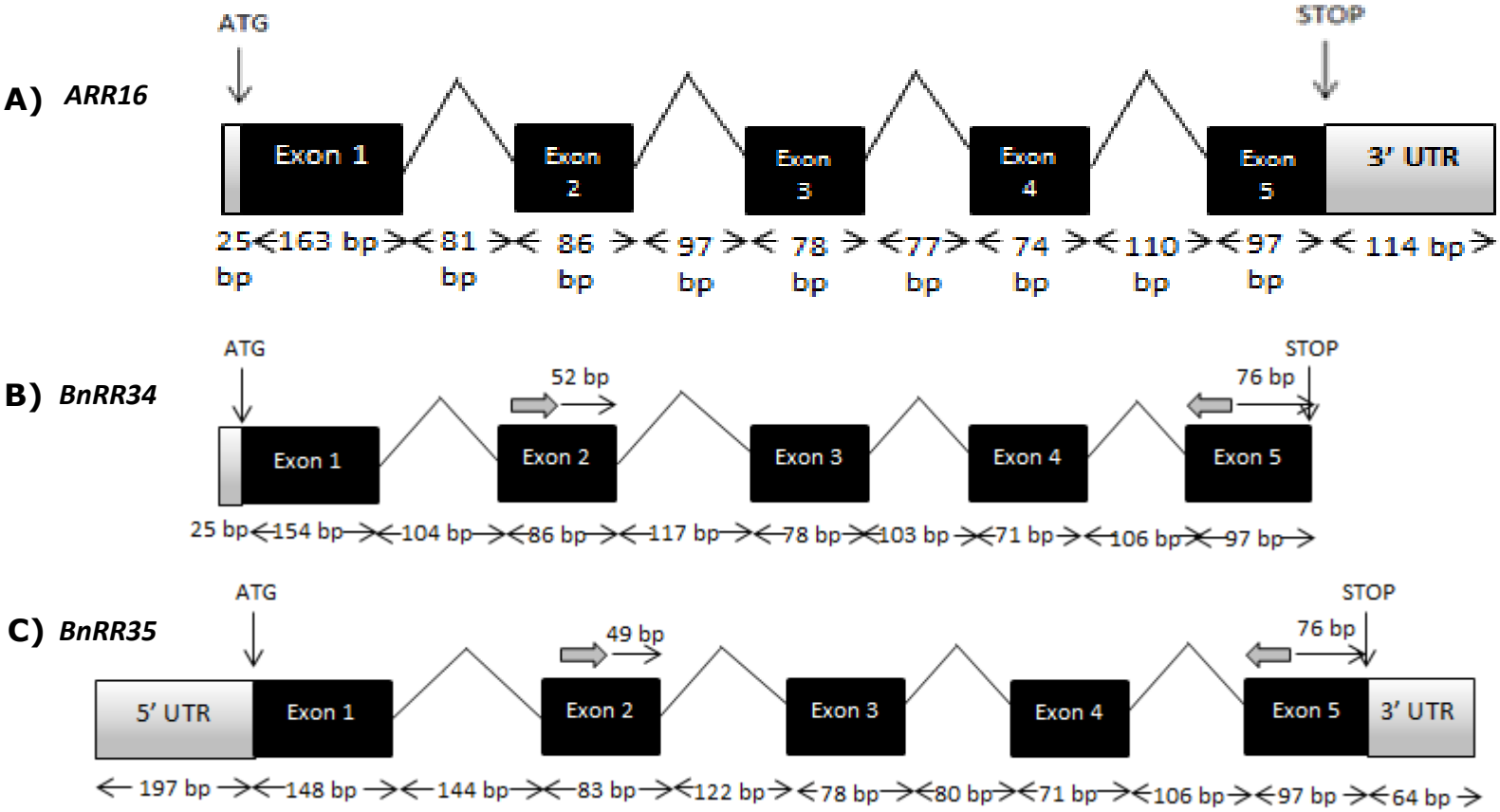
Figure 3.3. Amino acid alignment of GARP domain in (**A**) *ARR12* and its putative *B. napus* orthologues *BnRR57* and *BnRR58* and (**B**) *ARR21* and its *B. napus* orthologues BnRR71 – BnRR74. Conserved amino acids found in Myb plant proteins highlighted in red (as identified by Hosoda et al., 2002).

3.4.2 Structural analysis of BnRRs

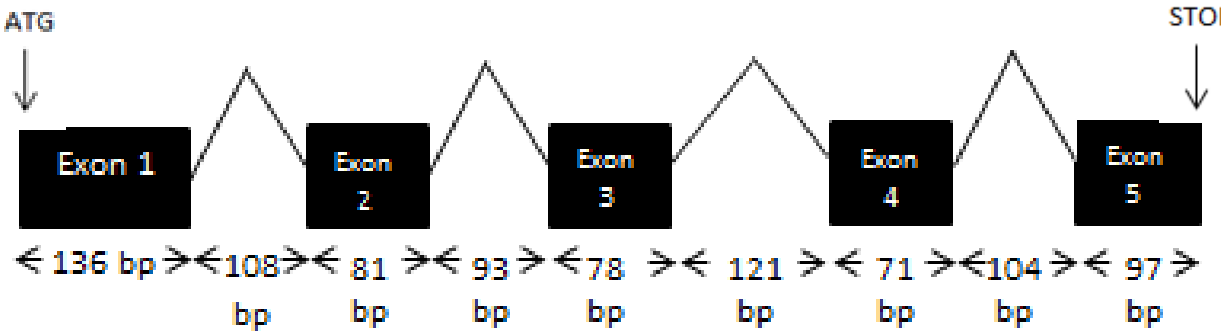
Structures of chosen *BnRR*s are displayed in **Fig. 3.4**. The type-A *ARR16* orthologues *BnRR34* and *BnRR35* and *ARR17* orthologues *BnRR36* - *BnRR39* all possess four introns and five exons. The exons within *BnRR34* and *BnRR35* are similar in size to *ARR16* whereas three out of the four introns are larger than their *Arabidopsis* orthologue.

The structure of *BnRR37* differs from the other *ARR17 BnRR* orthologues in that it possesses a larger first exon of 175 bp compared with 49 bp and smaller first intron of 56 bp contrasted to 129 bp. *ARR17* also contains a larger first exon of 136 bp but a 108 bp first intron. All other exons and introns within *BnRR36* – *BnRR39* are similar to *ARR17* in terms of size and structure. *BnRR57* and *BnRR58*, orthologues of *ARR12* are composed of six exons and five introns. These *BnRR*s are similar in structure to their *ARR* orthologue however their first and third introns are considerably larger than in *ARR12*.

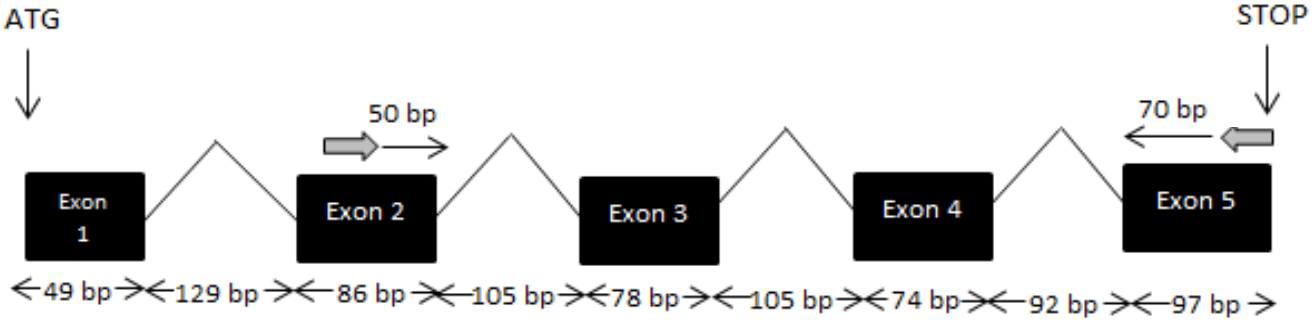
BnRR71 - *BnRR74*, are somewhat different to their *ARR21* orthologue in that they possess two additional introns and exons. *ARR21* contains a large fifth intron at 729 bp which is not present in any of the *B. napus* orthologues. Variability in structure between each of the *B. napus ARR21* orthologues also exists with differences in both exon and intron size. For example the first intron in *BnRR73* is approximately 200 bp smaller than the intron within *BnRR71*, *BnRR72* and *BnRR74*.


A) ARR16
ATG
STOP
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
3' UTR
25 bp
163 bp
81 bp
86 bp
97 bp
78 bp
77 bp
74 bp
110 bp
97 bp
114 bp
B) BnRR34
ATG
52 bp
76 bp
STOP
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
25 bp
154 bp
104 bp
86 bp
117 bp
78 bp
103 bp
71 bp
106 bp
97 bp
C) BnRR35
ATG
49 bp
76 bp
STOP
5' UTR
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
3' UTR
197 bp
148 bp
144 bp
83 bp
122 bp
78 bp
80 bp
71 bp
106 bp
97 bp
64 bp

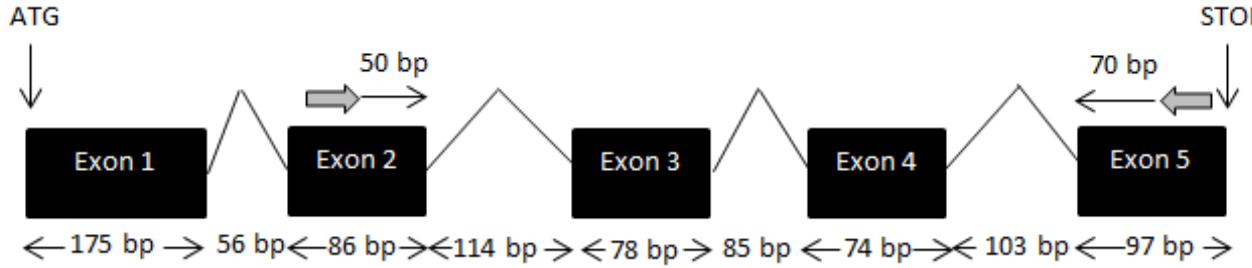
D) ***ARR17***



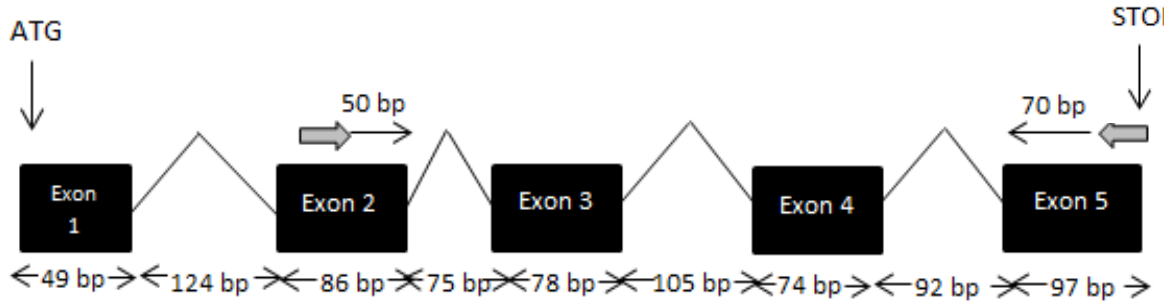
E) ***BnRR36***



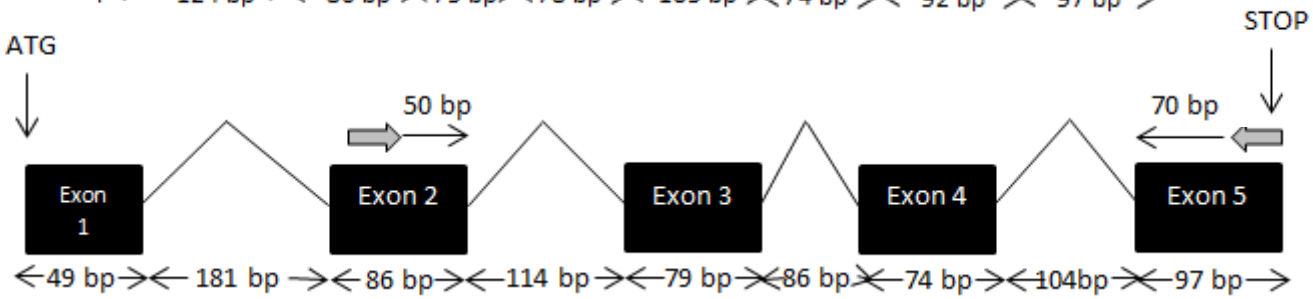
F) ***BnRR37***

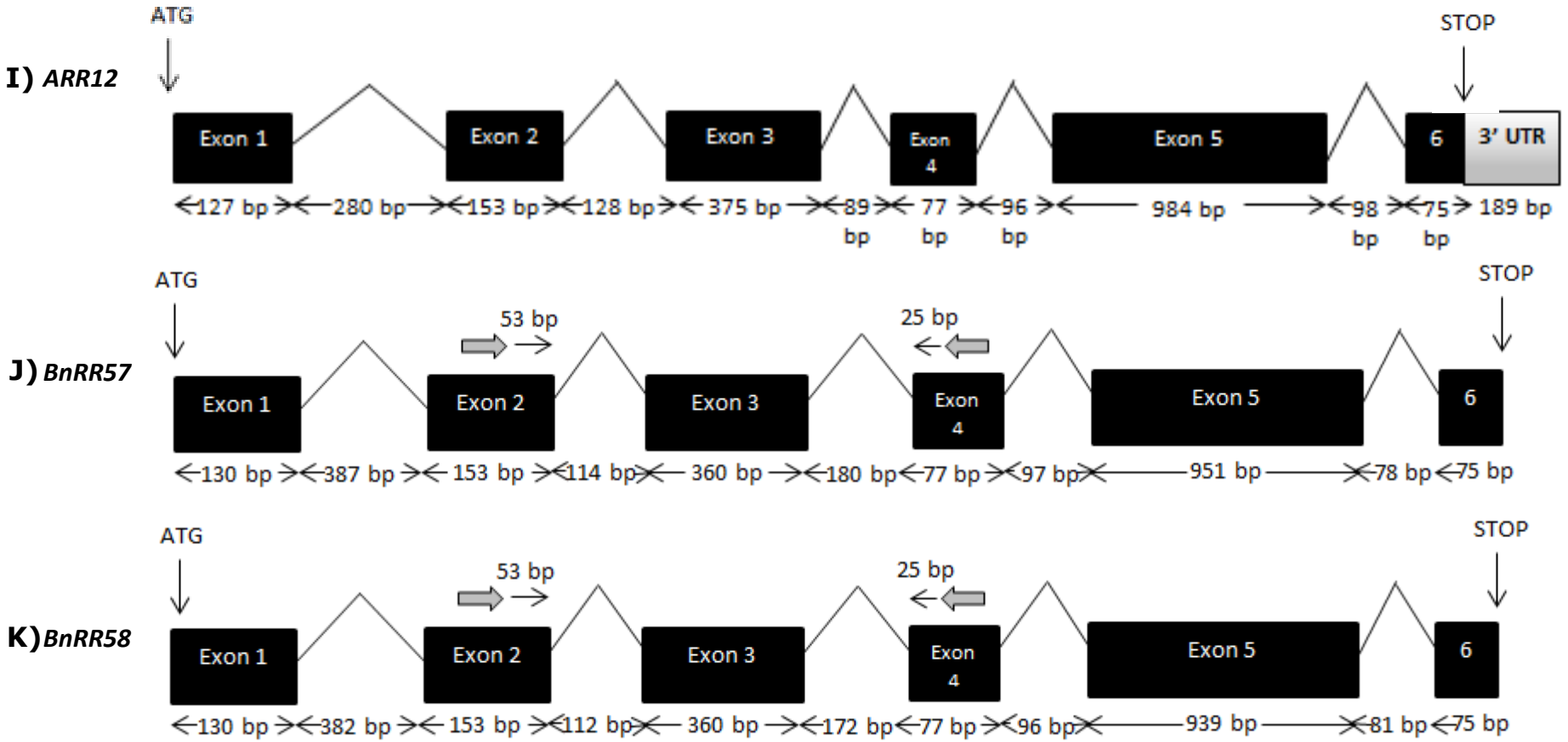


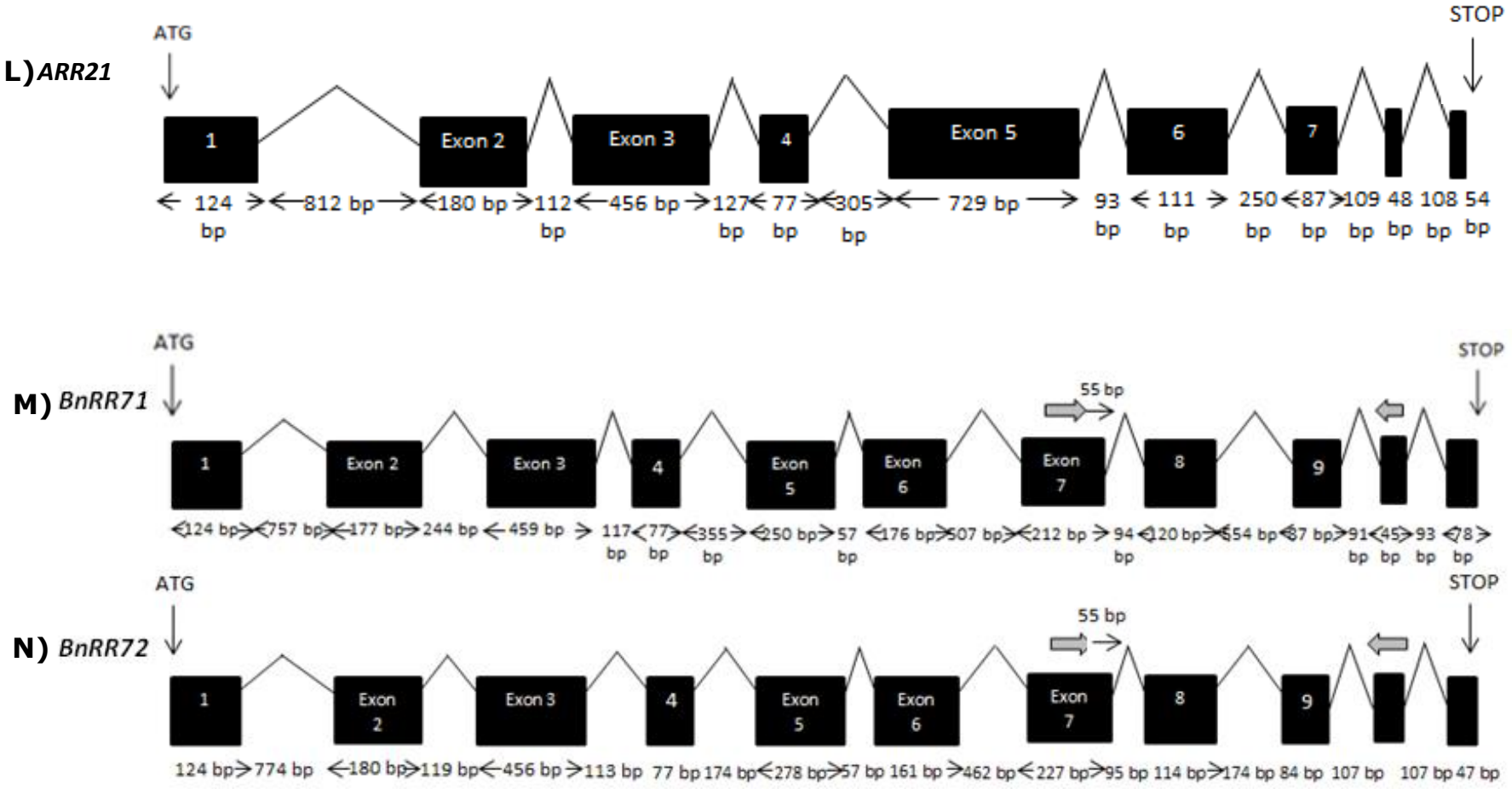
G) ***BnRR38***



H) ***BnRR39***




I) ARR12
ATG
STOP
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
6
3' UTR
127 bp
280 bp
153 bp
128 bp
375 bp
89 bp
77 bp
96 bp
984 bp
98 bp
75 bp
189 bp
J) BnRR57
ATG
STOP
53 bp
25 bp
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
6
130 bp
387 bp
153 bp
114 bp
360 bp
180 bp
77 bp
97 bp
951 bp
78 bp
75 bp
K) BnRR58
ATG
STOP
53 bp
25 bp
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
6
130 bp
382 bp
153 bp
112 bp
360 bp
172 bp
77 bp
96 bp
939 bp
81 bp
75 bp


L) ARR21
ATG
STOP
1
Exon 2
Exon 3
4
Exon 5
6
7
124 bp
812 bp
180 bp
112 bp
456 bp
127 bp
77 bp
305 bp
729 bp
93 bp
111 bp
250 bp
87 bp
109 bp
48 bp
108 bp
54 bp
M) BnRR71
ATG
STOP
55 bp
1
Exon 2
Exon 3
4
Exon 5
Exon 6
Exon 7
8
9
124 bp
757 bp
177 bp
244 bp
459 bp
117 bp
77 bp
355 bp
250 bp
57 bp
176 bp
507 bp
212 bp
94 bp
120 bp
54 bp
87 bp
91 bp
45 bp
93 bp
78
N) BnRR72
ATG
STOP
55 bp
1
Exon 2
Exon 3
4
Exon 5
Exon 6
Exon 7
8
9
124 bp
774 bp
180 bp
119 bp
456 bp
113 bp
77 bp
174 bp
278 bp
57 bp
161 bp
462 bp
227 bp
95 bp
114 bp
174 bp
84 bp
107 bp
107 bp
47 bp

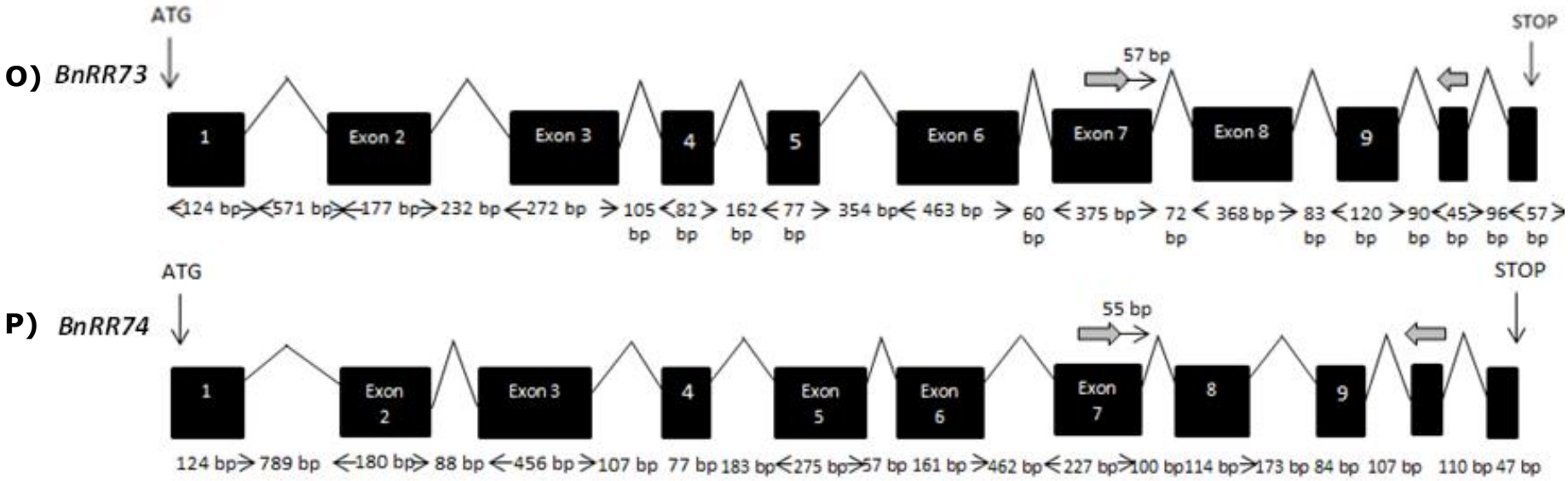


Figure 3.4. Predicted genomic structures of (**A**) *ARR16* (**B**) *BnRR34* (**C**) *BnRR35* (**D**) *ARR17* (**E**) *BnRR36* (**F**) *BnRR37* (**G**) *BnRR38* (**H**) *BnRR39* (**I**) *ARR12* (**J**) *BnRR57* (**K**) *BnRR58* (**L**) *ARR21* (**M**) *BnRR71* (**N**) *BnRR72* (**O**) *BnRR73* (**P**) *BnRR74.* Grey arrows above Brassica genes represent primer locations used for RT-PCR gene expression analysis presented in **Fig. 4.2, Chapter 4**. Untranslated regions (UTR) presented where information was available.

3.5 *ARR22* orthologues in *Brassicas*

The gene of primary interest in this study is the *Arabidopsis* type-C *ARR22* and its putative orthologues in *Brassica* species. An assessment of their genomic structures, synteny with *Arabidopsis* and amino acid sequences was hence performed.

3.5.1 Identification of Brassica ARR22 orthologues

Two putative orthologues of *ARR22* were identified both in *B. rapa* and *B. oleracea* (information obtained from the *Brassica* database, EnsemblPlants and Bolbase; http://brassicadb.org/brad/; http://plants.ensembl.org/; http://www.ocri-genomics.org/; January 2013). *BrRR39* (Bra001099) and *BoRR38* (Bol034163) are both located on chromosome 3 in *B. rapa* and *B. oleracea* respectively and *BrRR40* (Bra040204) and *BoRR39* (Bol001327) are both positioned on chromosome 5.

Knowing that two orthologues of *ARR22* existed in both *B. rapa* and *B. oleracea*, it was predicted that four *B. napus* orthologues would be distinguished within the databases. However only three were identified (*Brassica napus* Genome Browser http://www.genoscope.cns.fr/brassicanapus/; December 2015). *BnRR77* (BnaA05g33120D; **Fig. 3.3 E**) is located on chromosome A05, thought to originate from *BrRR40* (Bra040204) in *B. rapa*. *BnRR78*

(BnaC03g33640D) and *BnRR79* (BnaC05g47370D) are located on chromosome C03 and C05 and are believed to originate from *BoRR38* (Bol034163) and *BoRR39* (Bol001327) respectively. It was thus expected that an orthologue of *BrRR39* (Bra001099) existed in *B. napus* on chromosome A05. Gene expression analysis (see **Fig. 4.5** and **4.6, Chapter 4**) in fact revealed the presence of an additional transcript. Subsequent cloning sequencing of this transcript confirmed it to be the *B. napus* orthologue of *BrRR39* (Bra001099) absent from the databases.

3.5.2 Syntenic comparisons

Genes adjacent to *ARR22* and the putative *B. napus* orthologues were identified to analyse gene order and further deduce gene function. *ARR22* is situated on chromosome 3 in *Arabidopsis*. Genes situated within a 100 Kb region around *ARR22* were distinguished and compared with 100 Kb regions around *BnRR77*, *BnRR78* and *BnRR79* located on *B. napus* chromosomes A05, C03, C05 respectively (**Fig. 3.5 B, C, D**). As database information was lacking for the fourth predicted *B. napus* orthologue, the region around *BrRR39* located on chromosome 3 in *B. rapa* was analysed (**Fig. 3.5 A**).

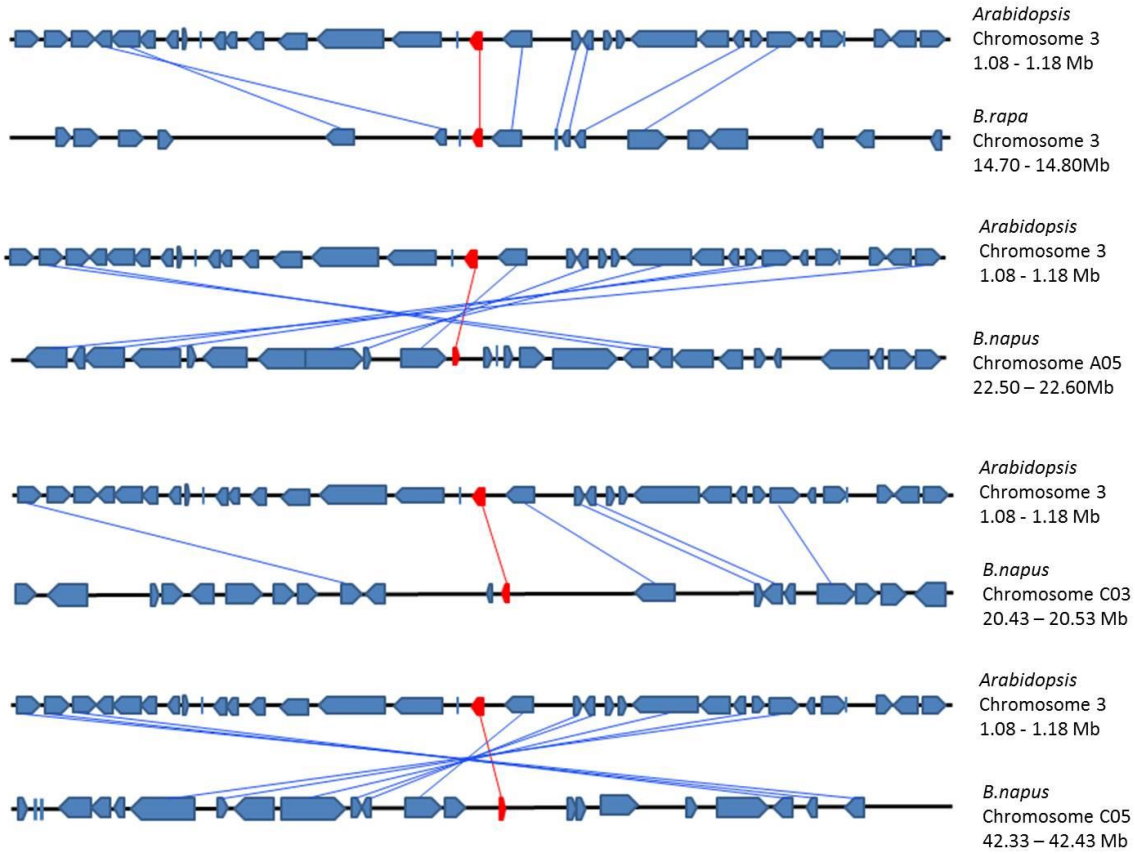


Figure 3.5. Syntenic comparison of chromosome 3 in *Arabidopsis* with (**A**) *B. rapa* chromosome 3; (**B**) *B. napus* chromosome A05; (**C**) *B. napus* chromosome C03; and (**D**) *B. napus* chromosome C05. Genes in red indicate *ARR22* and its putative *Brassica* orthologues. Blue lines indicate syntenic genes. Figure displayed within a 100 kb region.

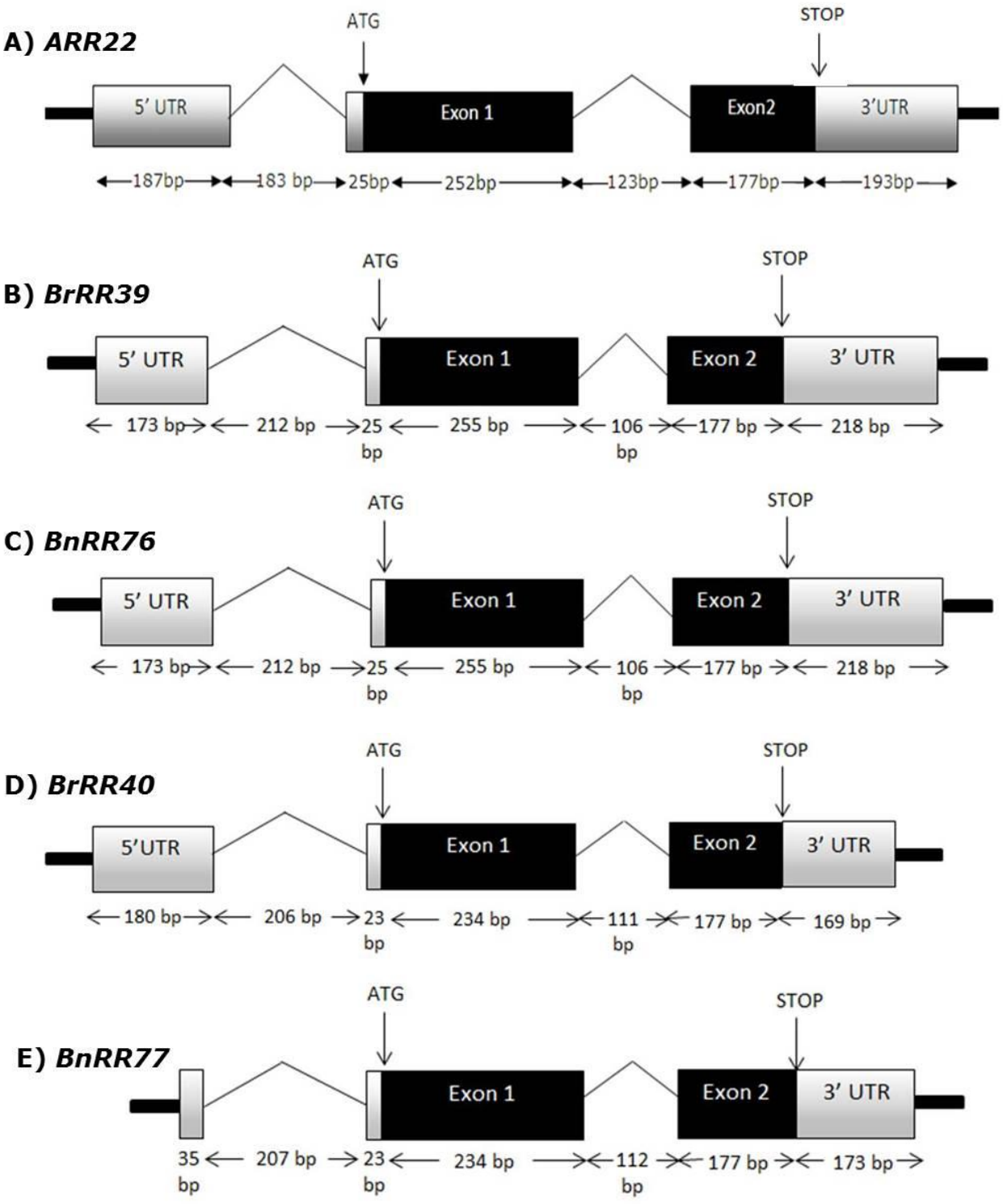
On *B. rapa* chromosome 3 seven syntenic regions were distinguished. On chromosome C03 in *B. napus* only five regions were identified. However on chromosomes A05 and C05 eight and nine syntenic regions were observed respectively. Moreover the orientation of chromosomes A05 and C05 in *B. napus* was inverted in comparison to *Arabidopsis* chromosome 3 and *B. rapa* chromosome 3. Three genes upstream of *ARR22* (At3g04290, At3g04300 and At3g04310) were conserved and within the same order on *B. rapa* chromosome 3 and *B. napus*

chromosomes C03 and C05. These genes encode a Li-tolerant lipase and two proteins of unknown function. At3g04370 was also a gene identified to be conserved on all *Brassica* chromsomes studied although not in the same position. This gene encodes a plasmodesmatal protein.

3.5.3 Genomic structure characterisation

Previous work has shown that *ARR22* contains two introns; one (183 bp) situated within the 5' UTR 25 bp up from the ATG start codon and one (123 bp) within the ORF (Gattolin et al., 2006). In line with the objective of the study to characterise putative *Brassica* orthologues of *ARR22*, the predicted gene structures of these were analysed.

As presented in **Fig. 3.6**, putative *Brassica* orthologues of *ARR22* also contain two introns; one located within the 5' UTR (23 bp – 26 bp up from the ATG start codon) and one within the open reading frame. The sizes of the 5' UTR introns within the *Brassica* orthologues are larger than that of *ARR22* (201 bp – 212 bp compared with 183 bp). On the other hand, introns located within the open reading frame of the *Brassica* orthologues are smaller than *ARR22* (106 – 112 bp compared with 123 bp) with the exception of *BoRR39* which is predicted to possess a larger open reading frame intron (131 bp).


A) ARR22
ATG
STOP
5' UTR
Exon 1
Exon2
3'UTR
187bp
183 bp
25bp
252bp
123bp
177bp
193bp
B) BrRR39
ATG
STOP
5' UTR
Exon 1
Exon 2
3' UTR
173 bp
212 bp
25 bp
255 bp
106 bp
177 bp
218 bp
C) BnRR76
ATG
STOP
5' UTR
Exon 1
Exon 2
3' UTR
173 bp
212 bp
25 bp
255 bp
106 bp
177 bp
218 bp
D) BrRR40
ATG
STOP
5'UTR
Exon 1
Exon 2
3' UTR
180 bp
206 bp
23 bp
234 bp
111 bp
177 bp
169 bp
E) BnRR77
ATG
STOP
Exon 1
Exon 2
3' UTR
35 bp
207 bp
23 bp
234 bp
112 bp
177 bp
173 bp

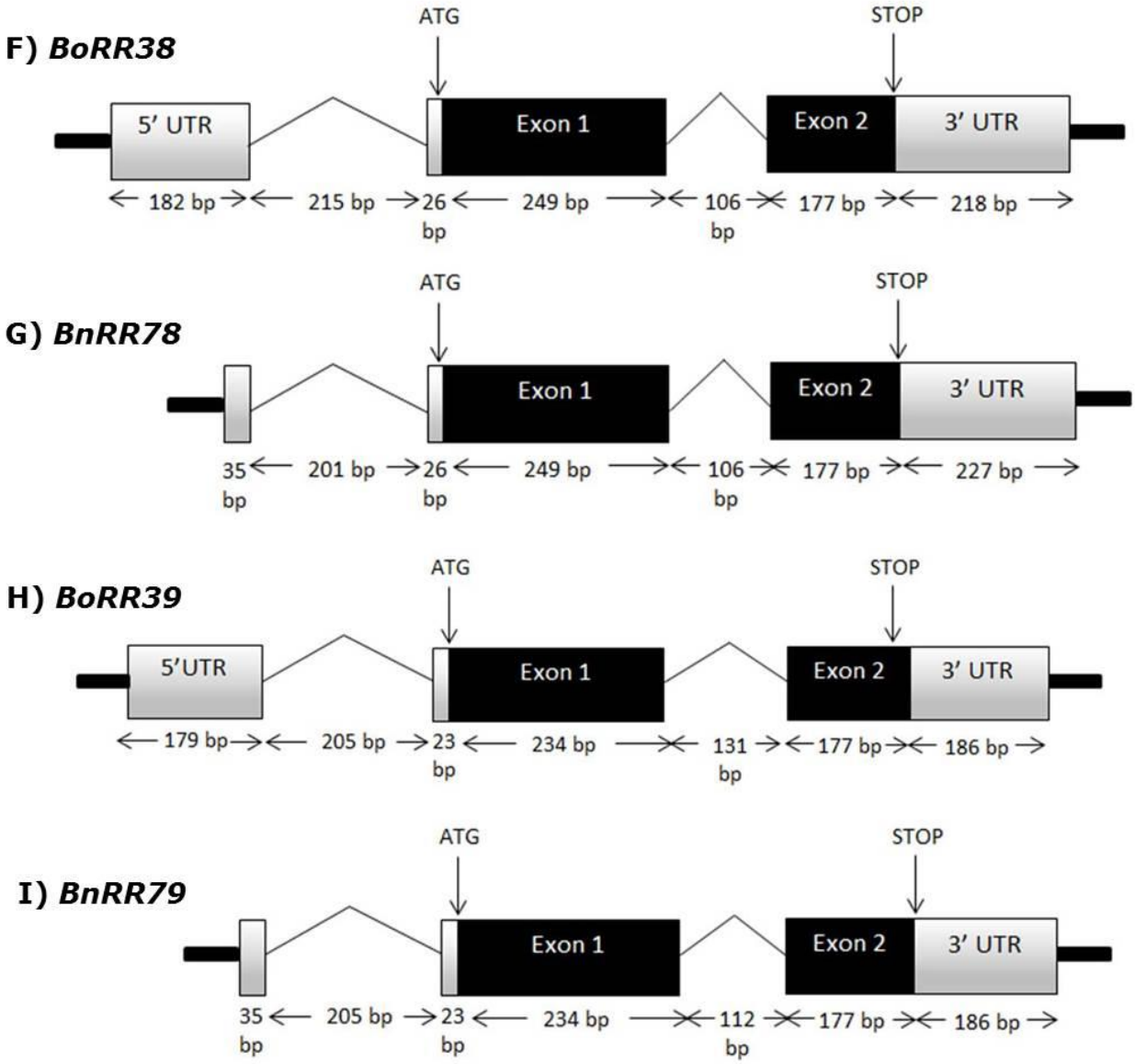


Figure 3.6. Predicted genomic structures of (**A**) *ARR22* (**B**) *BrRR39* (Bra001099) (**C**) *BnRR76* (BnaA03gXXXXD [Identified]) (**D**) *BrRR40* (Bra040204) (**E**) *BnRR77* (BnaA05g33120D) (**F**) *BoRR38* (Bol034163) (**G**) *BnRR78* (BnaC03g33640D) (**H**) *BoRR39* (Bol001327) (**I**) *BnRR79* (BnaC05g4737D). Full 5′ UTR information was absent for *B. napus* genes.

The size of exon one varies between the *Brassica* genes. A larger first exon size of 255 bp is observed in *BrRR39* (Bra001099) and *BnRR76* (BnaA03gXXXXD [identified]) while a smaller size of 234 bp is observed in *BrRR40* (Bra040204) and *BnRR77* (BnaA05g33120D). In a similar

pattern, a larger (249 bp) exon exists in *BoRR38* (Bol034163) and *BnRR78* (BnaC03g33640D) while a smaller (234 bp) exon exists in *BoRR39* (Bol001327) and *BnRR79* (BnaC05g4737D). The size of exon two is consistent with *ARR22* in all *Brassica* orthologues (177 bp).

3.5.4 Sequence alignment

A nucleic acid alignment was carried out on *ARR22* and the putative *Brassica* orthologues to determine any differences within their sequences (**Fig. 3.7;** for full genomic alignment see **Appendix II**). Within *BrRR39* and its identified *B. napus* orthologue *BnRR76* a small extra region of six nucleic acids (**Fig. 3.7** highlighted in turquoise) was observed which was absent in all other sequences. A further additional region of sixteen nucleic acids (**Fig. 3.7.** highlighted in green) was identified in *BnRR39*, *BnRR76*, *BoRR38* and its *B. napus* orthologue *BnRR78* as well as within *ARR22*.

Transcription of the fully processed *ARR22* mRNA transcript produces a 142 amino acid polypeptide (Gattolin et al., 2006). Coding DNA sequences for each of the putative *Brassica* orthologues of *ARR22* were converted into amino acid sequences using an *in silico* sequence conversion tool (http://in-silico.net/tools/biology/sequence_conversion). *BrRR39* produces a polypeptide of 143 amino acids which is predicted to be the same for its *B. napus* orthologue *BnRR76* while *BrRR40* and its *B.*

napus orthologue *BnRR77* produce a slightly smaller 136 amino acid polypeptide.

Figure 3.7. Nucleic acid alignment of *ARR22* and putative response regulator orthologues in *B. rapa (BrRR); B. oleracea (BoRR); and B. napus (BnRR)*. Region shown is part of exon one. Coding region in uppercase. Start codon highlighted in red. Areas of interest that have been referred to in the text are highlighted in turquoise and green. Alignment was carried out using the Clustal Omega web service (McWilliam et al., 2013). *BnRR76 is sequenced cDNA hence lacks the 5' UTR. Asterisks (*) indicate fully conserved regions.

BoRR38 and *BnRR78* produce a 141 amino acid polypeptide while *BoRR39* and *BnRR79* also produce a 136 amino acid polypeptide. As differences were observed in polypeptide sizes it was expected that the extra nucleic acids within exon one were contributing to the protein sequences. To determine their location and examine amino acid sequence similarity an amino acid alignment was carried out (**Fig. 3.8 A**). All *Brassica* orthologues of *ARR22* contained the conserved DDK motif present in all RRs. Within *BrRR39* and *BnRR76* the extra six

nucleic acids situated 8 bp down from the ATG site within exon one of the coding region resulted in the addition of two amino acids; serine (S) and threonine (T; **Fig. 3.8 A** highlighted in turquoise). Similarly the region of sixteen additional nucleic acids, 32 bp down from the ATG, within *ARR22*, *BoRR38*, *BnRR78*, *BrRR39* and *BnRR76* results in the addition of five additional amino acids: threonine (T); lysine (K); serine (S); valine (V); and glutamic acid (E) as well as a change at amino acid 19 from isoleucine (I) to valine (V). These amino acids are absent in *BrRR40*, *BoRR39* and their respective orthologues *BnRR77* and *BnRR79* in *B. napus.*

A)

B)

BrRR40	100%							
BoRR39	100%	100%						
BnRR79	100%	100%	100%					
BnRR77	100%	100%	100%	100%				
ARR22	81.25%	81.25%	81.25%	81.25%	100%			
BoRR38	81.94%	81.94%	81.94%	81.94%	88.88%	100%		
BnRR78	83.33%	83.33%	83.33%	83.33%	90.27%	97.91%	100%	
BrRR39	78.47%	78.47%	78.47%	78.47%	85.41%	95.13%	94.44%	100%
	BrRR40	**BoRR39**	**BnRR79**	**BnRR77**	**ARR22**	**BoRR38**	**BnRR78**	**BrRR39**

Figure 3.8. (**A**) Amino acid alignment of *ARR22* and putative orthologues in *Brassica* species. Regions of interest, discussed in the text are highlighted in turquoise and green. The DDK motif characteristically found in RRs is highlighted in red. (**B**) Amino acid similarity (%) between each sequence. *BnRR76* is a partial predicted amino acid sequence from a sequenced product and was removed from the sequence similarity analysis.

Analysis of the amino acid sequences was carried out using a sequence identity and similarity tool (**Fig. 3.8 B** http://imed.med.ucm.es/Tools/sias.html) and shows that *ARR22* is 81.25% similar to the *Brassica* orthologues. High similarity is observed between the *Brassica* genes although *BrRR39* is only 78.47% similar to *BrRR40*, *BoRR39*, *BnRR79* and *BnRR77* owing to the extra amino acids aforementioned.

3.6 Discussion

Plant multi-step phosphorelay systems are fundamental signalling systems that allow plants to respond to phytohormones and changes in

their environment (Hutchison and Kieber 2002*;* Hwang et al., 2002). Response regulators are the key components that regulate downstream signalling events. Analysis of the *Arabidopsis* genome has revealed that there are 24 ARRs which are categorized into three main groups referred to type-A, -B and –C (Schaller et al., 2007). The roles of type-A and type-B ARRs are relatively well defined. While type-A ARRs are implicated in several signalling pathways they are generally considered as partially redundant negative regulators of cytokinin signalling (Brandstatter and Kiever 1998; Taniguchi et al., 1998, Kiba et al., 1999; D'Agostino et al., 2000; Kiba et al., 2003; To et al., 2004). Type-B ARRs on the other hand contain DNA binding domains which allow them to function as transcription factors for the positive regulation of cytokinin signalling (Hwang and Sheen 2001; Mason et al., 2005; Yokoyama et al., 2007; Argyros et al., 2008; Ishida et al., 2008b). The precise role and mechanisms of the two type-C ARRs *ARR22* and *ARR24,* conversely, are comparatively unknown with no clarified involvement within hormone signalling. Work on *ARR22* however has shown that it appears to have a role associated with seed wounding and possibly assimilate partitioning (Gattolin et al., 2006).

A number of response regulators have been isolated and examined in crop species such as maize, rice, soybean and wheat (Asakura et al., 2003; Hirose et al., 2007; Le et al., 2011; Gahlaut et al., 2014). The

objective of this study was to identify response regulator genes in *Brassica* species.

3.6.1 Identification of BrRRs, BoRRs and BnRRs

In the simple diploid *Arabidopsis* genome 24 *ARR*s exist but within crop genomes that have been studied the number of response regulator genes seems to vary. For example in soybean there are 36 *GmRR*s (Mochida et al., 2010) with the number of orthologues for each *ARR* ranging from one to seven. Within the hexaploid wheat genome 45 *TaRR*s have been identified (Gahlaut et al., 2014). A study in Chinese cabbage (*B. rapa*) identified 42 *BrRR*s (Liu et al., 2014) which is in line with the findings of this present study. In *B. oleracea* this analysis identified 41 *BoRR* genes.

It is unsurprising that a higher number of *BrRR* and *BoRR* genes exist as *Brassica* genomes have not only experienced three rounds of whole genome duplication but both *B. rapa* and *B. oleracea* have additionally undergone a whole genome triplication (WGT) event after divergence from *Arabidopsis* approximately 9 – 15 million years ago (Wang et al., 2011; Cheng et al., 2014; Liu et al., 2014). It would hence be reasonable to assume that each species possesses three copies of each *Arabidopsis* gene. However this assumption was not observed with the number of *BrRR* and *BoRR* orthologues ranging from one to three. It is in fact believed that the *B. rapa* genome has undergone extensive

fractionation to reduce gene number (Wang et al., 2011; Cheng et al., 2012; Mun et al., 2009). Hence the findings of this *in silico* analysis of *Brassica* RRs are consistent with this concept. Interestingly the process of fractionation was not random with genes involved in signal transduction and stress response retained (Blanc and Wolfe 2004; Cheng et al., 2012; Rizzon et al., 2006). It could be predicted that the process of WGT would have also provided a number of genes with evolved or novel functions. The number of *ARR* orthologues identified within allotetraploid *B. napus* was, somewhat unsurprisingly, the sum of the *BrRR* and *BoRR* genes as a result of the hybridization of the *B. rapa* (A) and *B. oleracea* (C) genomes, predicted to have occurred ~10,000 years ago (Nagaharu 1935).

The phylogenetic analysis presented in this study was based on the amino acid sequences of *ARR*s, *BrRR*s and *BoRR*s. Overall, the outcome displayed that no new groups or sub-groups of *Brassica* RRs have been formed and the same pattern occurs as in *Arabidopsis* (Kiba et al., 2004).

3.6.2 Structural differences in BnRRs

From examination and comparison of the predicted gene structures of chosen *BnRR*s this study distinguished some differences in exon and intron number and size between *BnRR*s and *ARR*s. Few studies have fully examined this occurrence. Whether these differences provide

functional significance is hence unclear. Studies examining particular groups of genes in Brassica have acknowledged small changes in exon number and size. For example, glutamine synthetase genes in *B. napus* were observed to possess one less exon than their *Arabidopsis* orthologues (Orsel et al., 2014). Within *B. rapa* glucosinolate biosynthesis genes, while exon number was comparable to *Arabidopsis*, a small exon deletion resulted in a truncated protein (Zang et al., 2009). Addition of exon coding regions is assumed to potentially alter the function of the protein or its role within the signalling pathway.

3.6.3 SAC29 and characterising type-C BnRRs

Initially this study was based on the finding of an individual putative orthologue of the type-C *ARR22* in *B. napus* known as *SAC29* (Whitelaw et al., 1999). Over the course of the overall study further information on the *B. napus* sequence became publicly available allowing for the identification of four *B. napus* putative orthologues of *ARR22* (*BnRR76* – *BnRR79*). Two of these were identified as originating from *B. rapa* (*BnRR76* and *BnRR77*) and two from *B. oleracea* (*BnRR78* and *BnRR79*). While genomic structures were relatively similar between genes, prominent differences were observed within and between the sequences. One *B. rapa* orthologue (*BrRR39*) and one *B. oleracea* (*BoRR38*), and consequently two *B. napus* (*BnRR76* and *BnRR78*), genes contain an additional sixteen nucleic acid sequence within the coding region which contributes to the addition of five amino acids. This

sequence is also observed within *ARR22*. Interestingly this sequence is lacking from *BnRR77* and *BnRR79*, originating from *BrRR40* and *BoRR39* respectively which could have occurred during diploidization events after the split from *Arabidopsis*. Although it is unclear what the addition (or lack) of amino acid residues contributes, this observation raises the possibility of differences in gene function or mechanism of action. The receiver domain of response regulators functions as the site of phosphorylation (Imamura et al., 1999). Although little work has examined the structures of receiver domains in plants, in bacteria certain features of the receiver domain amino acid sequences contribute to a specific structure and consequently function (Bourret 2010). As the additional amino acids seen in the Brassica genes and *ARR22* are present within this region, it is possible that lack of these residues alters the protein configuration.

3.6.4 Synteny comparisons between Arabidopsis and B. napus

Analysing chromosomal synteny can assist in revealing the evolution of related species as well as the functions of syntenic genes (Tang et al., 2008). Shared, or conserved, synteny describes the preservation of genes, or genomic fragments, on chromosomes in different species that have evolved from a common ancestor (Lyons et al., 2008). Syntenic genes are orthologous and hence normally have equivalent functions. Previous comparative analyses have revealed high conservation of gene order between *Arabidopsis* and *Brassica* species (Town et al., 2006).

However considerable gene loss and rearrangements have also occurred (Kowalski et al., 1994; Lukens et al., 2003; Park et al., 2005). Although it is not apparent that rearrangements have occurred during the hybridization of the *Brassica* A and C genomes (Rana et al., 2004).

In this study some microsynteny was observed between the studied region of *Arabidopsis* chromosome 3 and *B. rapa* chromosome 3 and *B. napus* chromosomes A05 and C03. It was also apparent that some reshuffling of genes has occurred during the evolution of *Brassica* in addition to gene loss or rearrangement.

3.7 Conclusions

This *in silico* analysis is the first study to uncover the presence of eighty-three response regulators in *B. napus* and contributes to the knowledge of *Brassica* genome evolution. Results of the phylogenetic analysis are consistent with that seen in *Arabidopsis* with *Brassica* response regulators split into the three groups, type-A, -B and –C. *B. napus* orthologues of two type-A and two-B ARRs were chosen for further analyses. Small differences were observed in genomic structure but this information is insufficient to deduce alterations in function. Previous work revealing the existence of *SAC29*, a putative type-C *ARR22* orthologue, has been considerably expanded with the identification of four *B. napus* orthologues (*BnRR76* – *BnRR79*). Striking differences within their nucleic and amino acid sequences have alluded

to the possibility of altered function, expression or roles. The spatial and temporal expression patterns of these *BnRR*s will be examined in the next chapter (Chapter 4) with the aim of dissecting their possible contribution to plant growth and development.

Chapter 4:

Analysis of Gene and Protein Expression

4.1 Introduction

Seed development is a complex yet organised biological process involving coordinated expression of an array of genes. Generally there are four discrete stages: embryo patterning; embryo growth; seed maturation, in which storage products such as proteins and lipids accumulate; and seed desiccation (Dong et al., 2004; Fei et al., 2007; Goldberg et al., 1989).

Previous work in *Arabidopsis* has shown that the type-C response regulator *ARR22* is expressed in flowers and developing siliques (Gattolin et al., 2006). Due to the possession of two introns, located within the 5' UTR and open reading frame, *ARR22* produces four splice variants which accumulate to different proportions throughout silique development. Histochemical localization of *ARR22* revealed intense expression at the seed:funiculus junction in response to wounding leading to the hypothesis that *ARR22* is post-transcriptionally up-regulated after seed damage has occurred (Gattolin et al., 2006). Furthermore, microarray data has shown that seed storage protein genes are down-regulated rapidly within 90 mins post wounding while seed protease genes are up-regulated, suggesting that *ARR22* is implicated in assimilate partitioning (Naomab, 2008).

In the previous chapter, an *in silico* analysis uncovered four putative orthologues of *ARR22* in *B. napus* (*BnRR76* – *BnRR79*). Each of these orthologues contains two introns akin to *ARR22* and exhibit 82% amino acid similarity with *ARR22*. On the basis of this information it was hypothesised that these genes may be expressed in a similar manner to *ARR22* and may exhibit a similar function in seeds. As previous work has not analysed the expression of *ARR22* at the protein level, an antibody was also designed to examine the protein expression of the putative *ARR22* orthologues in *B. napus* (see **Fig. 2.2; Chapter 2** for design).

The key objectives of this study were hence to determine the spatial and temporal gene and protein expression of type-C orthologues of *ARR22* in *B. napus* vegetative and reproductive tissues throughout development and in response to wounding. The expression of SSP and cysteine protease genes was analysed in *B. napus* seeds pre- and post-wounding up to 120 mins post-wounding. The developmental silique and seed stages studied can be seen in **Fig. 4.1 A** and **B**.

Putative *B. napus* orthologues of the genes encoding type-A response regulators *ARR16* and *ARR17* and type-B response regulators *ARR12* and *ARR21* were additionally chosen to study for gene expression analysis on the basis of gene expression data in seeds (see **section 3.4**; **Chapter 3**).

1553

A)

1554

B)

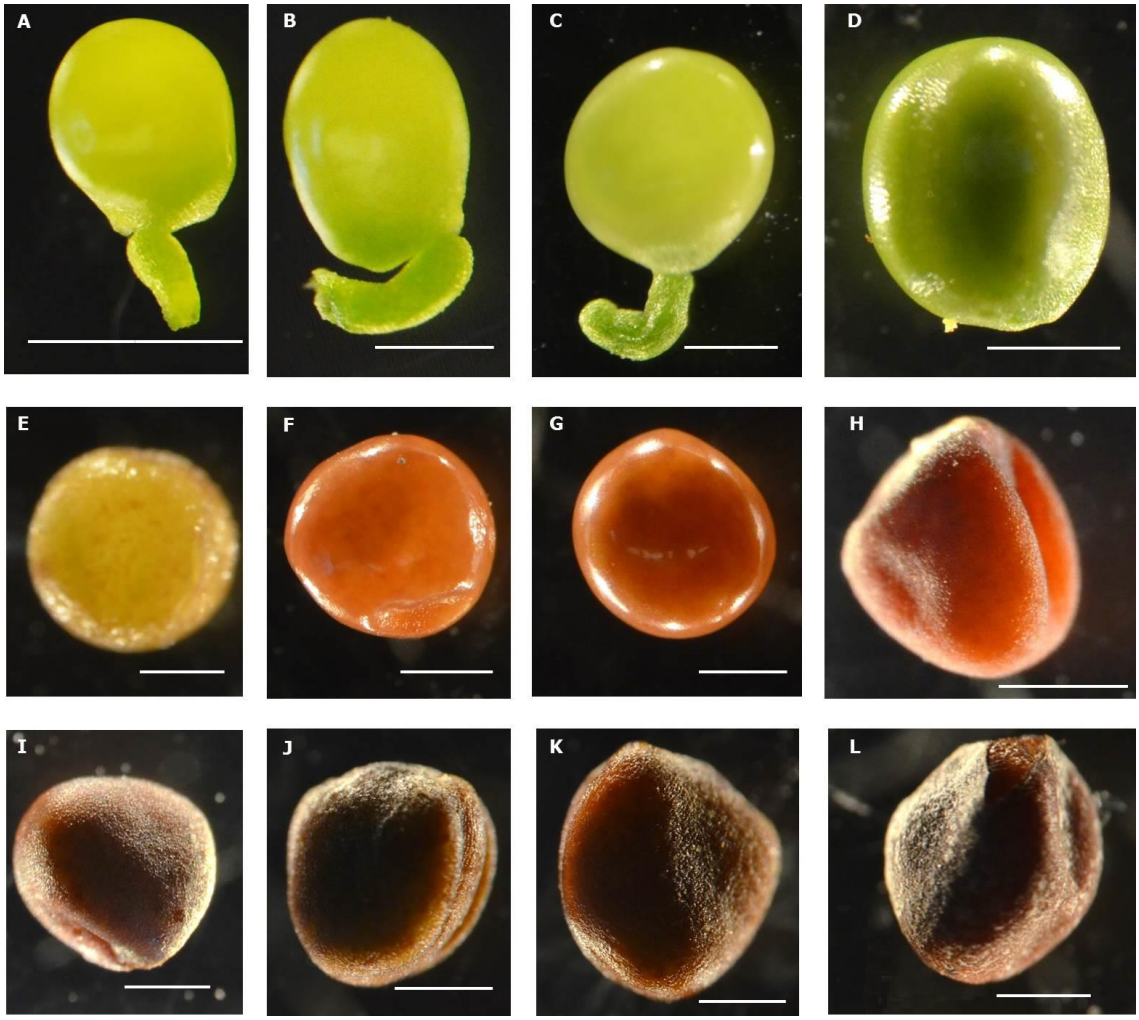


Figure 4.1. (**A**) *B. napus* silique morphology throughout development. (**B**) *B. napus* seed morphology throughout development. A: 5 DAF; B: 10 DAF; C: 15 DAF; D: 20 DAF; E: 25 DAF; F: 30 DAF; G: 35 DAF; H: 40 DAF; I: 45 DAF; J: 50 DAF; K: 55 DAF; L: 60 DAF. Bar = 1 mm.

4.2 Reverse transcription PCR analysis of type-A and type-B *BnRR* gene expression

The expression of putative *B. napus* type-A *ARR16* orthologues (*BnRR34* and *BnRR35)* and *ARR17* (*BnRR36* - *BnRR39*) along with type-B *ARR12* (*BnRR57* and *BnRR58*) and *ARR21* (*BnRR71* - *BnRR74*) was examined in buds, flowers and seeds (**Fig. 4.2**). Primers were designed and

positioned to amplify transcripts of all orthologues where appropriate (for primer positions see **Fig. 3.2; Chapter 3**).

All genes were expressed in early seed stages (10 – 30 DAF) although *BnRR36* – *BnRR39* gene expression was low in 20 and 30 DAF **Fig. 4.2**). Expression of all genes was low or absent in seeds 40 – 60 DAF. *BnRR71* – *BnRR74* expression was not observed in buds or flowers whereas transcripts of all other genes were amplified. Expression in buds was low for *BnRR57* - *BnRR58*.

Alternative splicing was observed for putative *ARR17* orthologues *BnRR36* – *BnRR39* (**Fig. 4.2**). Three transcripts were produced: a fully processed transcript (316 – 317 bp); a transcript retaining two introns (507 – 535 bp); and a transcript retaining three introns (618 – 621 bp). The fully processed transcript is highly expressed in buds, flowers and seeds 10 DAF. At 20 DAF only the fully processed transcript is expressed but at a low level. At 30 DAF all transcripts are expressed at a low level but with the transcript retaining two introns at a slightly higher level. The transcript retaining three introns is most expressed in seeds 10 DAF. For all other genes analysed only fully processed transcripts were observed.

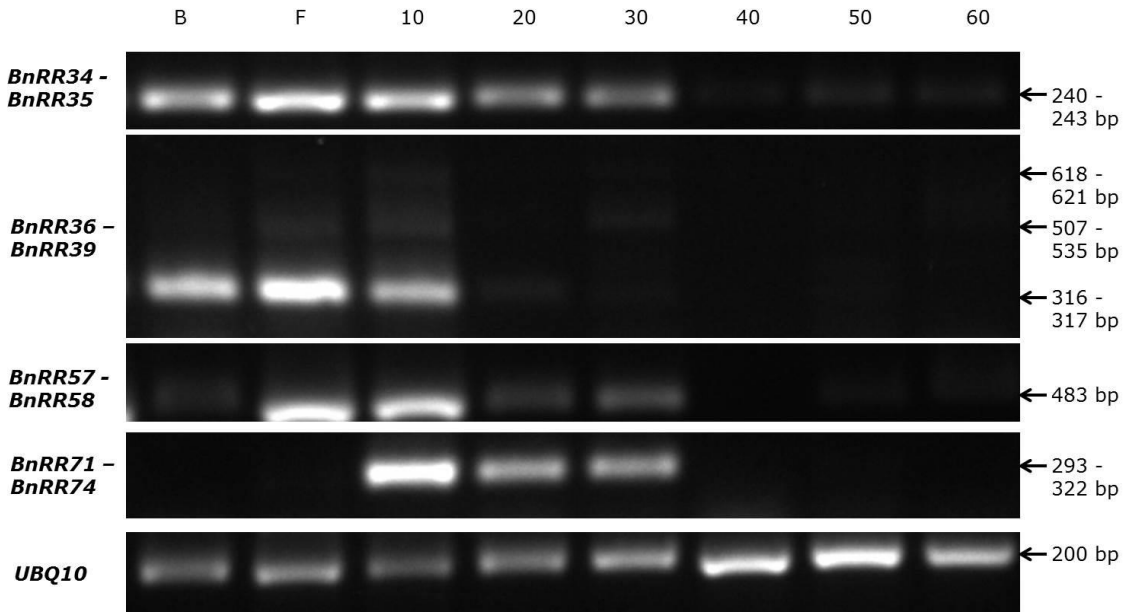


Figure 4.2. RT-PCR analysis of type-A *ARR16* putative orthologues *BnRR34 and BnRR35*; *ARR17* putative orthologues *BnRR36* - *BnRR39*; type-B *ARR12* putative orthologues *BnRR57* and *BnRR58*; and *ARR21* putative orthologues *BnRR71* - *BnRR74 in B. napus* buds (B), flowers (F) and seeds 10 – 60 DAF. *UBQ10* used as a housekeeping gene. Disparity in transcript sizes due to exon and intron size differences.

4.3 Spatial and temporal reverse transcription PCR analysis of putative *ARR22* orthologues in *B. napus*

As sequence information was originally lacking for *ARR22* putative orthologues in *B. napus* and *B. oleracea*, this analysis commenced by using primers based on *B. rapa* sequence information; specifically *BrRR40* due availability of 5’ UTR sequence information. Two forward primers were designed; one situated within the 5’ UTR and another within the ORF in exon 1 (**Fig. 4.3**).

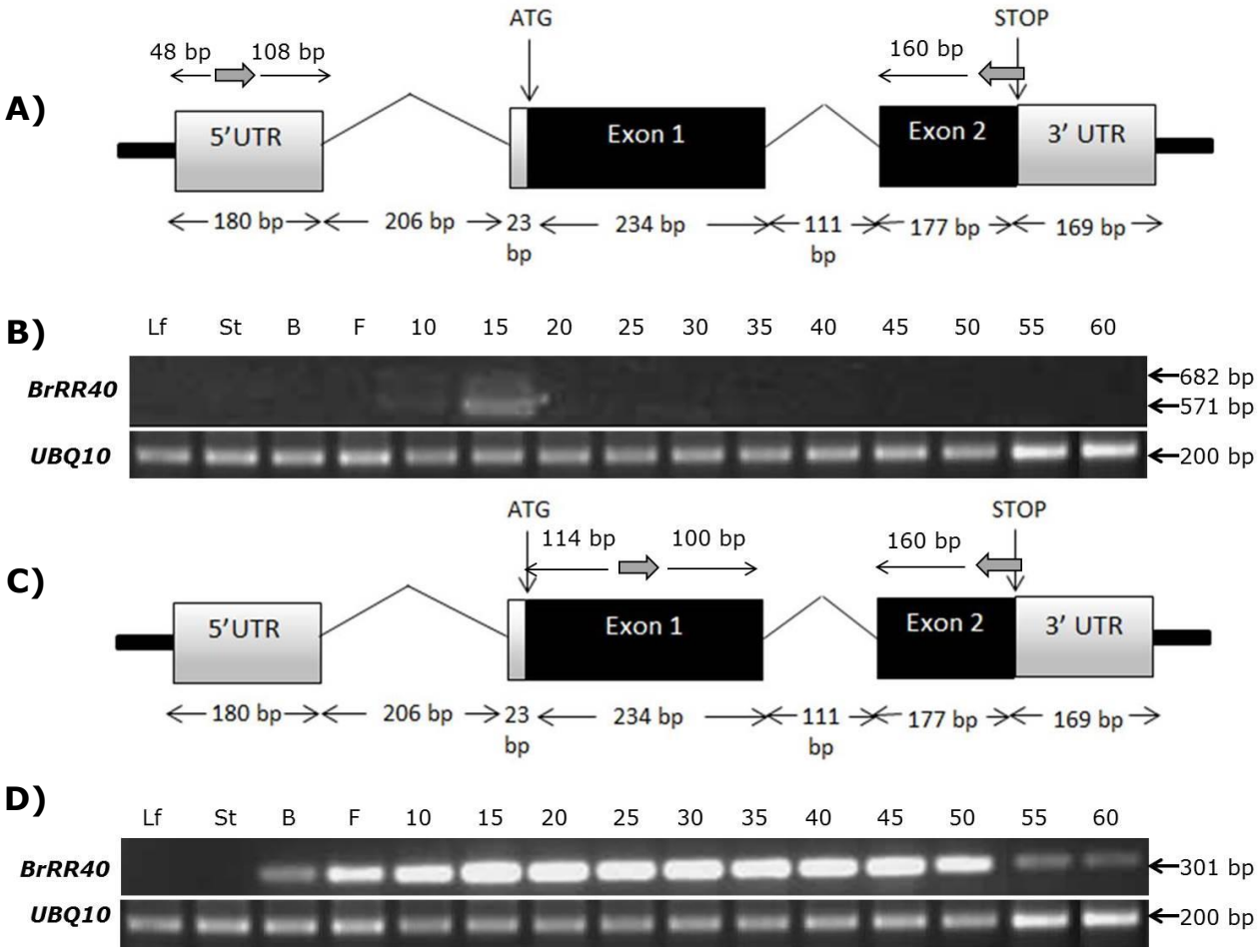


Figure 4.3. (**A**) Genomic structure of *BrRR40* showing position of forward primer situated within 5' UTR. Grey arrows represent primers (**B**) RT-PCR analysis of the putative *BrRR40* orthologue in *B. napus* using forward primer situated within 5' UTR in leaves (Lf), stem (St), buds (B), flowers (F) and seeds 10 – 60 DAF (**C**) Genomic structure of *BrRR40* showing position of forward primer situated within ORF. Grey arrows represent primers (**D**) RT-PCR analysis of putative *BrRR40* orthologue in *B. napus* using forward primer situated within ORF in leaves (Lf), stem (St), buds (B), flowers (F) and seeds 10 – 60 DAF.

When the forward primer situated within the 5' UTR was used, expression was confined to 10 and 15 DAF only (**Fig. 4.3 B**). Two splice variants were also observed; a fully processed transcript (571 bp) and a transcript predicted to contain the intron within the ORF (682 bp). In contrast, when RT-PCR analysis was carried out using the primer

situated within the ORF a transcript without the ORF intron was expressed in buds, flowers and all seed stages (10 – 60 DAF) although expression was comparatively lower in seeds 55 and 60 DAF.

4.3.1 Differential expression of putative ARR22 B. rapa and B. oleracea orthologous transcripts in B. napus

Following the gene expression analyses in section 4.3, primers were used to amplify the putative *ARR22* orthologue in *B. oleracea* genomic DNA for sequencing (see **Appendix III**). Comparison of the *B. rapa* and *B. oleracea* genomic sequences revealed nucleic acid differences, allowing primers to be designed to amplify transcripts from each species in *B. napus* (**Fig. 4.4 A**).

PCR analysis confirmed that the primers designed to isolate *B. rapa* and *B. oleracea* transcripts were able to amplify these transcripts specifically in *B. rapa* and *B. oleracea* respectively as well as in *B. napus* (**Fig. 4.4 B**). RT-PCR analysis using these primers in *B. napus* revealed that both transcripts are predominantly expressed in seeds 10 – 35 DAF (**Fig. 4.4 C**). Low expression of both was observed in buds and flowers. Expression of the *B. oleracea* transcript was, although low, was observed in seeds 40 – 50 DAF. Expression of this transcript is then absent in seeds 55 and 60 DAF whereas expression of the *BrRR40* transcript is absent in 40 and 45 DAF seeds but low expression of the transcript at 50 and 60 DAF seed.

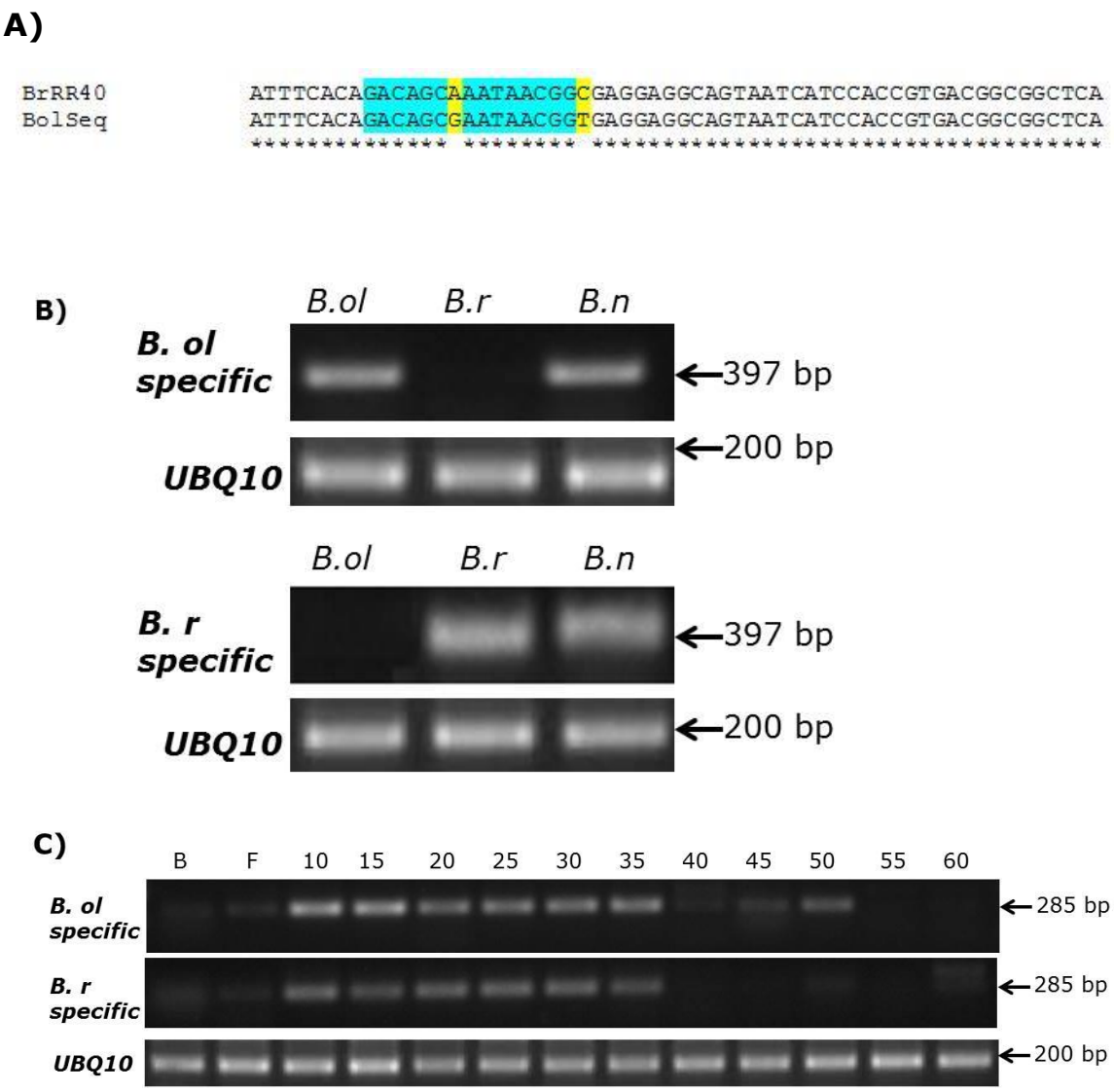


Figure 4.4. (**A**) Design of a forward primer to amplify orthologous transcripts from *B. rapa* and *B. oleracea* separately in *B. napus*. (**B**) Control PCR using specific primers on genomic DNA from *B. oleracea* (*B.ol*), *B. rapa* (*B.r*) and *B. napus* (*B.n*). (**C**) RT-PCR analysis using specific primers in *B. napus* buds (B), flowers (F) and seeds 10 – 60 DAF. *UBQ10* used as housekeeping control.

4.3.2 RT-PCR analysis of BnRR76 – BnRR79

During the course of the study database sequence information became available on *ARR22* putative orthologues in *B. napus* (Chalhoub et a., 2014). This allowed primers to be designed that spanned both known

introns (see **Fig. 4.5 A** for primer positions) to amplify these transcripts via RT-PCR.

Expression was observed in buds, flowers and seeds 5 – 40 DAF (**Fig. 4.5 B**). Very low expression was seen in seeds 45 – 55 DAF. The amplification of two transcripts (sized 344 bp and 367 bp) which appears as a double band occurred at 5, 10, 15, 20, 35 and 45 DAF. Amplification of only the smaller 344 bp transcript occurred in buds and 40 DAF seeds. Amplification of only the larger transcript occurred in seeds 25 and 30 DAF. A transcript size difference between *B. oleracea* and *B. rapa* and *B. napus* was observed when these primers were used on genomic DNA (**Fig. 4.5 C**).

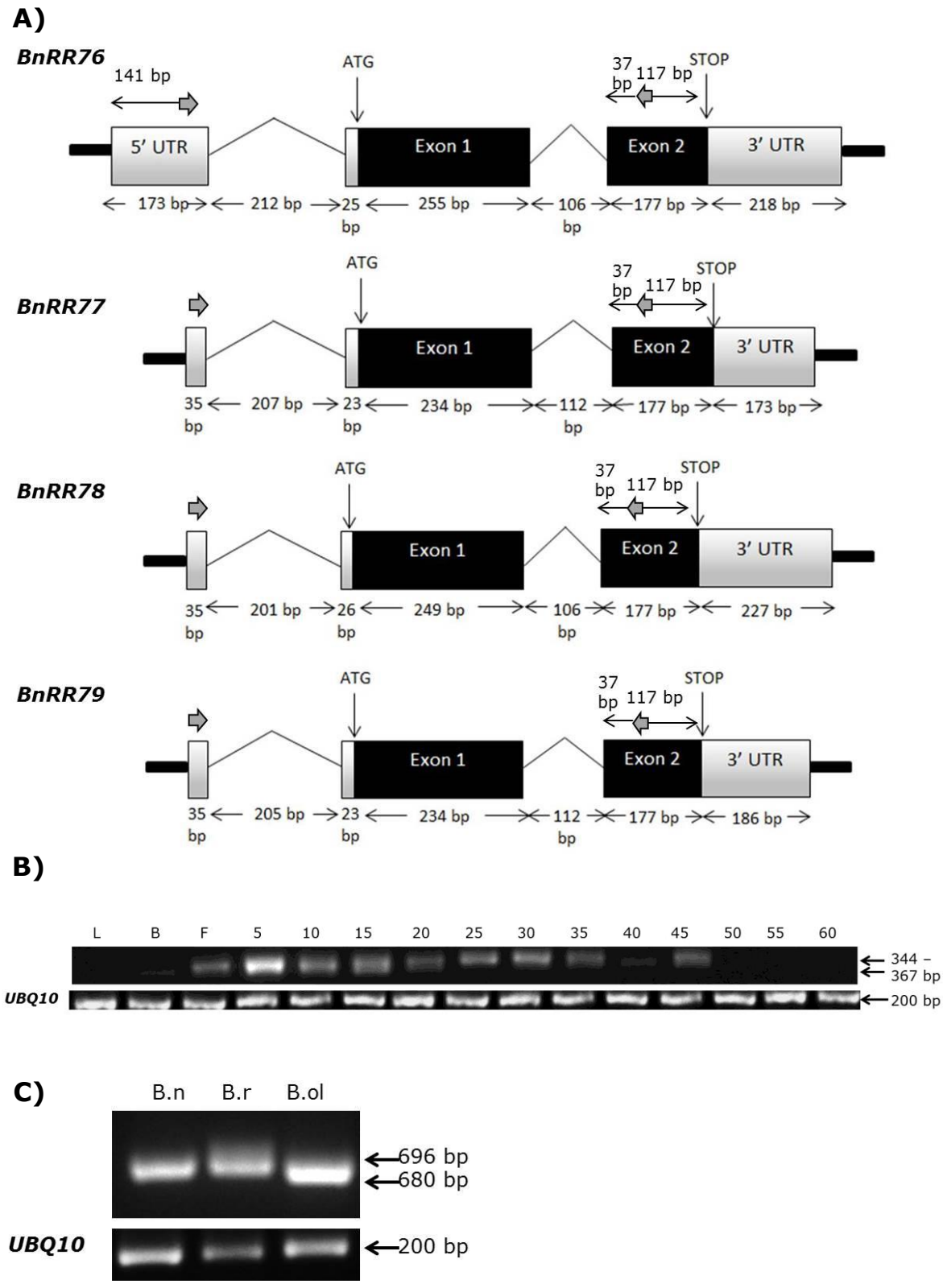


Figure 4.5. (**A**) Genomic structures of putative *ARR22* orthologues in *B. napus* showing design of primers (grey arrows). (**B**) RT-PCR analysis in *B. napus* leaves (L), buds (B), flowers (F) and seeds 5 – 60 DAF. (**C**) PCR using same primers on genomic DNA from *B. napus* (B.n), *B. rapa* (B.r) and *B. oleracea* (B.ol). *UBQ10* used as housekeeping control.

4.3.3 Amplification of the putative BrRR39 orthologue in B. napus

The size difference observed in **Fig 4.5 C** was dissected through sequence alignment of the putative *ARR22* orthologues in *B. rapa*, *B. oleracea* and *B. napus* and revealed the presence of an additional short sequence of nucleic acids in *BrRR39*. A forward primer was designed to amplify this transcript in *B. napus* (**Fig. 4.6 A**). PCR using gDNA extracted from *B. oleracea*, *B. rapa* and *B. napus* confirmed that this primer functioned specifically in *B. rapa* and *B. napus* (**Fig. 4.6 B**). A 121 bp transcript was amplified in flowers and seeds 5 – 55 DAF. No transcript was present in buds or 60 DAF seeds.

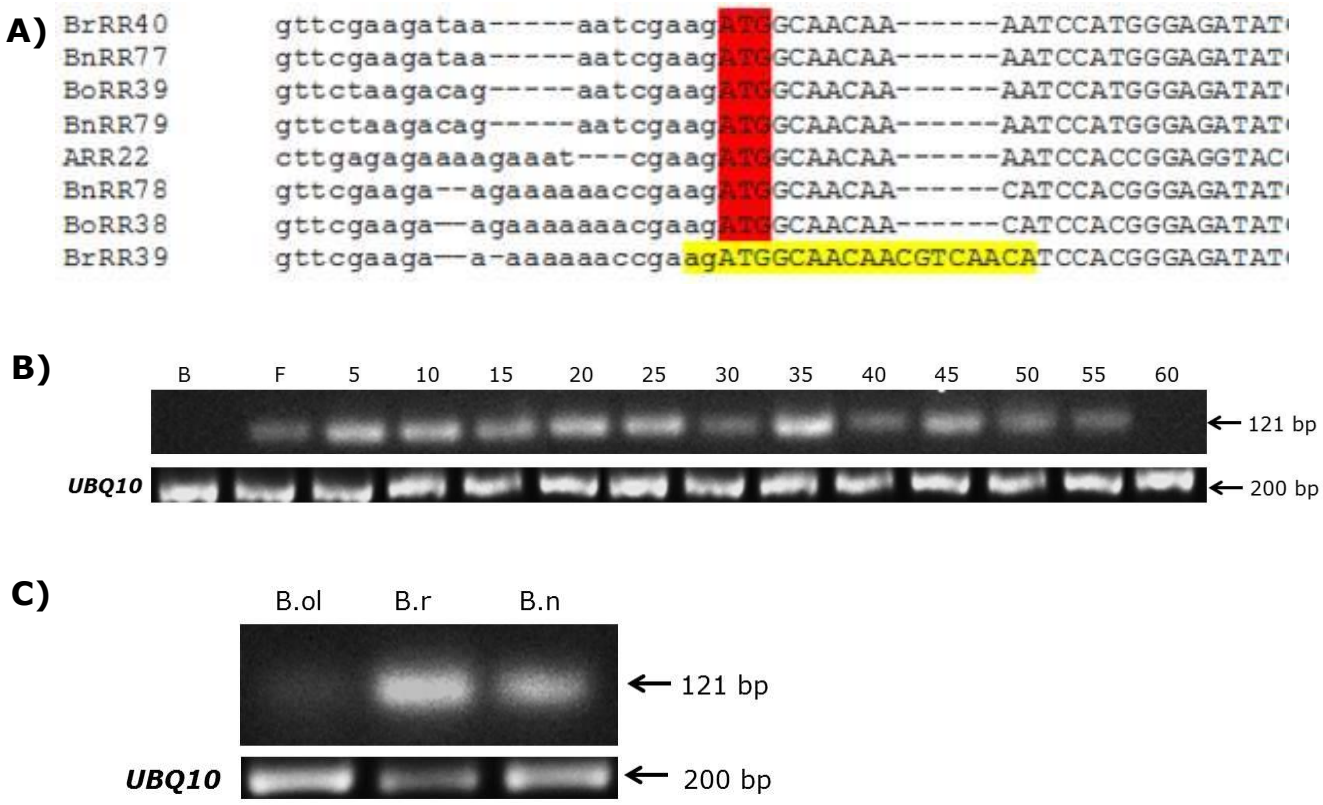


Figure 4.6 (**A**) Design of forward primer (highlighted in yellow) to amplify putative *BrRR39* orthologue in *B. napus* (**B**) RT-PCR amplification of the putative *BrRR39* orthologue in *B. napus* buds (B), flowers (F) and seeds 5 – 60 DAF. (**C**) PCR control using *BrRR39* primer on gDNA extracted from *B. oleracea* (B.ol), *B. rapa* (B.r) and *B. napus* (B.n). *UBQ10* used as housekeeping control.

4.4 Separate amplification of *BnRR76 – BnRR79* transcripts

4.4.1 Amplification of BnRR76 and BnRR78

Sequence analysis revealed that *BnRR76* and *BnRR78* contain an additional sixteen nucleic acid sequence (see **section 3.5.5; Chapter 3**) whereas *BnRR77* and *BnRR79* lack this sequence. It was hypothesised that the larger transcript amplified in **Fig 4.5 B** contained this extra sequence of nucleic acids. A primer was hence designed over this sequence to amplify *BnRR77* and *BnRR79* transcripts specifically.

Expression was observed in flowers and seeds 5 to 60 DAF. Expression was highest in seeds 5 to 25 DAF and 35 DAF. Expression was lowest in seeds 40 DAF and in 50 to 60 DAF.

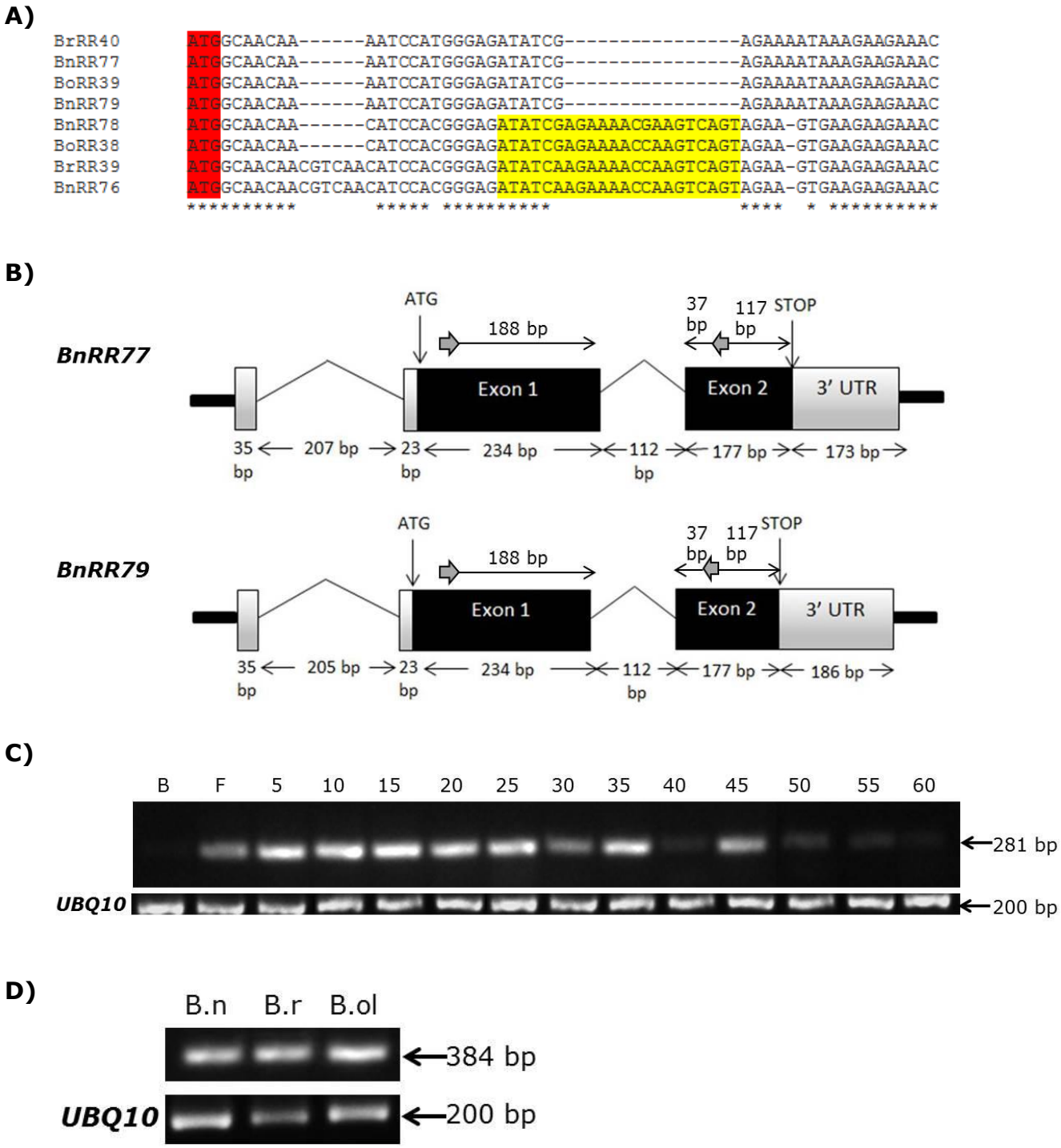


Figure 4.7. (**A**) Forward primer (highlighted in yellow) designed to amplify *BnRR77* and *BnRR79* transcripts containing identified extra nucleic acids. (**B**) Genomic structures of *BnRR77* and *BnRR79* showing design of primers (grey arrows). (**C**) RT-PCR analysis of BnRR77 and BnRR79 gene expression in *B. napus* buds (B), flowers (F) and seeds 5 – 60 DAF. (**D**) PCR control using *BnRR77* and *BnRR79* primer on gDNA

extracted from *B. napus* (B.n), *B. rapa* (B.r) and B. oleracea (B.ol). *UBQ10* used as housekeeping control.

4.4.2 Amplification of BnRR77 and BnRR79

A forward primer was subsequently designed (**Fig. 4.8 A** and **B**) to specifically amplify the *BnRR77* and *BnRR79* transcripts that do not contain the extra sequence of sixteen nucleic acids. RT-PCR analysis revealed amplification of two transcripts. A transcript of 377 bp containing the 112 bp intron present within the ORF was amplified in buds, flowers and in seeds 15 to 60 DAF with the exception of 50 DAF seeds (Fig. C). Amplification of a processed transcript (265 bp), not containing the intron, was observed in seeds 5 – 50 DAF. In buds, flowers and seeds 55 and 60 DAF only the 377 bp transcript was expressed. In 5, 10 and 50 DAF seed only the 265 bp transcript was present. Expression levels of the two transcripts varied throughout seed development. In 15 and 35 DAF the processed transcript was predominantly expressed while in 25, 30 and 45 DAF the unprocessed transcript appeared to be expressed at a higher level.

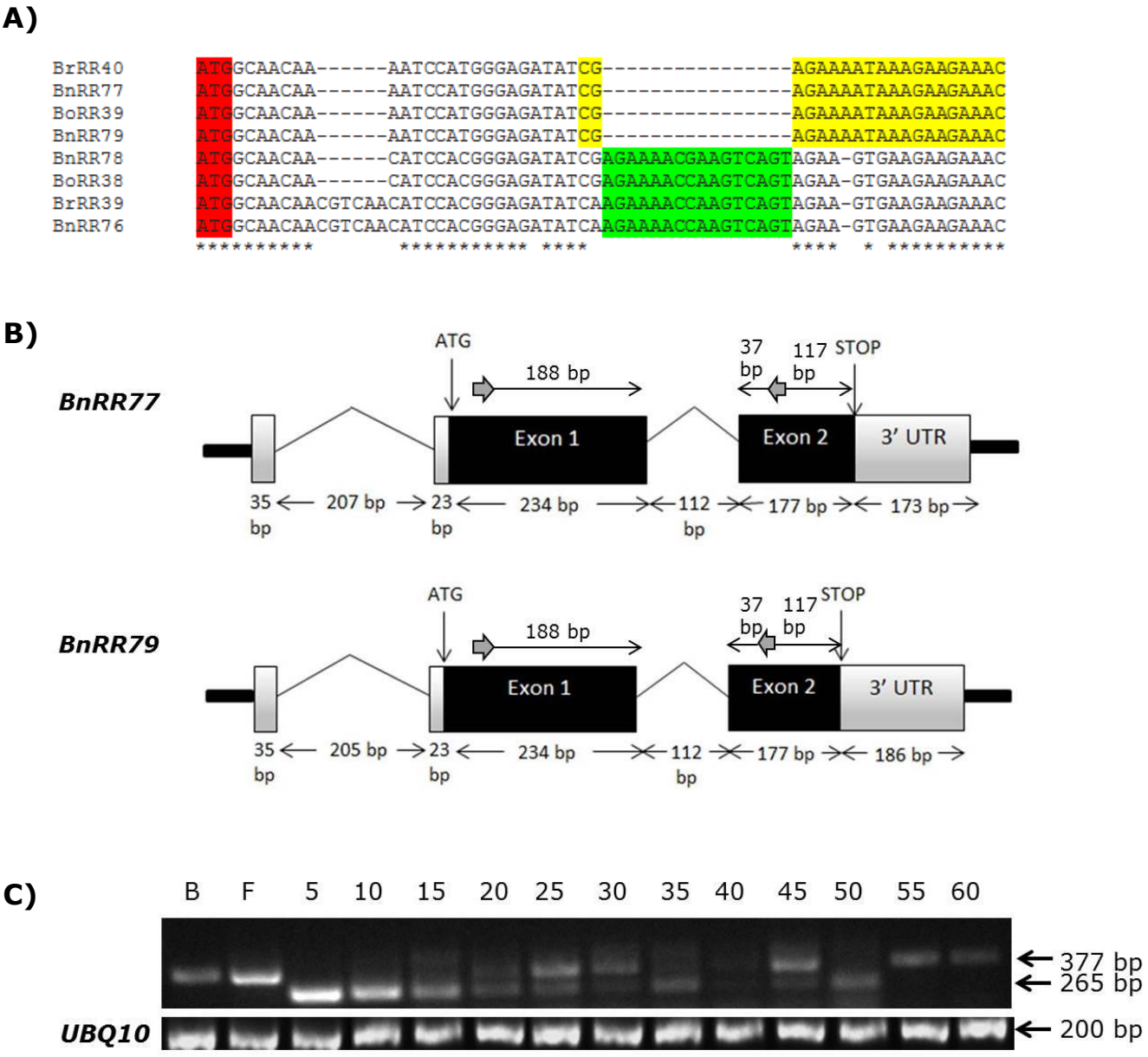


Figure 4.8. (**A**) Design of forward primer (highlighted in yellow) to amplify only transcripts that do not contain extra sequence of nucleic acids (highlighted in green). (**B**) Genomic structure of *BnRR77 and BnRR79* showing position of primers (grey arrows). (**C**) RT-PCR amplification of transcripts in *B. napus* buds (B) flowers (F) and seeds 5 – 60 DAF. *UBQ10* used as housekeeping control.

4.5 RT-PCR analysis of gene expression post-wounding

B. napus seeds were wounded on the plant at 20 and 35 DAF. These stages were chosen on the basis of high gene expression analysis of the putative *BrRR40* orthologue in *B. napus* observed between 15 and 45 DAF seed (**Fig. 4.2 D**). These stages also offer different points within the maturation phase of oilseed development in which lipids and SSPs accumulate (Huang et al., 2013; Obermeier et al., 2009). Wounded seeds were then left for 5 to 120 mins to analyse the effect of wounding on the expression of putative *B. napus ARR22* orthologues; and SSP and cysteine protease genes.

4.5.1 Effect of wounding on BnRR76 – BnRR79 gene expression

Primers as designed in **Fig. 4.2 C** and **Fig. 4.5 A** were utilised to analyse the gene expression of putative *ARR22* orthologues in *B. napus* post-wounding. When primers positioned within the 5' UTR were used expression was up-regulated in 20 DAF from 5 mins post-wounding (**Fig. 4.9 A**). In both 20 and 35 DAF expression was highly up-regulated at 80 and 120 mins (**Fig. 4.9 A**). An 8.8 and 7.5 fold change was quantified in comparison to the control using ImageJ at 80 and 120 mins respectively in 20 DAF seeds. In 35 DAF seed, 3.7 and 2.8 fold changes were observed at 80 and 120 mins respectively. When primers designed within the ORF were utilised no change in gene expression was

observed in comparison to control unwounded seeds in both 20 and 35 DAF seed (**Fig. 4.9 B**).

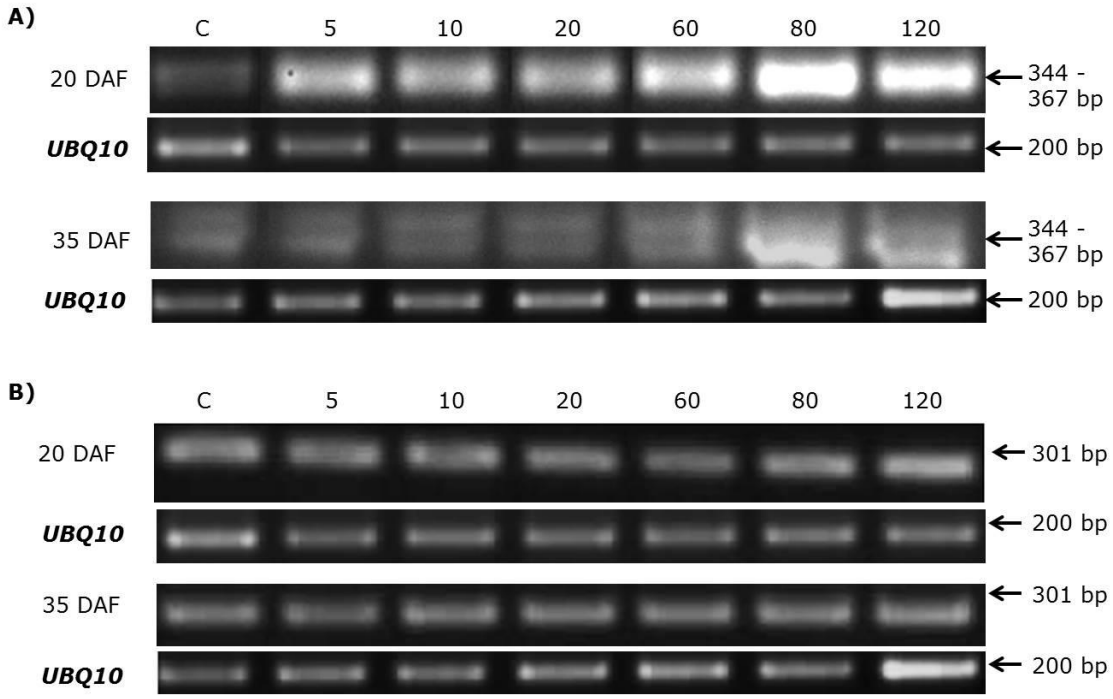


Figure 4.9. RT-PCR analysis of *BnRR76 – BnRR79* gene expression post-wounding in 20 and 35 DAF seeds (**A**) Using primers positioned within 5' UTR (**B**) Using primers positioned within ORF; control unwounded (C), 5 – 120 mins. *UBQ10* used as housekeeping control.

4.5.2 Effect of wounding on SSP and protease gene expression

Genes encoding SSPs and a cysteine protease were chosen from wounded Arabidopsis seed microarray data previously carried out (Naomab, 2008). Genes that were selected exhibited the biggest fold changes in gene expression 90 mins post-wounding. Putative *B. napus* orthologues of these genes were identified to study and included napin A and a cysteine protease. Genes were also selected from serial analysis

of gene expression data (LongSAGE; Obermeier et al., 2009) and these included seed specific protein and cruciferin. Gene expression was analysed in unwounded seeds throughout development and in 20 and 35 DAF seeds post-wounding.

Napin A expression was observed in buds, flowers and in all seeds 10 – 60 DAF (**Fig. 4.10 A**). In wounded seeds 20 and 35 DAF there was no change in napin A gene expression when compared with control unwounded seeds (**Fig. 4.10 B**).

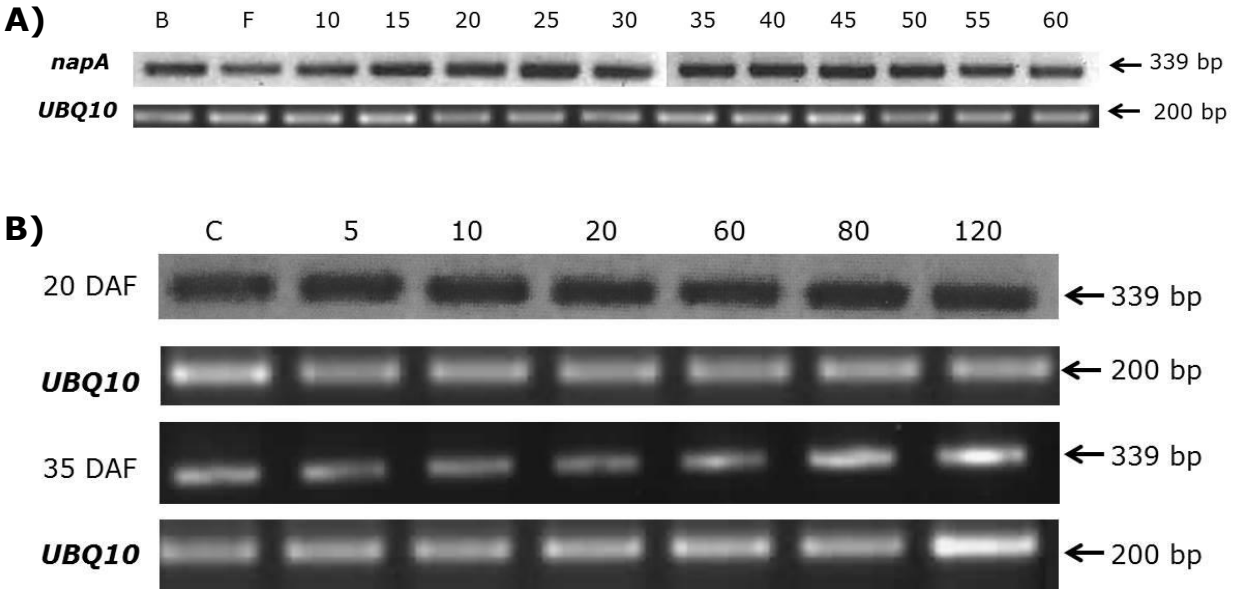


Figure 4.10. RT-PCR analysis of SSP napin A (napA) gene expression (**A**) Throughout development in unwounded buds (B), flowers (F), and seeds 10 – 60 DAF (**B**) In 20 and 35 DAF seeds post wounding; control unwounded (C), 5 – 120 mins. *UBQ10* as housekeeping control.

Similarly cysteine protease expression was observed in buds, flowers and in all seed stages 10 – 60 DAF (**Fig. 4.11 A**). Cysteine protease

gene expression did not appear to change in response to wounding in 20 or 35 DAF seed (**Fig. 4.11 B**).

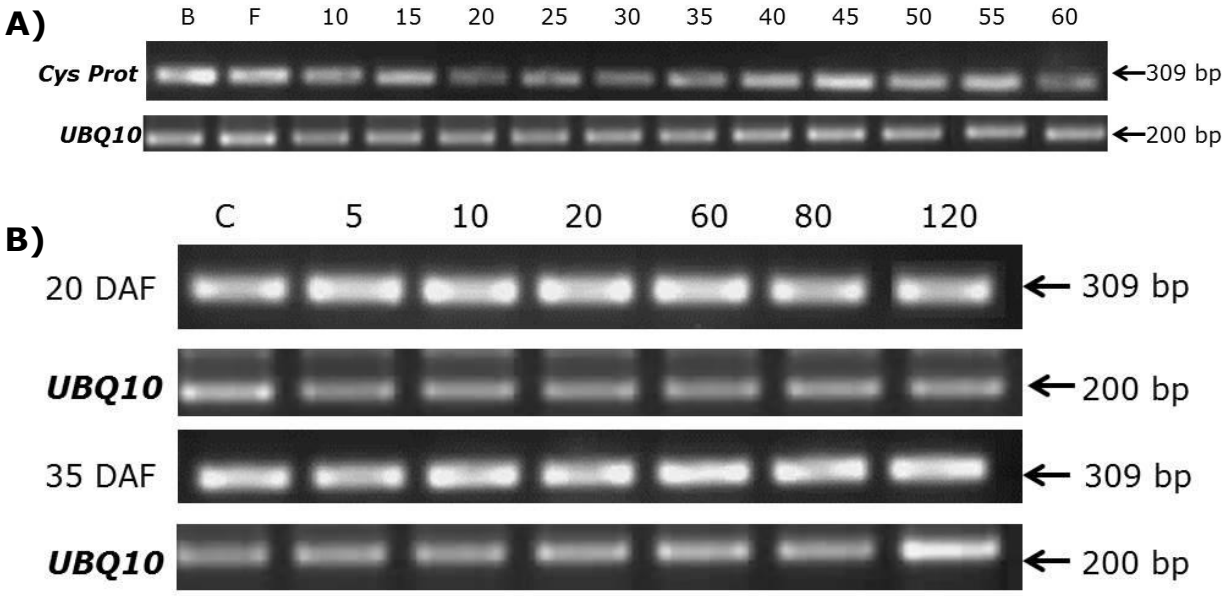


Figure 4.11. RT-PCR analysis of cysteine protease gene expression (**A**) Throughout development in unwounded buds (B), flowers (F), and seeds 10 – 60 DAF (**B**) In 20 and 35 DAF seeds post wounding; control unwounded (C), 5 – 120 mins. *UBQ10* as housekeeping control.

The gene expression of seed specific protein was analysed in buds, flowers and seeds 10 – 60 DAF (**Fig. 4.12 A**). Expression was highest in 20 and 30 DAF. No difference in gene expression was observed in 20 and 35 DAF post-wounding (**Fig. 4.12 B**).

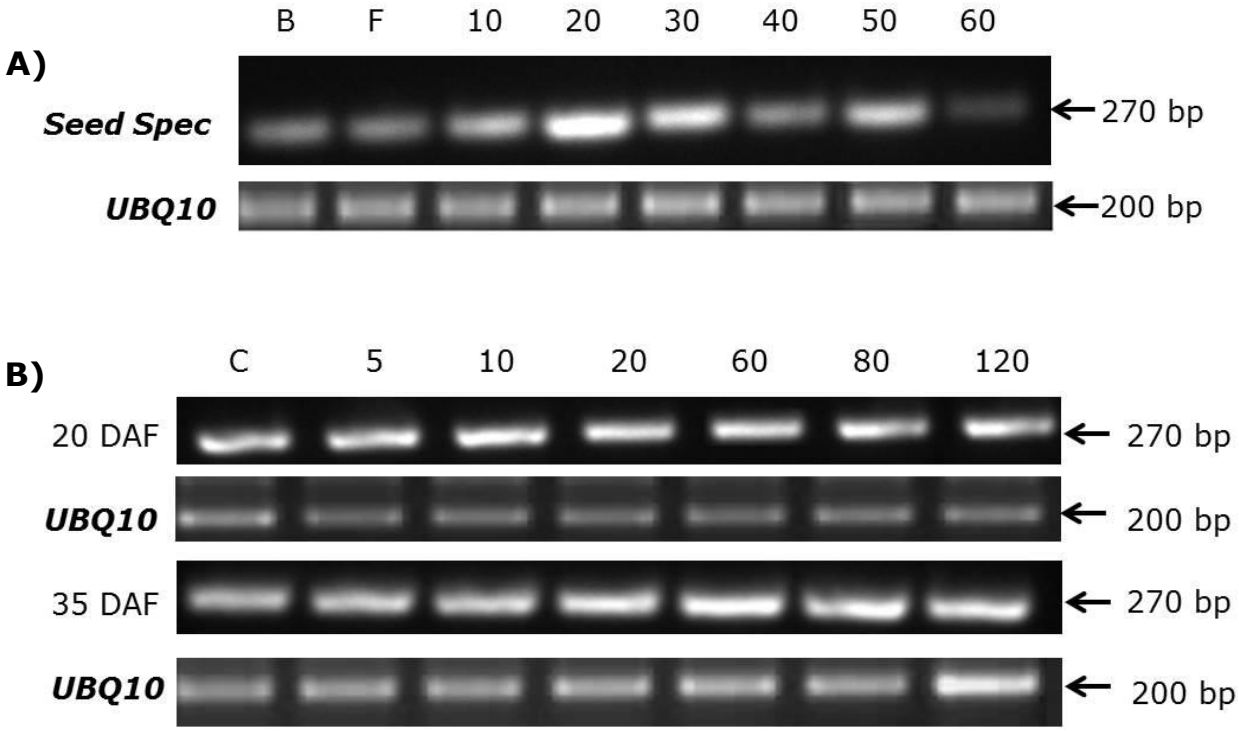


Figure 4.12. RT-PCR analysis of seed specific protein gene expression (**A**) Throughout development in unwounded buds (B), flowers (F), and seeds 10 – 60 DAF (**B**) In 20 and 35 DAF seeds post wounding; control unwounded (C), 5 – 120 mins. *UBQ10* as housekeeping control.

Cruciferin expression was observed in flowers and seeds 10 – 60 DAF but not in buds (**Fig. 4.13 B**). In 20 DAF seed no effect was observed post-wounding. In 35 DAF wounded seeds expression appeared up-regulated 20 – 120 mins post-wounding when compared with the unwounded control (**Fig. 4.13 B**).

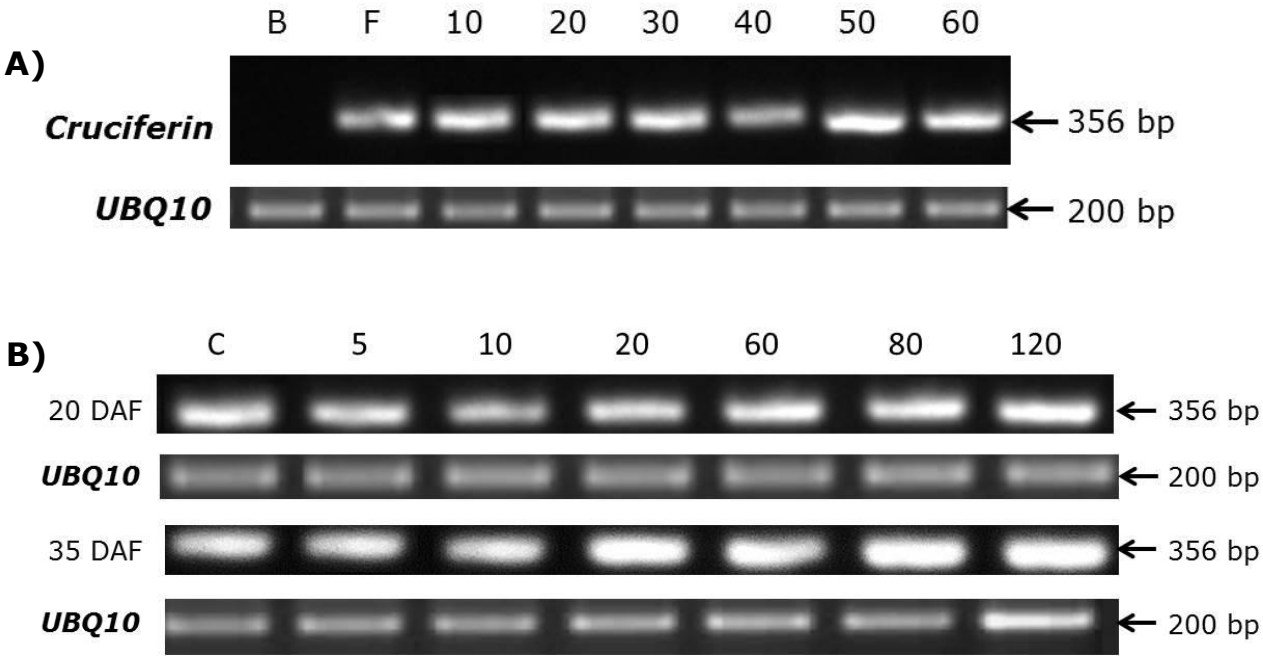


Figure 4.13. RT-PCR analysis of cruciferin gene expression. (**A**) Throughout development in unwounded buds (B), flowers (F), and seeds 10 – 60 DAF (**B**) In 20 and 35 DAF seeds post wounding; control unwounded (C), 5 – 120 mins. *UBQ10* as housekeeping control.

4.6 Protein analysis of putative *ARR22* orthologues in *B. napus*

An antibody designed (see **Fig. 2.2; Chapter 2**) on a fifteen amino acid sequence present in both *ARR22* and putative orthologues in *B. napus* was used to elucidate the protein expression of *BnRR76* – *BnRR79* in *B. napus* seeds.

4.6.1 Analysis of BnRR76 – BnRR79 protein expression

Dot blot analysis was chosen to study the expression of *BnRR76 – BnRR79* protein expression in seed stages 5 to 35 DAF (**Fig. 4.14**). High expression was observed in seeds 5 – 20 DAF. Expression was lower in 35 DAF seed.

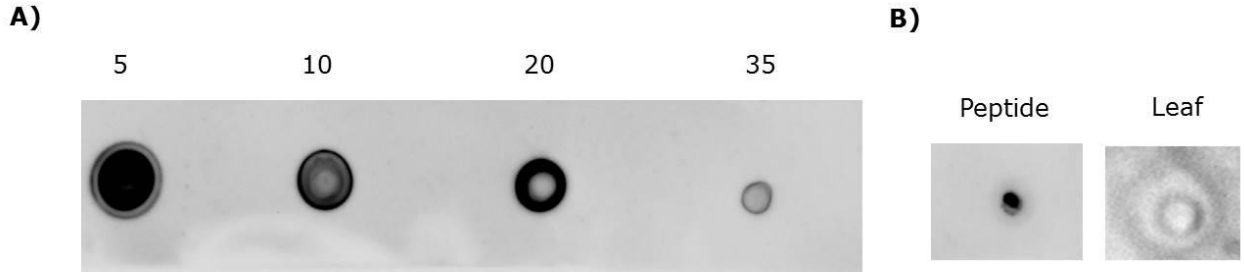


Figure 4.14. (**A**) Dot blot analysis of protein expression in 5 – 35 DAF *B. napus* seeds. (**B**) Peptide positive control and leaf negative control.

4.6.2 Protein expression post-wounding

As dot blot analysis showed high protein expression at 20 DAF, Western blot analysis was focussed on this seed stage. Seeds 20 DAF were wounded and protein expression analysed at 60 and 120 mins. These times were chosen around the 90 mins time point as analysed previously (Naomab, 2008) since no data were available to indicate temporal differences between transcript expression and translation. The expression of the 15 KDa protein appeared up-regulated in seeds 60 mins in post-wounding but then appeared decreased at 120 mins (**Fig. 4.15 A**).

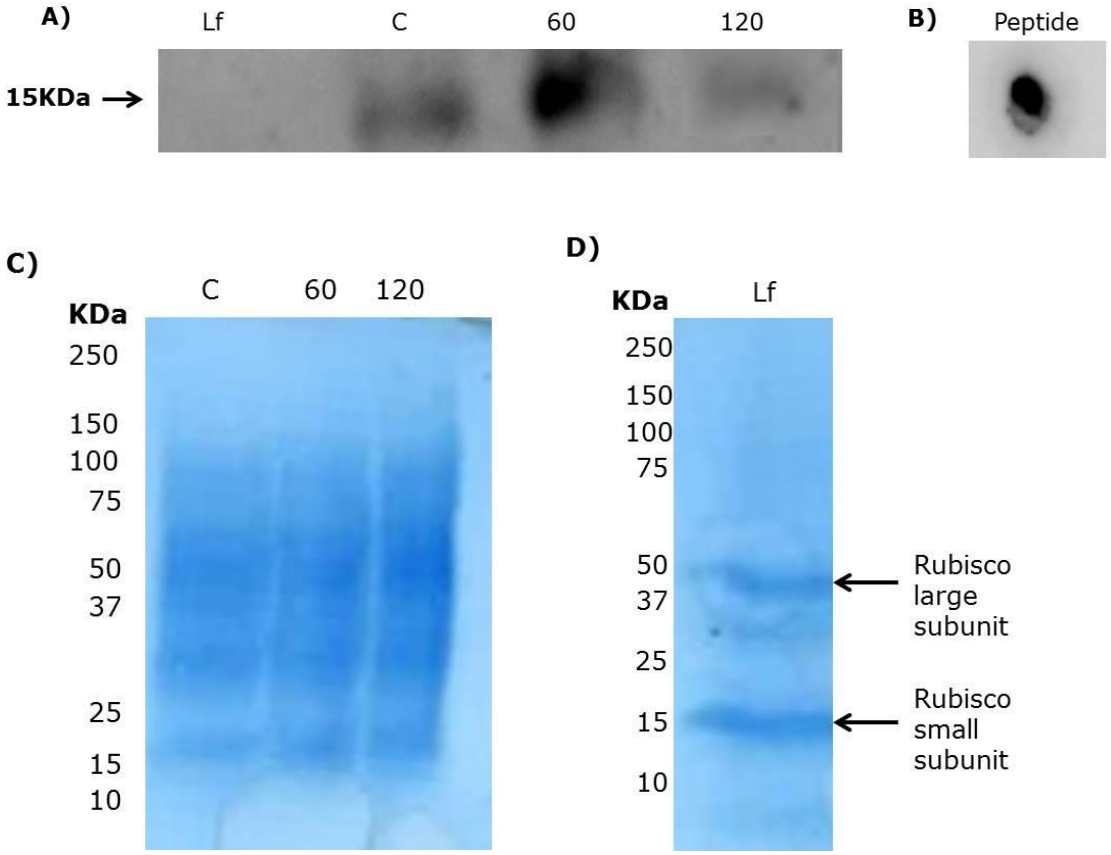


Figure 4.15. Protein analysis of *putative ARR22 orthologues in B. napus* seeds post-wounding. (**A**) Western blot analysis of protein expression in *B. napus* leaf (L) and 20 DAF seeds; control unwounded (C) and post-wounding at 60 and 120 mins; 100 µg loaded. (**B**) Peptide control on dot blot. (**C**) Coomassie stain of 20 DAF samples to check loading. (**D**) Coomassie stain of leaf sample to check loading. Rubisco subunits labelled.

4.7 Discussion

Seed development and maturation in *B. napus* are key processes for the plant in which a range of lipids and proteins accumulate. Seed filling is a complex biological process with several integrated biosynthetic pathways and regulatory mechanisms which involve an assortment of genes and hormones (Niu et al., 2009; Song et al., 2015). *ARR22* has been hypothesised to be involved the partitioning of seed resources

(Gattolin et al., 2006). Studying the expression of genes and proteins that are potentially involved in the regulation of assimilate partitioning have fundamental applications, particularly for breeding to increase crop yields.

While the impact and responses of plants to environmental stresses and pathogen attack have been extensively studied (Reymond and Farmer 2008; Reymond et al., 2000; Savatin et al., 2014), little is known on how plants respond to mechanical wounding of the seed. Previous work in *Arabidopsis* has shown that puncturing the seed leads to an up-regulation in protease genes and a down-regulation in SSPs (Naomab, 2008).

This part of the study therefore aimed to probe the role of the putative *ARR22* orthologues and type-A and type-B BnRRs that are potentially involved in seed development and assimilate partitioning in the economically important crop *B. napus*. The impact of mechanical wounding during seed maturation was additionally analysed.

4.7.1 Expression profiles of type-A and type-B BnRRs during seed development

To date there have been no studies examining the gene expression of type-A or type-B RRs in *Brassica* species. For this study, putative *B. napus* type-A *ARR16* orthologues (*BnRR34* and *BnRR35)* and *ARR17*

(*BnRR36* - *BnRR39*) along with type-B *ARR12* (*BnRR57* and *BnRR58*) and *ARR21* (*BnRR71* - *BnRR74*) were analysed. In *Arabidopsis* previous work has shown that *ARR16* and *ARR17* genes are primarily implicated in the regulation of root development (Kiba et al., 2002; Ren et al., 2009). Meanwhile it has been demonstrated that *ARR12* has a role in cytokinin response in roots and is involved in mediating the effects of drought (Nguyen et al., 2016; Yokoyama et al., 2007) and *ARR21* has been identified to be predominantly expressed in reproductive organs and siliques (Horak et al., 2003; Tajima et al., 2004).

Interestingly, the results of the present study show that the putative *B. napus* type-A and type-B orthologues analysed are all expressed in seeds while putative orthologues of type-A *ARR16*, *ARR17* and type-B *ARR12* are additionally expressed in buds and flowers. With the exception of *ARR21*, these observations have not been identified in *Arabidopsis*. It could be speculated that expression of these allows for a strengthening in the regulation of cytokinin networks, particularly within the remobilization of resources from petals which are much larger in *B. napus*.

The expression of all of these genes was detected in seeds during the early stages of seed development with high expression particularly observed at 10 DAF. This stage is believed to be a key period in seed pattern formation and cell differentiation (Dong et al., 2003) and hence

suggests a novel function for these *BnRR*s however further in depth characterisation of these is needed to verify this. *ARR21* has previously been shown to be expressed in *Arabidopsis* floral organs (Tajima et al., 2004) yet RT-PCR analysis in *B. napus* showed the absence of the expression of putative *ARR21* orthologues *BnRR71* - *BnRR74* in *B. napus* flowers. As *ARR21* appears only to be expressed at the junction of the pedicel and in sepals/ carpels in *Arabidopsis*, this may not present enough tissue for a transcript to be detected in *B. napus* flowers and hence floral organs would need to be dissected out for further investigation. An alternative explanation may be in that the location and timing of *BnRR71* - *BnRR74* expression has become restricted to early stage siliques and has taken on a more silique specific role.

Among the expression profiles of the *BnRR*s studied, the putative orthologues of *ARR17* (*BnRR36* - *BnRR39*) exhibited alternative splicing. Although the fully processed transcript was predominantly expressed, unprocessed transcripts containing introns were expressed in buds, flowers and in seeds. The primers designed to amplify *BnRR36* – *BnRR39* spanned three introns. As these genes contain an additional intron upstream within the ORF it would be interesting to analyse whether a transcript containing this is additionally expressed. The results of this study are consistent with those of a microarray meta-analysis in *Arabidopsis* which identified alternative splicing in genes involved in cytokinin signalling and metabolism, including *ARR17*

(Bhargava et al., 2013). Intriguingly *ARR16* was also seen to exhibit splicing (Bhargava et al., 2013) however in this study the putative B. napus orthologues *BnRR34 and BnRR35* did not exhibit this as shown by RT-PCR analysis.

4.7.2 Distinguishing and dissecting the gene and protein expression patterns of BnRR76 – BnRR79

Previous analysis of *SAC29*, one of the putative orthologues of *ARR22* in *B. napus,* focussed on expression between 20 and 60 DAF (Whitelaw et al., 1999). RT-PCR analysis confirmed that expression of putative *ARR22* orthologues in *B. napus* is highest in seeds but expression can also be observed in buds and flowers.

Amongst the transcripts expressed it appeared that there were different expression patterns of genes originating from *B. rapa* and *B. oleracea*. A similar observation was described by Chen et al. (2010) in which three *n*-Glycerol-3-Phosphate Acyltransferase 4 (*GPAT4*) genes of high sequence similarity, two of which were from *B. rapa* and one from *B. oleracea*, exhibited distinct spatial and temporal gene expression patterns as well as varying levels of polypeptide accumulation. Likewise, in wheat three *wheat LEAFY HULL STERILE1* (*WLHS1*) homeologs present on the A, B and D genomes appear to have different effects on flower development via varying expression levels through altered genetic, as well as epigenetic, regulation (Shitsukawa et al., 2007).

Thus the inheritance of distinct expression patterns from ancestral genomes appears to be a common occurrence in species that have undergone polyploidization. Interestingly expression of *BnRR76* – *BnRR79* at the protein level also revealed differences when compared to the level of gene expression. RT-PCR analysis had revealed high gene expression at 35 DAF in Fig. 4.3 D but the expression of protein appeared lower at this same stage. It hence may be that *BnRR76* and *BnRR78* do not in fact encode a protein despite being expressed at the transcript level. However transcript levels do not always represent protein expression levels. For example Hajduch et al. (2010) found a large number of conflicting transcript and protein expression levels during *Arabidopsis* seed filling. Similarly, the transfer of *Arabidopsis* plants from low or normal light to high light triggered changes in transcript levels and abundance within 6 h which did not match the rate in protein synthesis (Oelze et al., 2014).

It has previously been demonstrated that *ARR22* produces four transcriptional variants in *Arabidopsis* by retention and splicing of introns located within the 5' UTR and ORF (Gattolin et al., 2006). While RT-PCR profiling in *B. napus* did not produce completely comparable results, it is evident that the four putative orthologues *BnRR76* – *BnRR79* are indeed processed in different ways and intron retention does occur. This occurrence was notably observed for *BnRR77* and *BnRR79* with the expression of transcripts containing an intron within

the ORF or fully processed transcripts detected differentially throughout development. Strikingly the expression of fully processed *BnRR77* and *BnRR79* transcripts as well as transcripts containing the intron located in the ORF occurred only at 10 and 15 DAF respectively when the forward primer was positioned within the 5' UTR. Gene regulation, including the splicing of introns to generate mRNA occurs at the post-transcriptional level (Proudfoot et al., 2002). It is estimated that intron retention occurs in up to 30% of *Arabidopsis* genes and variant transcripts appear to be developmentally specific (Ner-Gaon et al., 2004; Stamn et al., 2005) which has certainly been established here in *B. napus*. It is largely unknown what the precise significance of intron retention is, but transcript stability and modification of biological function are possible reasons and potentially allowing for a more rapid response to an external stimulus such as wounding. Interestingly the appearance of two splice variants in *BnRR77* and *BnRR79* occurred predominantly throughout the seed maturation phase. This phenomenon may hence confer a regulatory role in seed filling particularly as alternative splicing of several metabolic and developmental genes in developing soybean seeds has been demonstrated by Aghamirzaie et al. (2013). Whether this occurs in *BnRR76* and *BnRR78* is yet to be clarified but nucleic acid differences are seen in their putative *B. rapa* and *B. oleracea* orthologues.

4.7.3 Effect of wounding on BnRR76 – BnRR79 gene and protein levels

Only the one study by Gattolin et al. (2006) has investigated the effect of mechanical wounding specifically on seed development. It is however well established that plant stress, induced by such cues as water deficit, salinity, temperature and mechanical wounding, can cause huge crop yield losses (Vinocour and Altman 2005; Vij and Tyagi 2007). Previously it was demonstrated that, while the gene expression of *ARR22* did not change in response to wounding, the splicing profile was altered with the frequency of transcripts containing introns increased (Naomab, 2008). The gene expression of *BnRR76 – BnRR79* post-wounding differed to the observation in *ARR22* in that there in fact appeared to be an up-regulation at 80 mins post-wounding. Moreover RT-PCR analysis showed that it was the fully processed transcript that was up-regulated in 35 DAF seed. This results is inconsistent not only with that of *ARR22* but with the large and growing body of evidence that shows alternative splicing playing a prominent feature in plant responses to stress (Reddy 2007; Staiger and Brown 2013; Thatcher et al., 2016). This mechanism allows the plant to rapidly alter gene expression and it is believed that expression changes in splicing proteins determine this (Staiger and Brown 2013). An explanation for the differing observation between *ARR22* and *BnRR76 – BnRR79* could be that polyploidy has generated a loss or reshaping in alternative splicing patterns which has been

demonstrated in *B. napus* by Zhou et al. (2011) as part of the so-called "transcriptomic shock".

It had been hypothesised that wounding could in fact promote expression of *ARR22* and its putative *B. napus* orthologues at the protein level without increasing the encoding transcript. This suggestion is supported by studies that have found mRNA levels do not always correlate with protein abundance (Gygi et al., 1999). For example in a proteomic study of leaf responses to wounding, a number of proteins were up and down regulated while the same pattern was not seen at the transcript level (Gfeller et al., 2011). RT-PCR analysis had indicated that wounding may be promoting the up-regulation of *BnRR76 – BnRR79* at the gene expression level. Western blot analysis indicated that in 20 DAF *B. napus* seed the expression of *BnRR76 – BnRR79* protein was up-regulated at 60 mins post-wounding while gene expression remained at a baseline level. In contrast at 120 mins the level of protein present appears to decrease while gene expression remains up-regulated. This suggests that *BnRR76 – BnRR79* protein is more rapidly produced in response to wounding than an alteration in gene expression. This strategy presumably allows the plant to quickly adapt to the stress response. Whether this rapid induction of protein expression implies that *BnRR76 – BnRR79* produces a mobile a signal or is made to interact with other proteins is unclear. Moreover the quick down-regulation of protein abundance possibly suggests the existence of a post-

translational process involving a feed-back loop between the levels of transcript and protein and protein degradation.

4.7.4 Effect of wounding on seed filling

Seed storage proteins accumulate during seed maturation and provide a nutrient resource for germinating embryos. Cruciferin (12S) and napin (2S) are two major SSPs in *B. napus* that constitute 60% and 20-30% of the total mature seed protein respectively (Lonnerdahl and Jansson 1972; Crouch and Sussex 1981; Ericson et al., 1986). Napins are a multigene family comprised of approximately 16 genes (Josefsson et al., 1987; Scofield and Crouch 1987). Gene expression of napin A was not only observed at all seed stages up to maturation but also in buds and flowers while napin mRNA has previously only been detected in seeds from around 20 DAF to 40 DAF, peaking at 30 DAF (DeLisle and Crouch 1989; Finkelstein et al., 1985), although expression of an embryo specific napin has been reported in buds and flowers (Namasivayam et al., 2008). Similarly, the expression of cruciferin was observed in flowers but this was likely to have been detected in the pollen as it has been speculated that cruciferin plays an additional role in pollen tube growth (Sheoran et al., 2009). SSPs were shown to be highly down-regulated 90 mins post-wounding in *Arabidopsis* (Naomab, 2008). In the present study the expression of SSPs chosen to study did not change in response to wounding even after 120 mins. A previous study has shown that mechanical wounding of *Arabidopsis* leaves induces the

expression of a number of genes which peaks at 90 to 120 mins (Reymond et al., 2000). Whether 120 mins provides a sufficient time point to observe a change in gene expression in *B. napus* is debatable as *Brassica* presents a larger system than *Arabidopsis.* Additionally, wounding of *Arabidopsis* seeds was undertaken on siliques that had been excised from the plant (Gattolin et al., 2006; Naomab, 2008) hence potentially providing a wound signal in itself. Studies have shown that wound induced responses can indeed be elicited in undamaged tissues located away from the site of wounding (Schilmiller and Howe 2005).

Cruciferin, in contrast to the expression of orthologous genes in *Arabidopsis*, appeared to be up-regulated in 35 DAF seed 80 mins post-wounding. While it is reasonable to suggest that qPCR is needed to quantify this, jasmonate and abscisic acid, hormones involved in plant wounding and stress responses, have been shown to induce cruciferin expression in seeds (Wilen et al., 1991). A number of genes implicated in protein degradation had previously been studied in *Arabidopsis* and were shown to be induced by wounding (Naomab, 2008). The expression of the gene encoding cysteine protease studied in *B. napus* was not wound induced, however it appeared that this enzyme was not seed specific. A number of seed storage processing enzymes exist in *Arabidopsis* such as vacuolar processing enzymes (VPEs) and aspartic proteases (Mutlu et al., 1999; Gruis et al., 2002) and some orthologues

have been identified in *Brassica* seeds (Wan et al., 2002; Obermeier et al., 2009). These therefore provide additional candidate genes to analyse. It has been predicted that ubiquitination plays a role in the wound induced degradation of SSPs in *Arabidopsis* (Naomab, 2008). Progress has yet to be made in the elucidation of ubiquitin activating ligases related to plant defense in *Brassica* species and hence it cannot be speculated whether this mechanism is implicated.

4.8 Conclusions

Overall this study has yielded results in *B. napus* that are inconsistent with the present knowledge on the role and general expression of *ARR22*. The outcome of the expression analyses suggests that the wound response in *B. napus* seeds may differ to that observed in *Arabidopsis*. The lack of change in SSP and protease expression suggests that these genes are either not affected by wounding of *B. napus* seeds or changes are induced post 120 mins. Furthermore, although there was strong expression of the putative *ARR22* orthologues *BnRR76 – BnRR79* during the seed maturation phase, their roles in seed development and metabolism are yet to be fully verified. It hence cannot be confirmed that these genes play a role in assimilate partitioning. The next phase of this study will therefore aid in determining whether *ARR22* has additional functions.

Despite this, analyses of type-A and type-B *BnRR*s have revealed for the first time a potential role for RRs in early seed development. Further analysis of hormone regulation, signalling and their gene interactions are required to support and investigate this.

Furthermore this study reports the differential regulation of transcripts present on two different genomes. While this has been described for other polyploids such as wheat (Shitsukawa et al., 2007), how abundant this phenomenon is for other genes in *B. napus* has not been established. It is likely that both genetic and epigenetic regulation governs their expression but understanding this control is crucial for future manipulation of such genes to avoid compromising plant fitness. This analysis has additionally revealed a further level of gene regulation complexity as seen by alterations in alternative splicing patterns between *Brassica* and *Arabidopsis*. There is growing evidence that demonstrates alternative splicing as an important influence on a variety of plant developmental and signalling mechanisms and it has been shown here to be a feature of *B. napus* seed development. It is believed that alternative splicing plays an important feature in the management of gene expression at the transcript level while increasing protein diversity (Reddy et al., 2013). Characterising how alternative splicing is regulated developmentally as well as in response to stress is a key avenue for crop improvement.

Finally this study also provides preliminary evidence of changes occurring to *BnRR76 – BnRR79* at the protein level in response to wounding which were not previously studied in *ARR22*. Identifying how wounding promotes rapid up-regulation as well as further quantification of level and timing will aid in elucidating the role of *ARR22* putative orthologues in *B. napus*.

Chapter 5:

Effects of dexamethasone (DEX) induced overexpression of *ARR22* in *Arabidopsis*

5.1 Introduction

The function of *ARR22* has previously been examined in *Arabidopsis* by both mutant analysis and overexpression under a 35S promoter (Gattolin et al., 2008; Horak et al., 2008). Insertion of T-DNA into the intron located within the ORF of *ARR22,* which produced two mutant alleles, *arr22-2* and *arr22-3,* resulted in the absence of a transcript in siliques but no differences in silique or seed development, morphology or metabolic phenotype could be detected in mutant lines when compared with the wild type (Horak et al., 2008). Similarly, a T-DNA insertion 75 bp downstream of the ATG site within the coding region did not reveal phenotypic effects on vegetative and reproductive growth or silique and seed development (Gattolin et al., 2008). However, when *ARR22* was ectopically expressed, an extreme dwarf phenotype with reduced flower number was observed (Gattolin et al., 2008).

More recently, Kang et al. (2013) have shown that overexpression of *ARR22* using a dexamethasone (DEX) inducible system results in enhanced drought, dehydration and cold tolerance in 10 – 12 d plants. The DEX pOp/*LhGR transcription activation system, placed under the control of a CaMV 35S promoter, was joined to an ARR22:GUS construct which was under the control of six copies of the lac operator (Kang et al., 2013).* It was also hypothesised that an Asp residue at amino acid 74 acts as putative phospho-accepting site. Therefore transgenic lines harbouring an Asp to Asn mutation ($ARR22^{D74N}$) were additionally

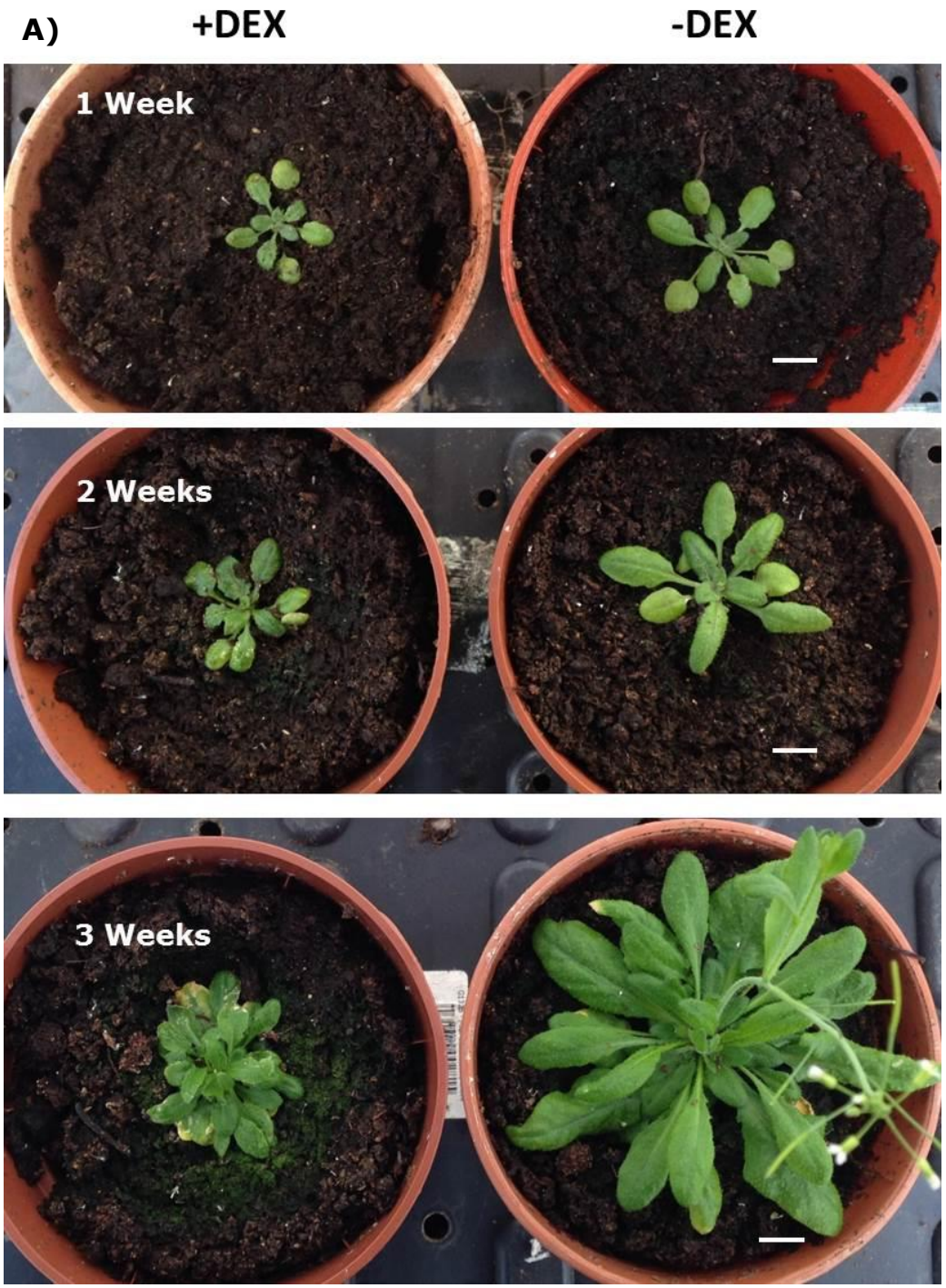
created. While RT-PCR, GUS and immunoblot analyses showed that *ARR22* transcript and protein levels were upregulated in response to DEX treatment in the *ARR22*^{D74N} lines, drought, dehydration and cold tolerance levels were comparable to the wild type.

Morphological effects of DEX induced *ARR22* overexpression have not previously been examined and hence for the present study transgenic *Pro*$_{35S}$*:ARR22:HA lines 11-7 and 15-5 and Pro*$_{35S}$*:ARR22*^{D74N}*:HA lines 17-3 and 20-3 were obtained for this purpose. Modification of amino acid 74 was confirmed by sequencing. The key objective of this study was therefore to observe the effect of DEX induced overexpression of ARR22 on physiological measurements while examining gene and protein expression.*

5.2 Effect of DEX induced *ARR22* expression on leaf and rosette development

To analyse whether overexpressing *ARR22* in a DEX-inducible manner had an effect on the phenotype and development of rosettes and leaves, plants were sprayed every day from 7 d post-germination for 3 weeks with (+) DEX or (-) DEX control. The phenotype of transgenic and ColWT plant rosettes is shown in **Fig. 5.1**. The rosette areas of transgenic plants sprayed with (+) DEX was seriously compromised in comparison with their (-) DEX controls. DEX treated *Pro*$_{35S}$*:ARR22:HA*

lines 11-7 and 15-5 exhibited a more bushy phenotype. ColWT plants sprayed with DEX or (-) DEX did not exhibit any phenotypic effects.



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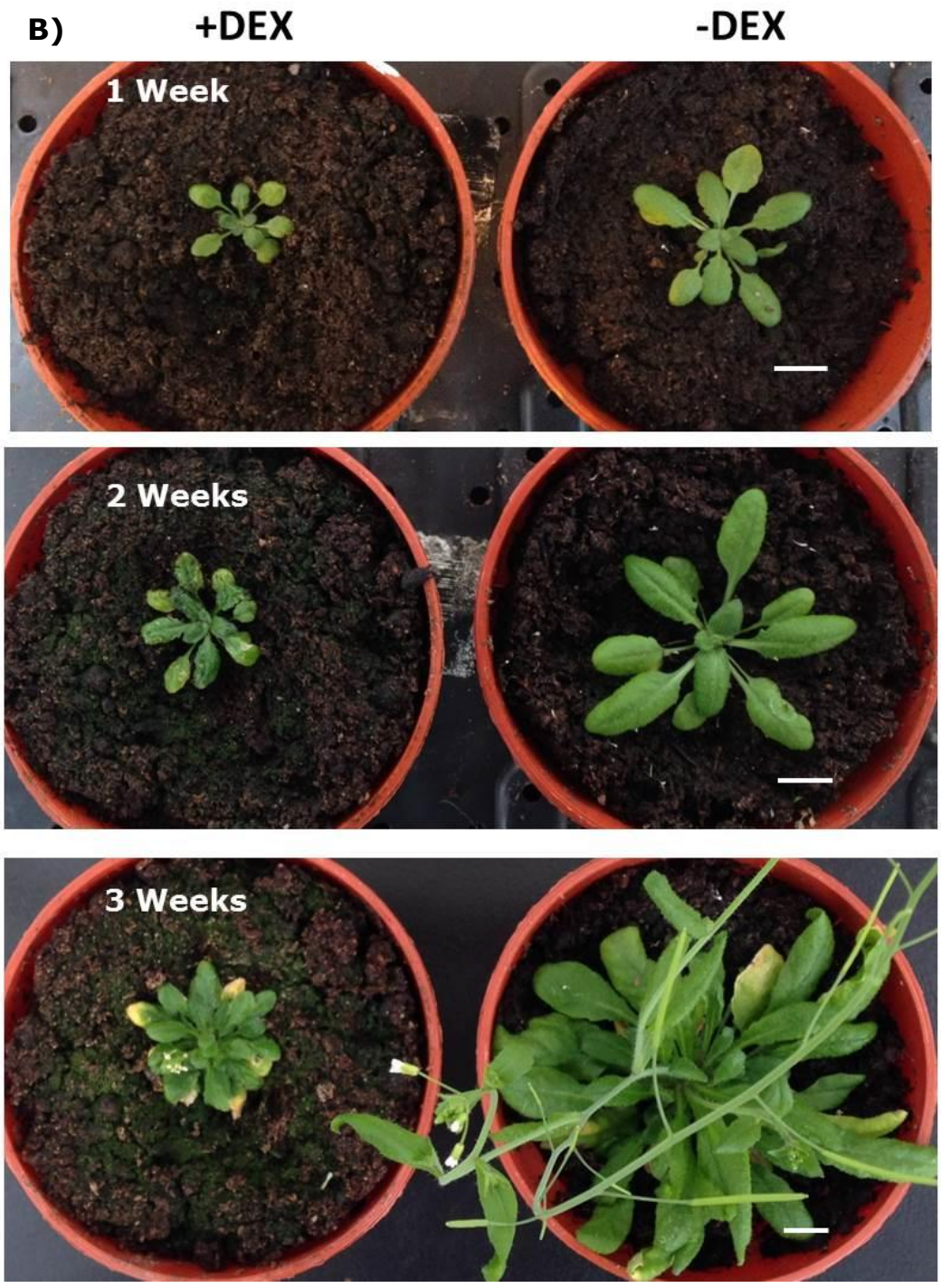

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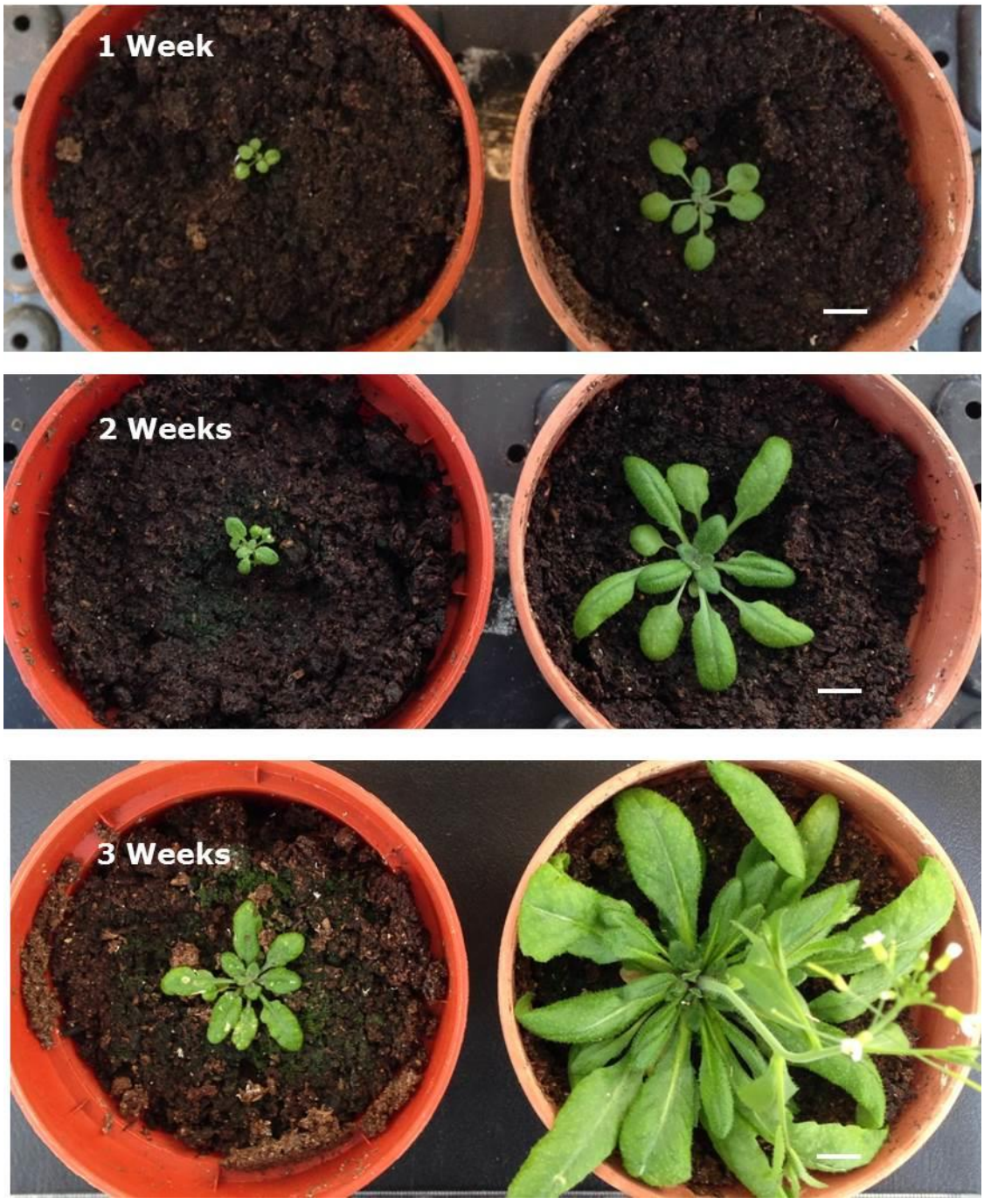
2191

C) +DEX -DEX



D)

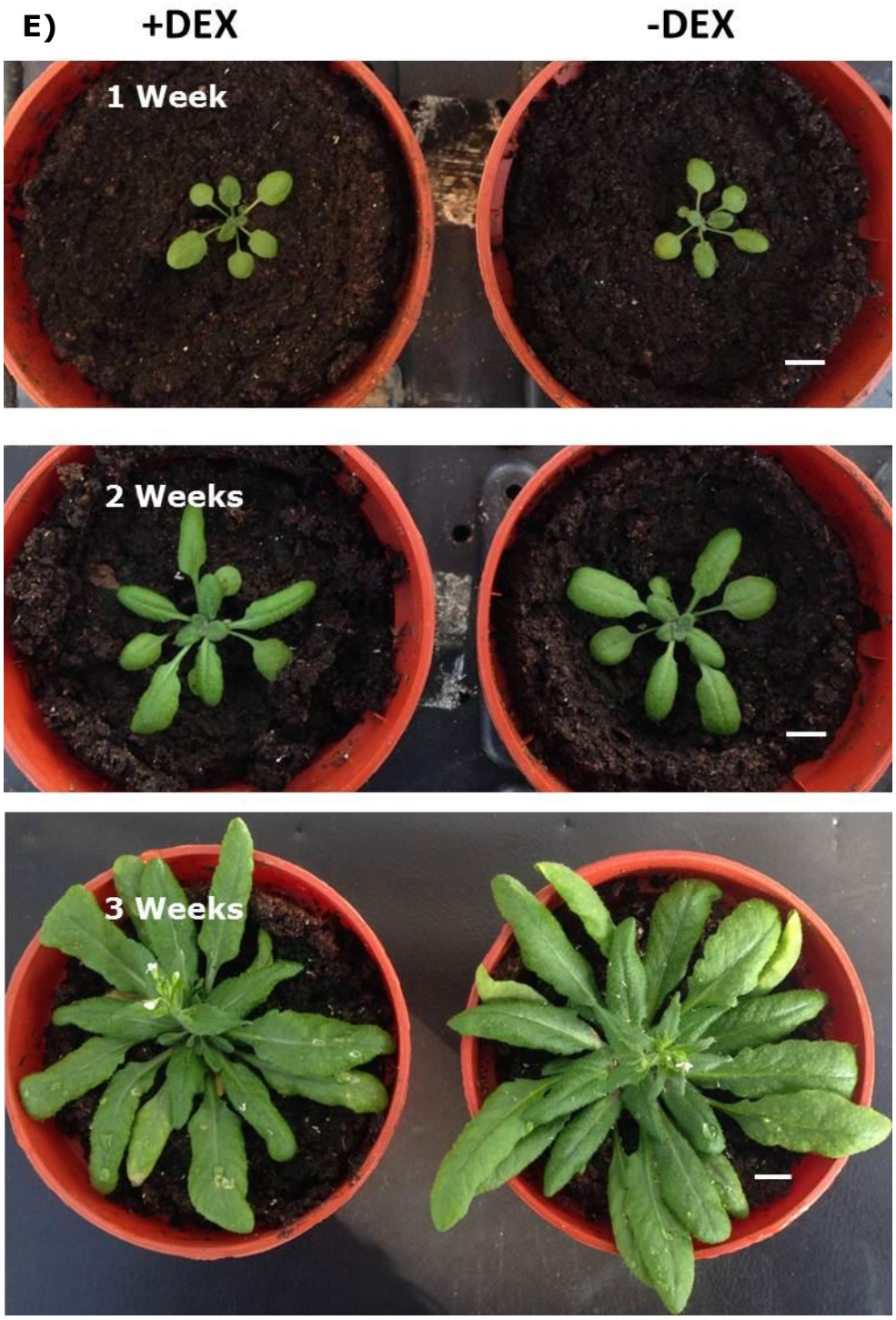


Figure 5.1. Effect of (+) DEX or (-) DEX control treatments for 3 weeks on rosette phenotype in (**A**) *Pro$_{35S}$:ARR22* line 11-7 (**B**) *Pro$_{35S}$:ARR22 line 15-5 (**C**) Pro$_{35S}$:ARR22^{D74N} line 17-3 (**D**) Pro$_{35S}$:ARR22^{D74N} line 20-3 and (**E**) ColWT plants. Bar = 1 cm.*

Rosette area differences were quantified by measuring the final rosette area after 3 weeks of spraying (**Fig. 5.2**). The rosette areas of transgenic lines treated with (+) DEX were significantly (p<0.01) smaller than the (-) DEX controls. No significant difference was observed between ColWT plants treated with (-) DEX control and (+) DEX. The rosette area of the (-) DEX control treated line 17-3 was significantly (p<0.05) larger than (-) DEX control ColWT. Conversely rosette area of the (-) DEX control treated line 11-7 was significantly (p<0.05) smaller than (-) DEX control ColWT.

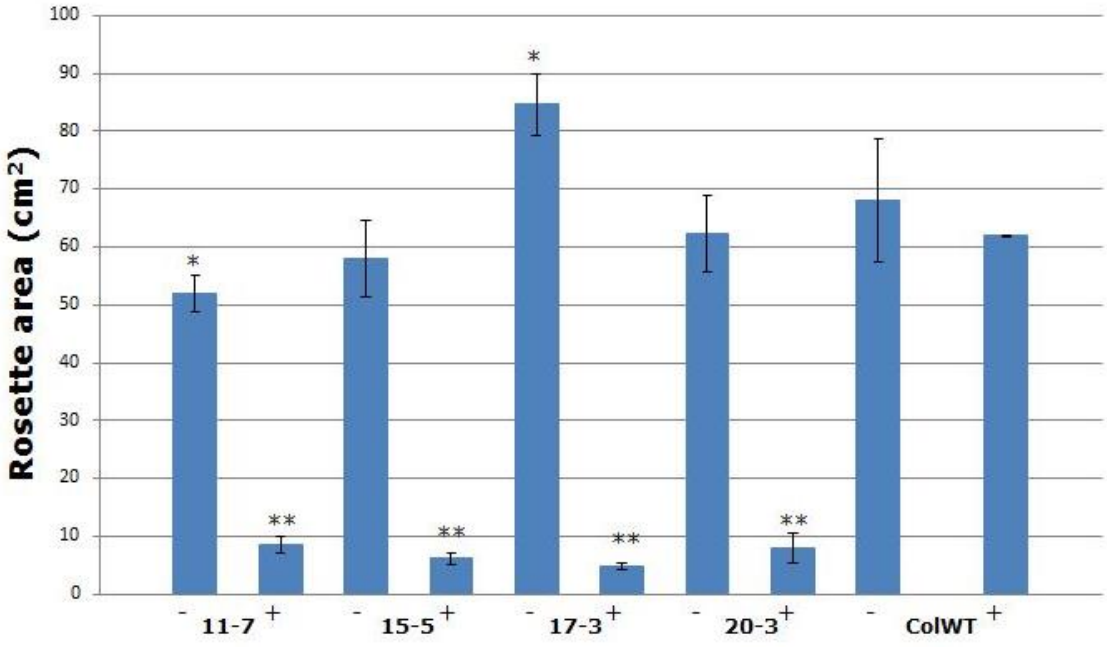
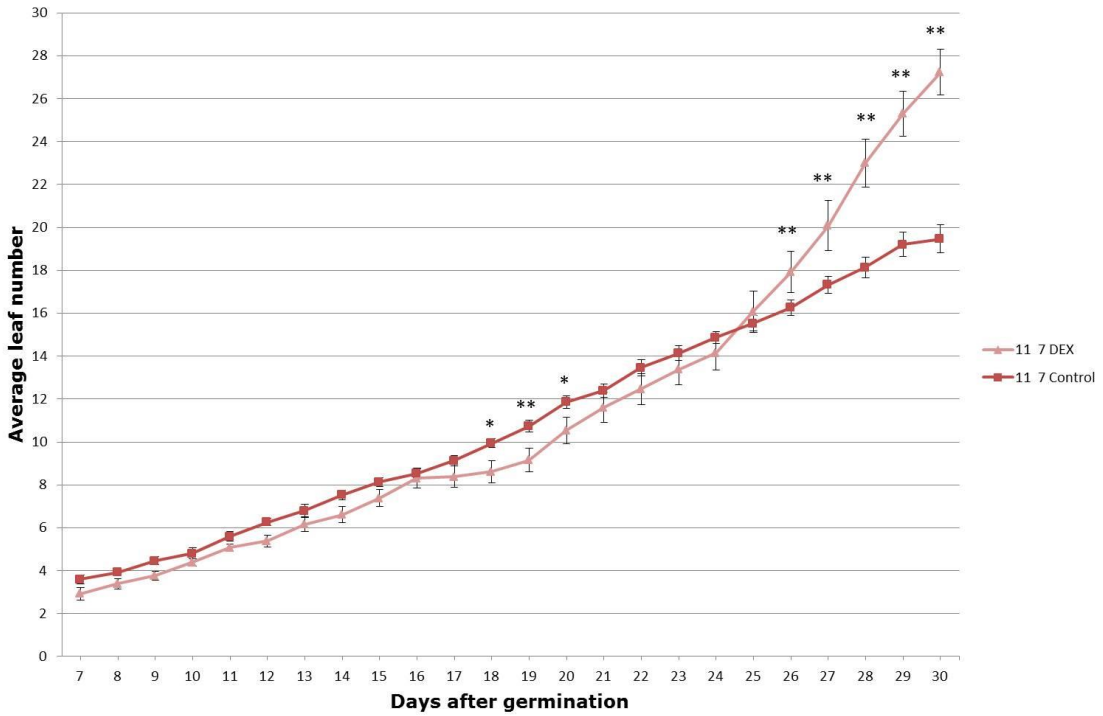


Figure 5.2. Effect of (+) DEX or DEX control (-) treatment on rosette area after 3 weeks of spraying. Statistically significant changes compared within lines and indicated with * when p<0.05 and ** when p<0.01. Error bars represent standard error of the mean; n=3.

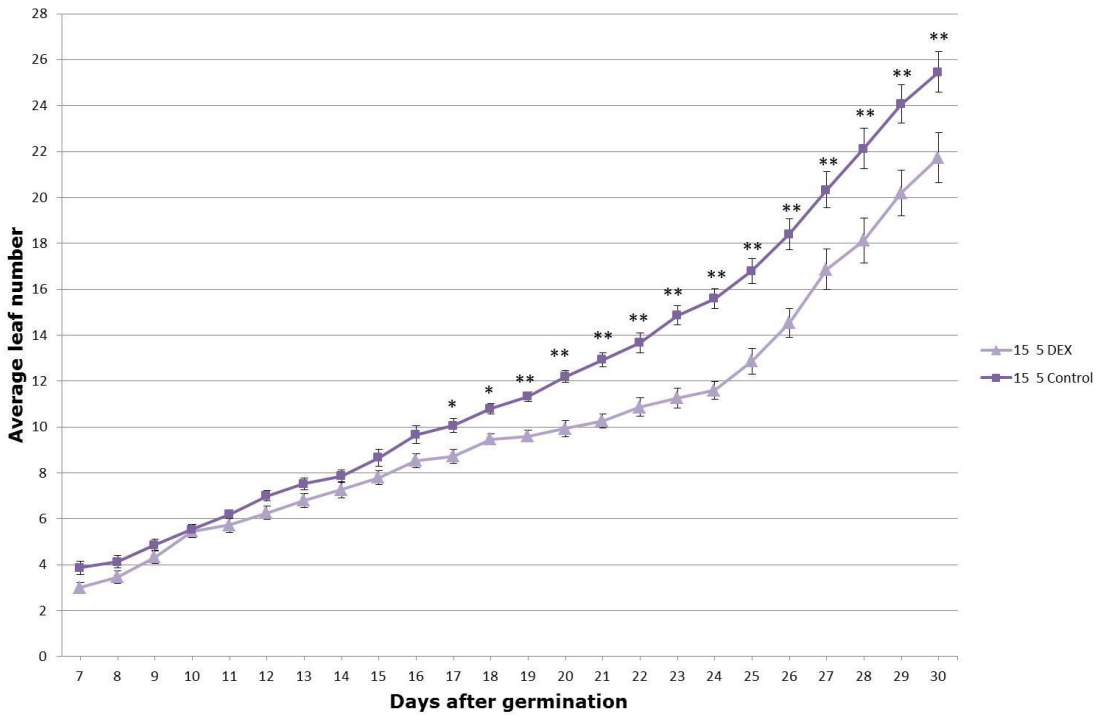
Leaf number was analysed by counting the number of visible leaves on (+) DEX treated and (-) DEX control plants every day for 3 weeks (**Fig.**

5.3). *Pro_{35S}:$ARR22^{D74N}$ lines 17-3 and 20-3 treated with (+) DEX were significantly ($p<0.01$) different in leaf number from 9 d of spraying when compared with the (-) DEX control treated plants (**Fig. 5.3 C** and **D**).* DEX treatment led to a reduction in the number of visible leaves. *Significant differences between (+) DEX treated Pro_{35S}:ARR22* lines 11-7 and 15-5 and the (-) DEX controls were observed later after 17 d of spraying (**Fig. 5.3 A** and **B**). Effects of DEX treatment on leaf number in line 11-7 were only, however, predominantly observed later from 26 d of treatment and appeared to increase leaf number. DEX treatment appeared to have a significant effect on ColWT by reducing leaf number after 27 d of spraying. Differences in leaf number were also observed between the (-) DEX control transgenic lines and ColWT (**Fig. 5.3 E**). In particular *Pro_{35S}:ARR22 line 15-5 had a higher leaf number over the course of the treatment. In contrast, Pro_{35S}:ARR22 line 11-7 and Pro_{35S}:$ARR22^{D74N}$ line 20-3 exhibited fewer leaves from 28 d.*

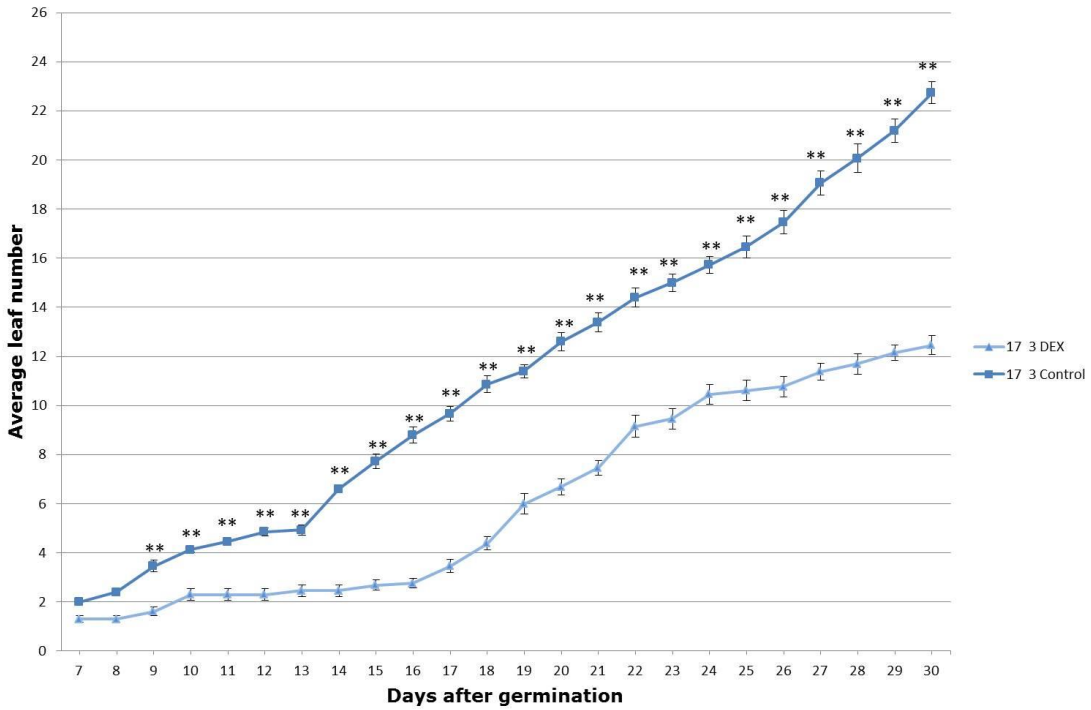
A)


Average leaf number
Days after germination
11 7 DEX
11 7 Control

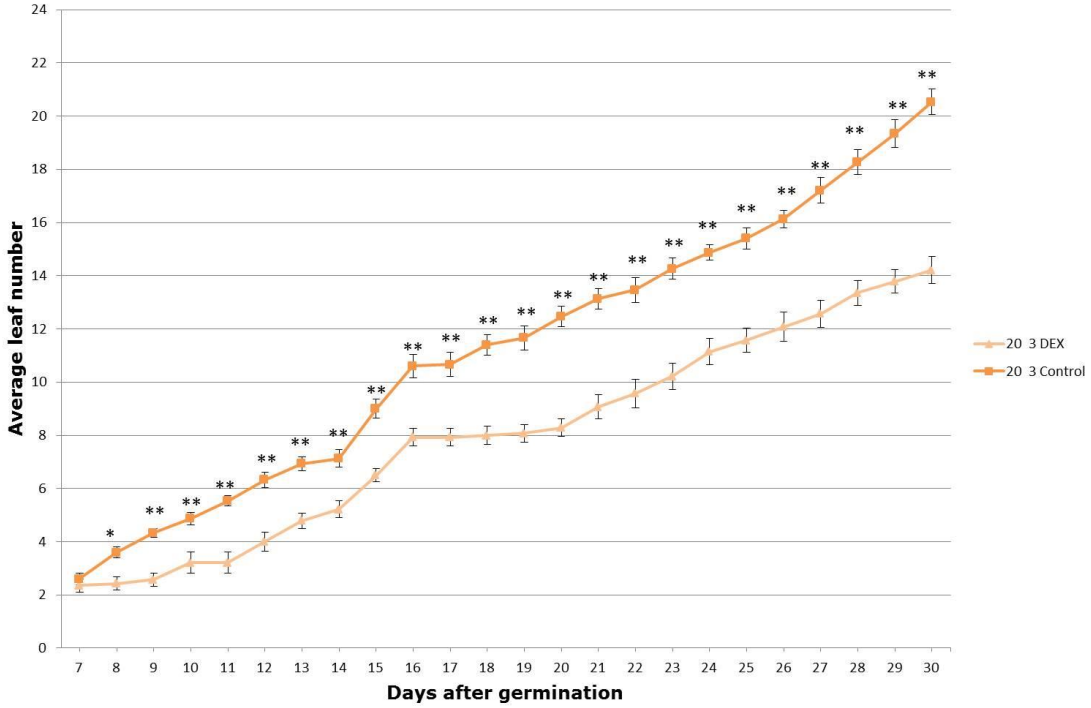
B)


Average leaf number
Days after germination
15 5 DEX
15 5 Control

C)


Average leaf number
Days after germination
17 3 DEX
17 3 Control

D)


Average leaf number
Days after germination
20 3 DEX
20 3 Control

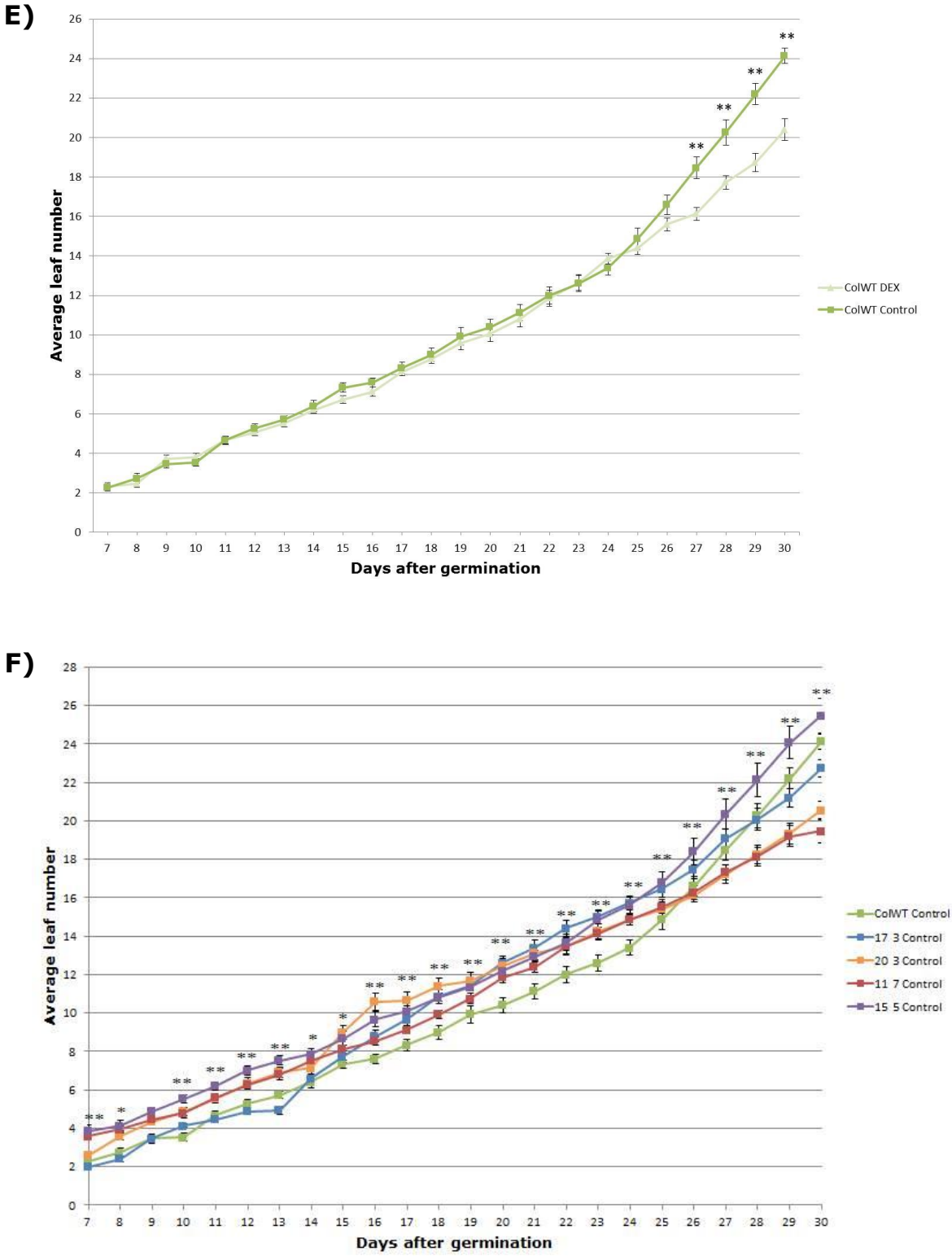


Figure 5.3. Effect of (+) DEX or (-) DEX control treatment on leaf number for 3 weeks. (**A**) *Pro$_{35S}$:ARR22* line 11-7 (**B**) *Pro$_{35S}$:ARR22 line 15-5 (**C**) Pro$_{35S}$:ARR22^{D74N} line 17-3 (**D**) Pro$_{35S}$:ARR22^{D74N} line 20-3 and (**E**) ColWT plants (**F**) Comparison of all (-) DEX controls.* Statistically significant changes compared within lines and between controls indicated with * when $p<0.05$ and ** when $p<0.01$. Bar indicates standard error of the mean; n=15.

After 3 weeks of treatment rosettes were dissected out to observe the phenotype of individual leaves (**Fig. 5.4**). The transgenic lines treated with (+) DEX had visibly smaller leaves compared to the (-) DEX controls and ColWT. The appearance of serrated leaves occurred in *Pro$_{35S}$:ARR22^{D74N} line* 20-3. In all transgenic lines treated with (+) DEX there was evidence of necrosis. No phenotypic effects were observed in the ColWT (+) DEX treated plants.

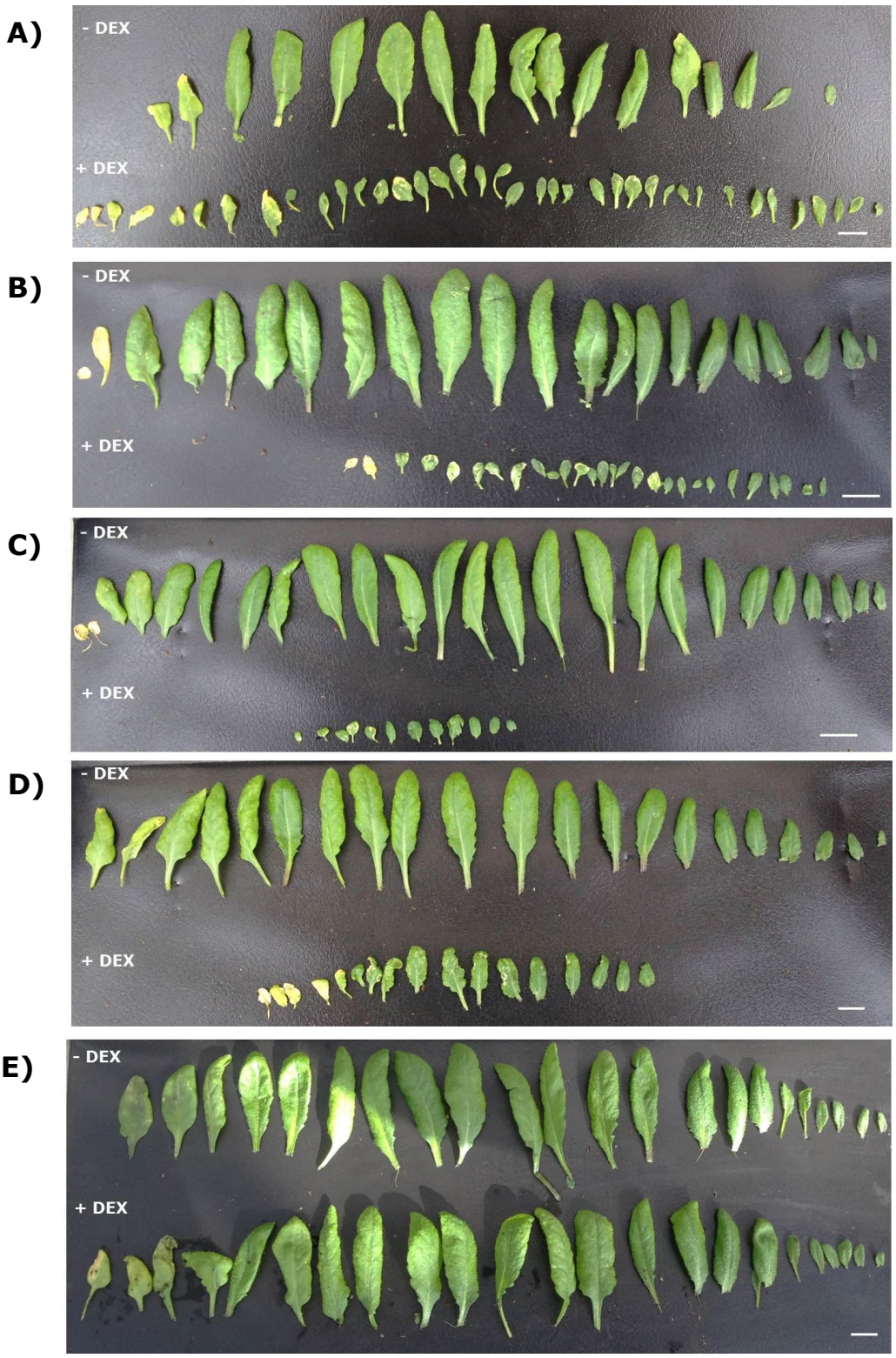


Figure 5.4. Leaf morphology of (**A**) *Pro$_{35S}$:ARR22* line 11-7 (**B**) *Pro$_{35S}$:ARR22 line 15-5 (**C**) Pro$_{35S}$:ARR22^{D74N} line 17-3 (**D**) Pro$_{35S}$:ARR22^{D74N} line 20-3 and (**E**) ColWT plants treated with (+) DEX or (-) DEX control. Bar = 1 cm.*

5.2.1 GUS analysis of ARR22 expression in response to DEX treatment

As the DEX-inducible system contains a *GUS* reporter gene histochemical analysis was performed on whole rosettes to visually observe the DEX induced reporter gene expression. More intense staining was observed in (+) DEX treated *Pro$_{35S}$:ARR22* lines 11-7 and 15-5 when compared with *Pro$_{35S}$:ARR22^{D74N} lines 17-3 and 20-3 (**Fig. 5.5**). Very little staining was detected in line 17-3. No staining was observed in ColWT.*

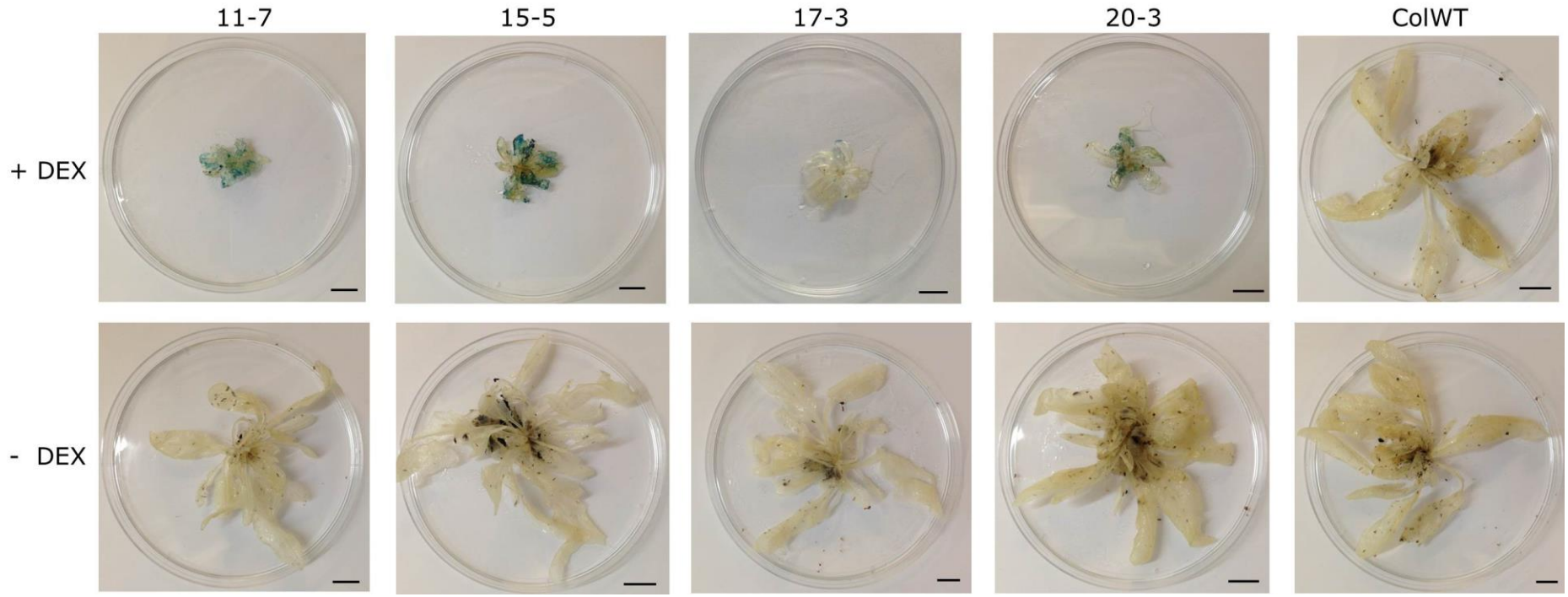


Figure 5.5. Expression of the *GUS* reporter gene in 3 week old plants treated with (+) DEX or (-) DEX control. Bar = 1 cm.

5.2.2 RT-PCR analysis of ARR22 expression in response to DEX treatment

To verify that the application of DEX leads to the upregulation of *ARR22* gene expression in the transgenic lines RT-PCR analysis was performed. Whole plants (full rosette and roots), that were sprayed every day with (+) DEX or (-) DEX control until flowering, were analysed (**Fig. 5.6 B**). Interestingly expression of *ARR22* was detected in transgenic lines treated with (-) DEX control. Expression was markedly lower in line 17-3 when compared with the other lines. The expression of *ARR22* was upregulated in all transgenic plants treated with (+) DEX when compared with the (-) DEX control. No transcript was detected in ColWT.

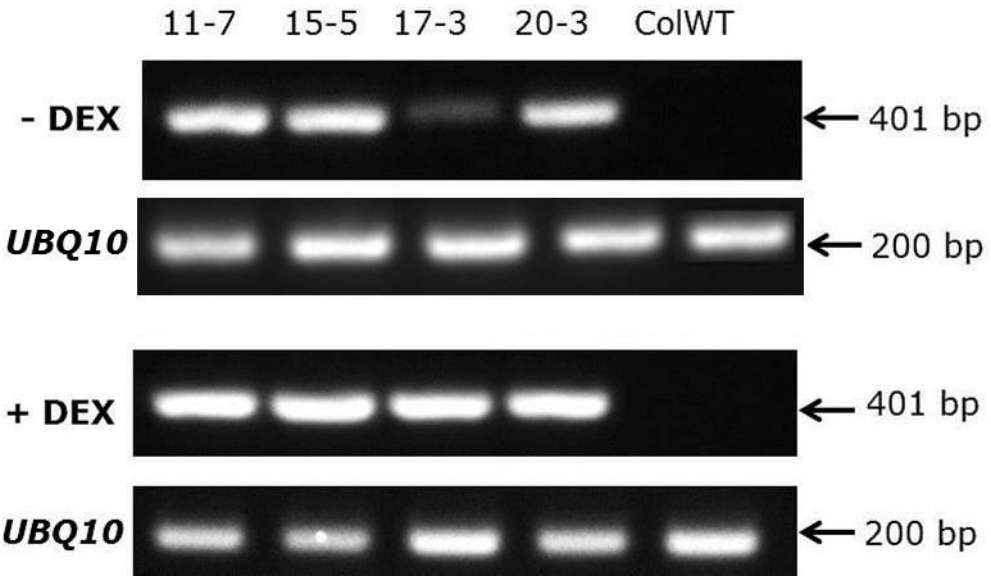


Figure 5.6. RT-PCR analysis of *ARR22* expression in transgenic plants and ColWT treated with (-) DEX control or (+) DEX for 3 weeks. *UBQ10* used as housekeeping gene.

5.2.3 Analysis of *ARR22* protein expression in response to DEX treatment

The expression of *ARR22* was subsequently examined at the protein level in whole plants (full rosette and roots) sprayed every day until flowering using dot blot analysis (**Fig. 5.7**). *ARR22* protein expression was not detected in (-) DEX control treated *Pro$_{35S}$:ARR22* lines 11-7 and 15-5 and *Pro$_{35S}$:ARR22^{D74N} line 17-3 whereas a low level of ARR22 protein was detected in Pro$_{35S}$:ARR22^{D74N} line 20-3. ARR22 protein accumulation was substantially upregulated in the transgenic lines treated with DEX. Intriguingly, a low level of ARR22 protein was detected in ColWT plants however DEX treatment did not initiate upregulation.*

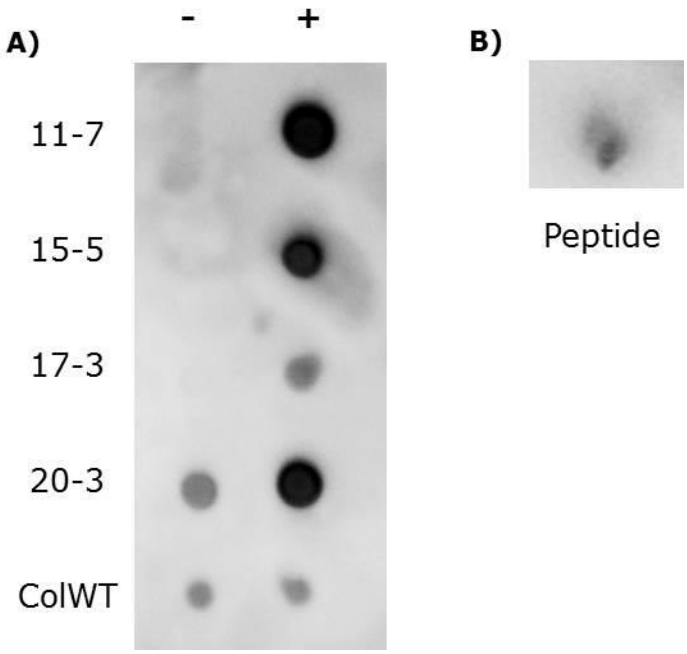


Figure 5.7. (**A**) Dot blot analysis of *ARR22* protein expression in 3 week old plants (whole rosette and roots) treated with (-) DEX control or (+) DEX. 20 µg protein applied. (**B**) Peptide control.

5.3 Effect of DEX application on root phenotype

Plants that had been sprayed for 3 weeks with (+) DEX or (-) DEX control from 7 d seedlings were excised from soil to observe the root phenotype. The roots of transgenic plants treated with (+) DEX were severely stunted compared to the (-) DEX controls (**Fig. 5.8**). DEX treatment did not appear to have an effect on root length in ColWT.

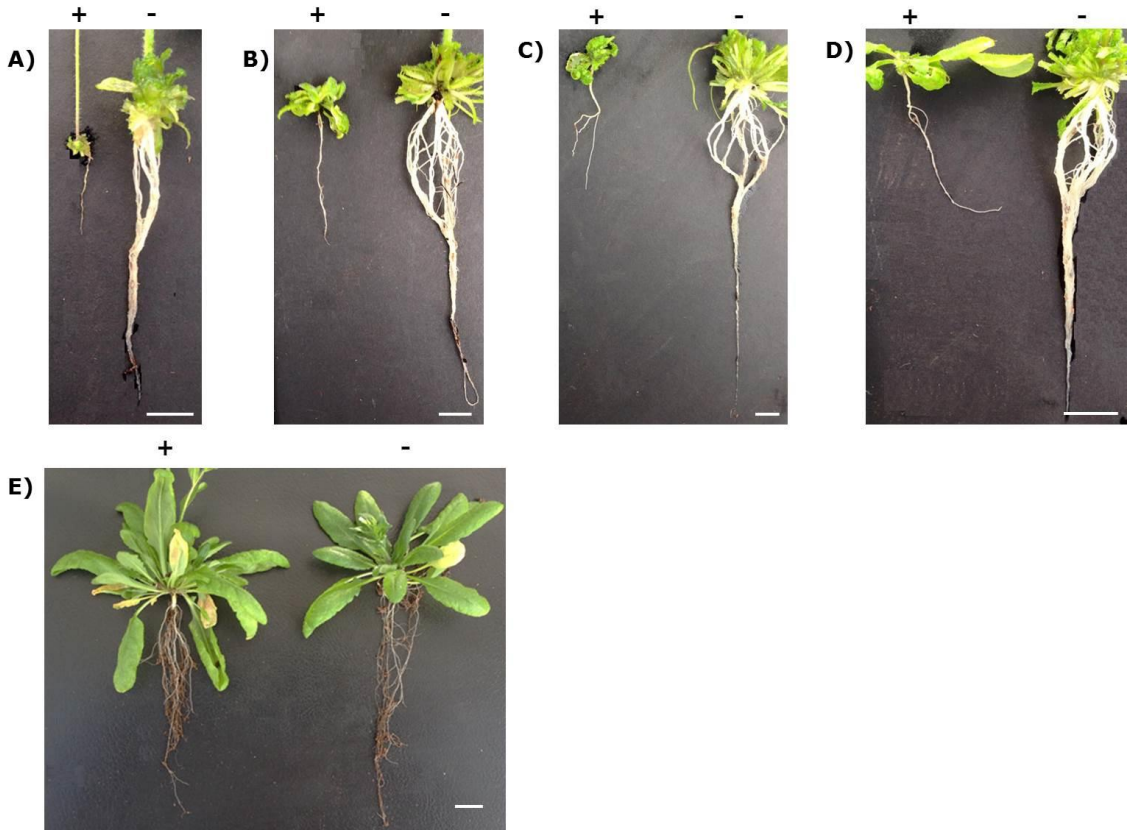


Figure 5.8. Root phenotype in (**A**) *Pro$_{35S}$:ARR22* line 11-7 (**B**) *Pro$_{35S}$:ARR22 line 15-5 (**C**) Pro$_{35S}$:ARR22^{D74N} line 17-3 (**D**) Pro$_{35S}$:ARR22^{D74N} line 20-3 and (**E**) ColWT plants treated with DEX (+) or (-) DEX control. Bar = 1 cm.*

The effect of DEX treatment on roots was quantified by measuring primary root length (**Fig. 5.9**). Root length was significantly shorter in

(+) DEX treated *Pro$_{35S}$:ARR22* line 11-7 (p<0.05) and *Pro$_{35S}$:ARR22^{D74N} lines 17-3 and 20-3 (p<0.01). No significant effect was observed in Pro$_{35S}$:ARR22* line 15-5 or ColWT. Transgenic (-) DEX controls were compared to ColWT and *Pro$_{35S}$:ARR22* lines 11-7 and 15-5 were significantly (p<0.05) shorter.

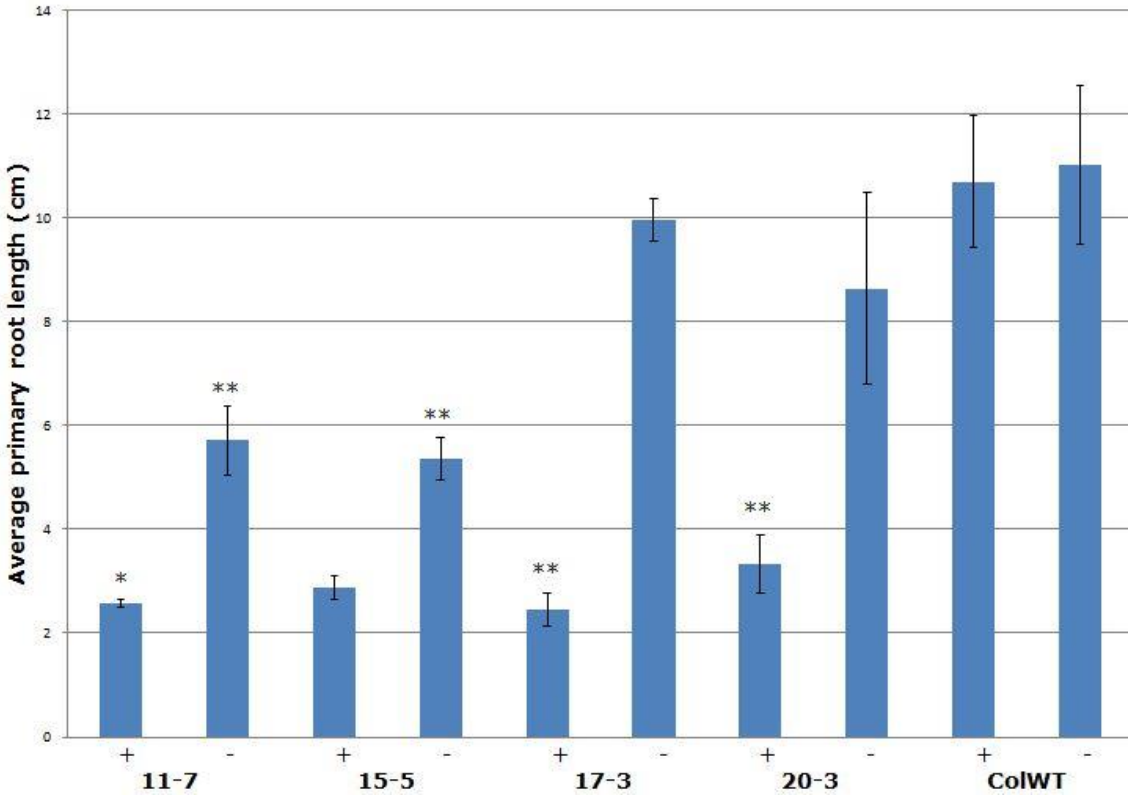
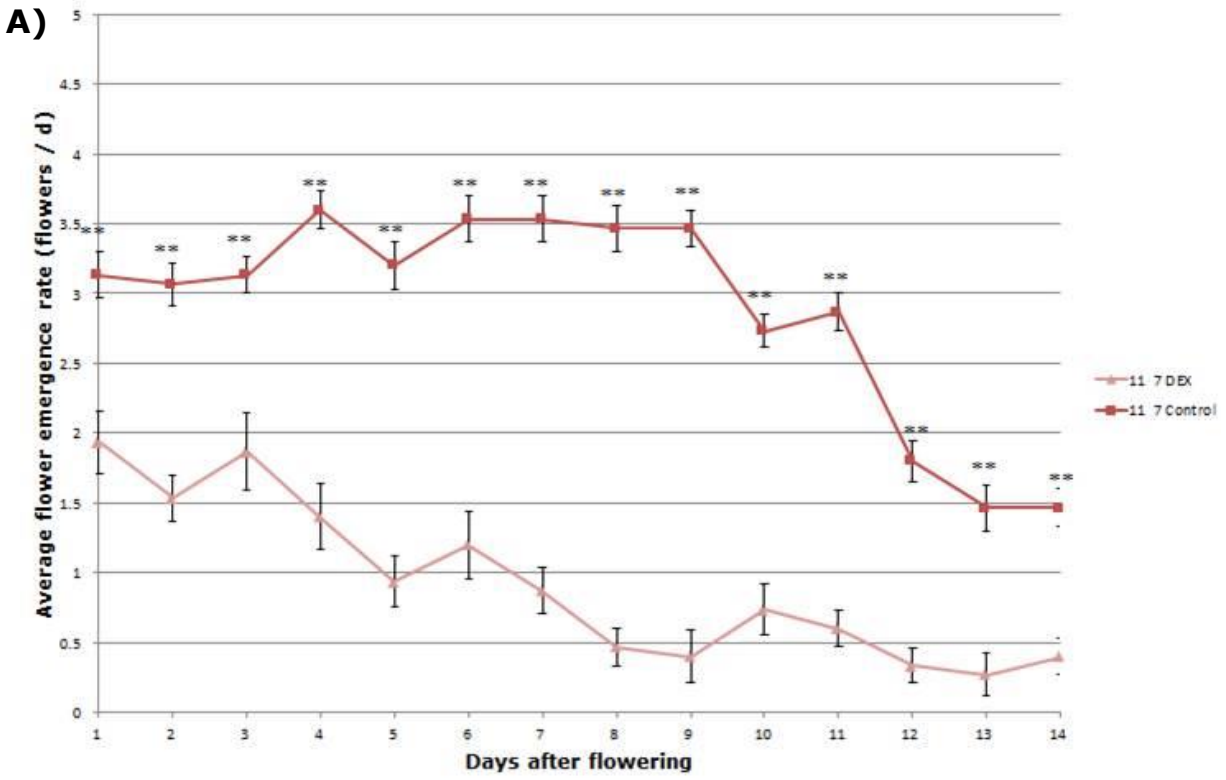


Figure 5.9. Effect of (+) DEX or (-) DEX control treatment on primary root length. Statistically significant changes compared within lines and between controls indicated with * when p<0.05 and ** when p<0.01. Bar indicates standard error of the mean; n=3.

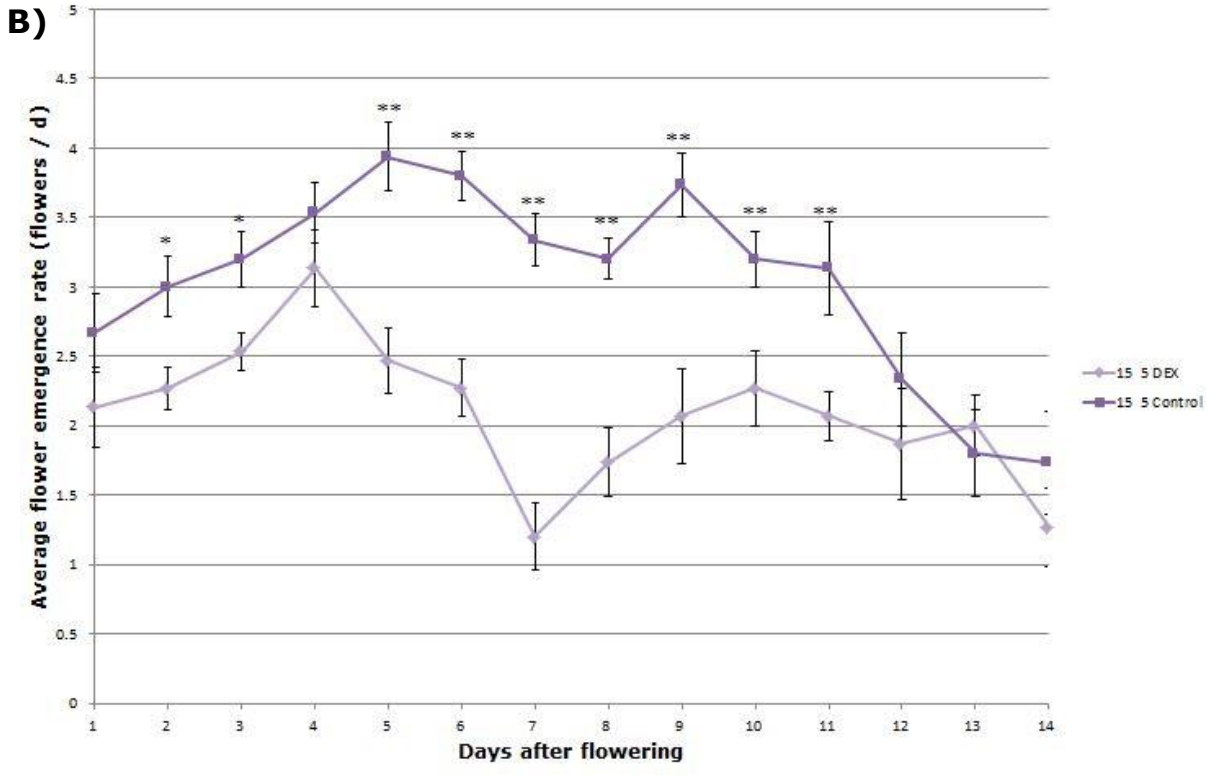
5.4 Effect of DEX induced *ARR22* expression post floral induction

To look at the effect of DEX treatment on post floral development, plants were sprayed every day for 2 weeks after floral induction had occurred. In (+) DEX treated *Pro$_{35S}$:ARR22* line 11-7 flower emergence

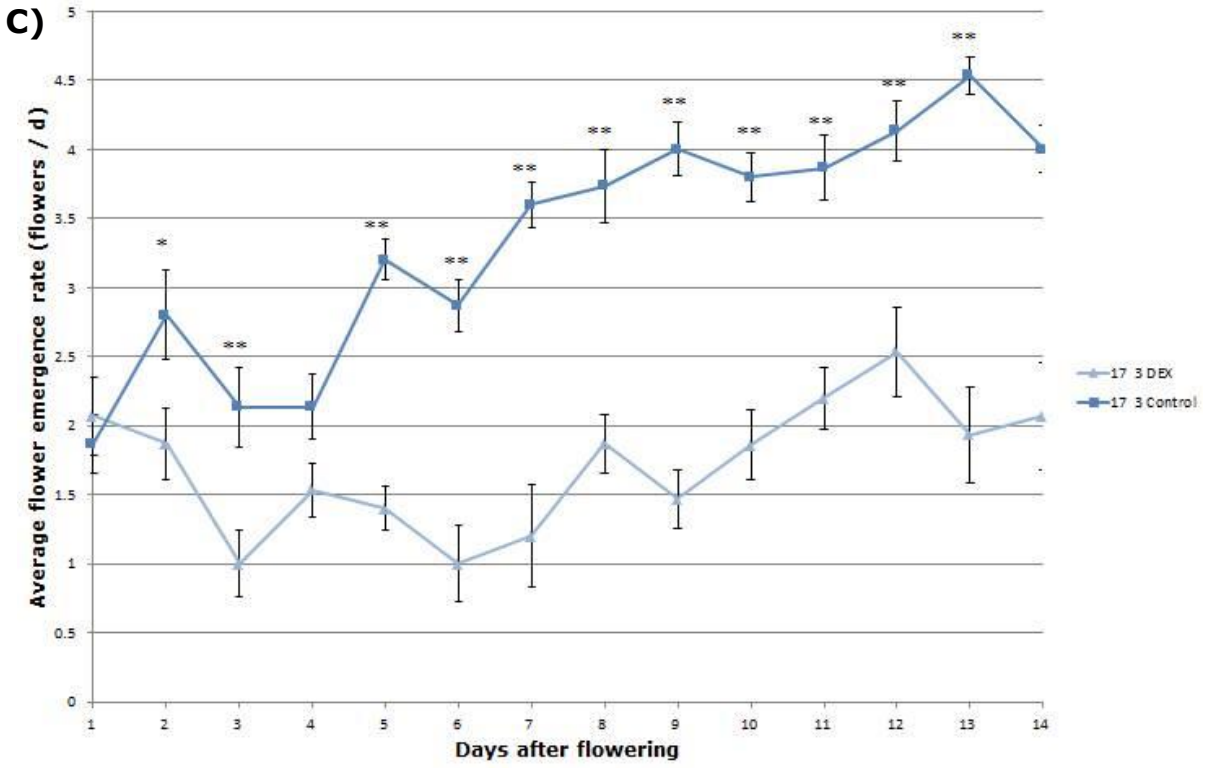
rate was significantly (p<0.01) lower than the (-) DEX control over the 2 weeks of treatment from 1 d (**Fig. 5.10 A**). In *Pro_{35S}:$ARR22^{D74N}$ line 20-3 a significant (p<0.01) difference in flower emergence was observed from 5 d (**Fig. 5.10 D**). A similar observation occurred in Pro_{35S}:$ARR22^{D74N}$ line 17-3 although a significant (p<0.05) effect was seen at 2 d.* In *Pro_{35S}:ARR22* line 15-5 flower emergence rate was significantly (p<0.01) altered between 5 and 11 d however the rate was comparable between (+) DEX treated and the (-) DEX control 12 – 14 d post floral induction/ treatment. In ColWT (+) DEX treatment, generally, had no effect on the flower emergence rate although a significant (p<0.05) effect was detected in DEX treated plants at 5 d (**Fig. 5.10 E**). The (-) DEX transgenic controls were also compared with (-) DEX ColWT (**Fig. 5.10 F**). Significant differences were particularly observed between ColWT and *Pro_{35S}:ARR22* lines 15-5 and 11-7. Flower emergence rate was higher in 15-5 and 11-7 than ColWT until 6 d. The rate was then lower in 15-5 and 11-7 10 d post floral induction. *Pro_{35S}:$ARR22^{D74N}$ line 17-3 remained comparable to ColWT with the exception at 2 d. Pro_{35S}:$ARR22^{D74N}$ line 20-3, however, had a lower rate when compared to ColWT at 9, 10 (p<0.05) and 12 d (p<0.01).*


A)
Average flower emergence rate (flowers / d)
Days after flowering
11_7 DEX
11_7 Control

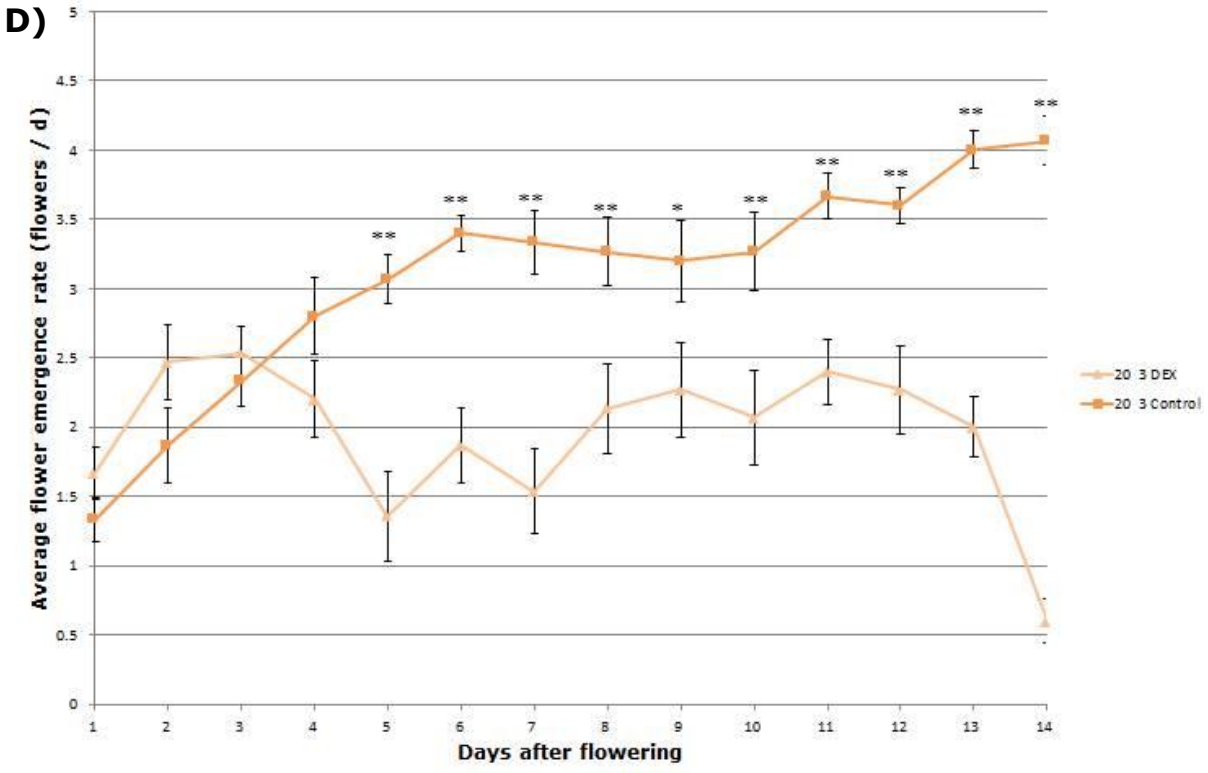
2345


B)
Average flower emergence rate (flowers / d)
Days after flowering
15_5 DEX
15_5 Control

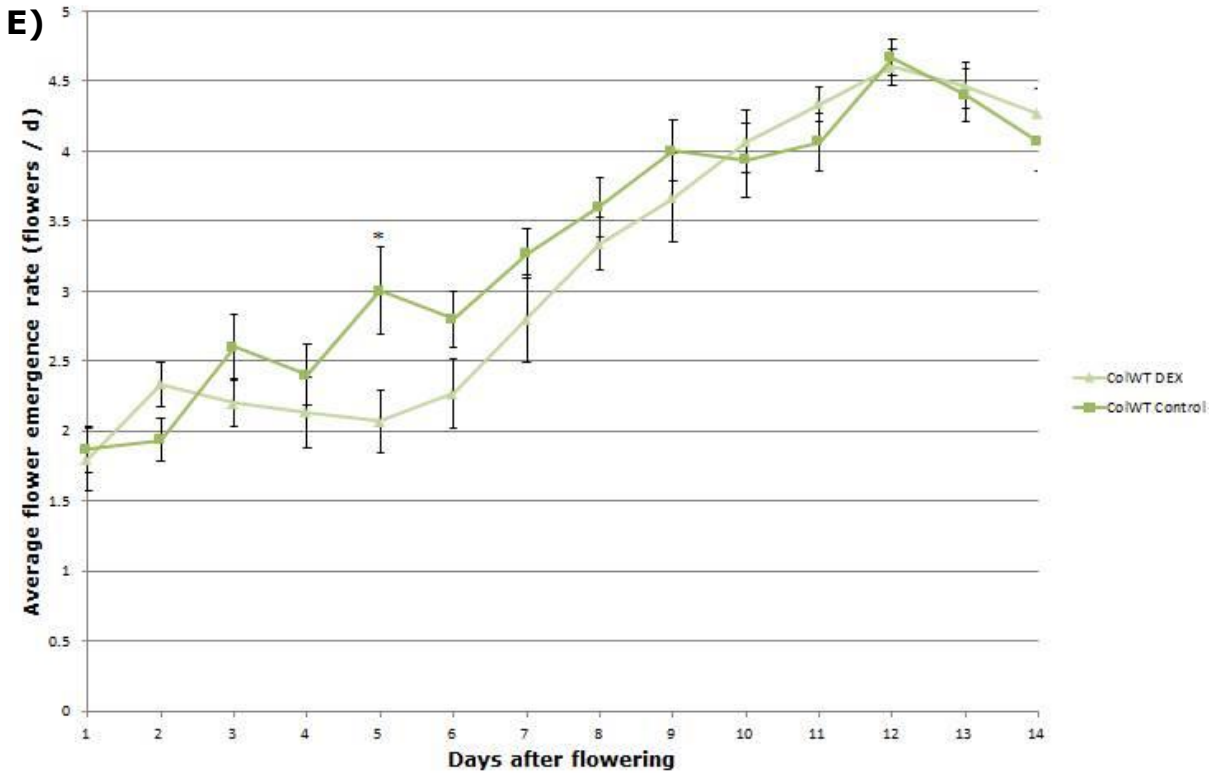
2346


C)
Average flower emergence rate (flowers / d)
Days after flowering
17 3 DEX
17 3 Control

2347


D)
Average flower emergence rate (flowers / d)
Days after flowering
20 3 DEX
20 3 Control

2348



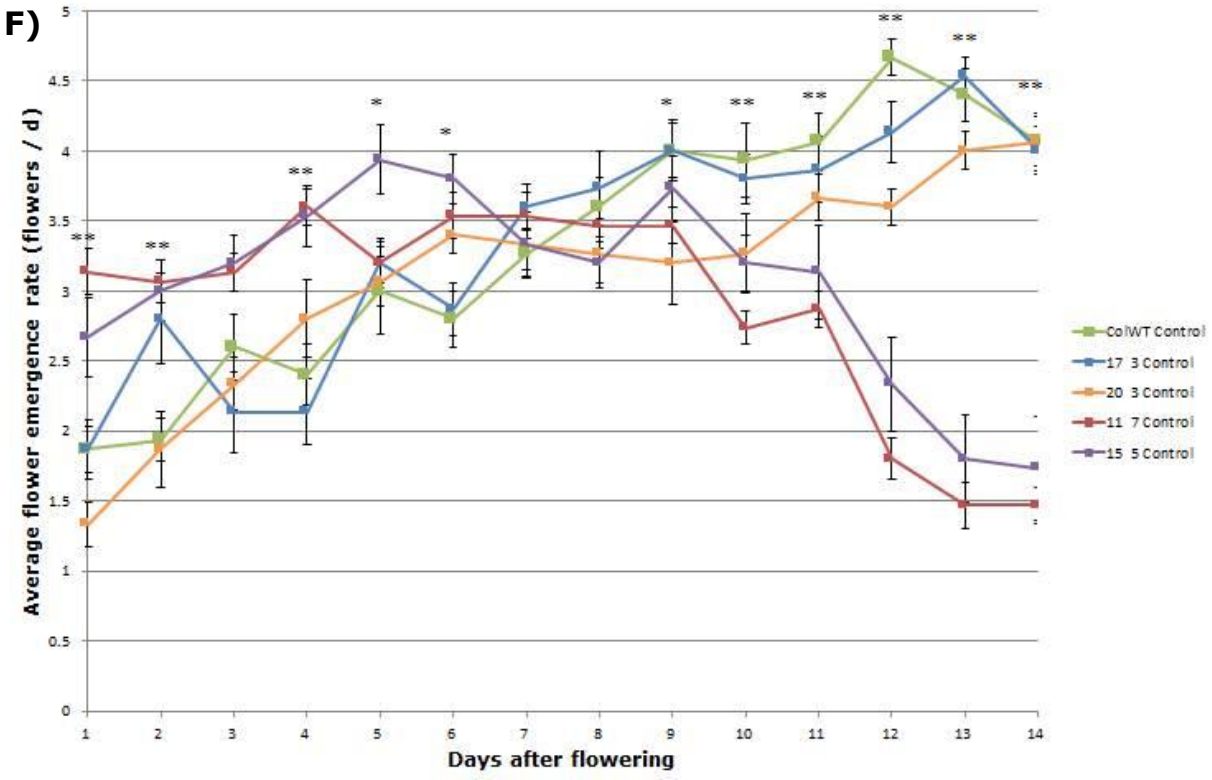


Figure 5.10. Effect of (+) DEX or (-) DEX treatment for 2 weeks on flower emergence rate in (**A**) *Pro35S:ARR22* line 11-7 (**B**) *Pro35S:ARR22* line 15-5 (**C**) *Pro35S:ARR22D74N* line 17-3 (**D**) *Pro35S:ARR22D74N* line 20-3 and (**E**) ColWT plants (**F**) Comparison of all (-) DEX controls.

Statistically significant changes compared within lines and between controls indicated with * when $p<0.05$ and ** when $p<0.01$. Error bars represent standard error of the mean; n=15.

The height of the primary inflorescence was measured after 2 weeks of treatment with (+) DEX or (-) DEX control. Transgenic and ColWT plants treated with DEX were significantly ($p<0.01$) smaller in height compared with the (-) DEX controls (**Fig. 5.11**). When the (-) DEX transgenic controls were compared with the (-) DEX ColWT control, only *Pro$_{35S}$:ARR22* line 11-7 was significantly ($p<0.01$) different in height.

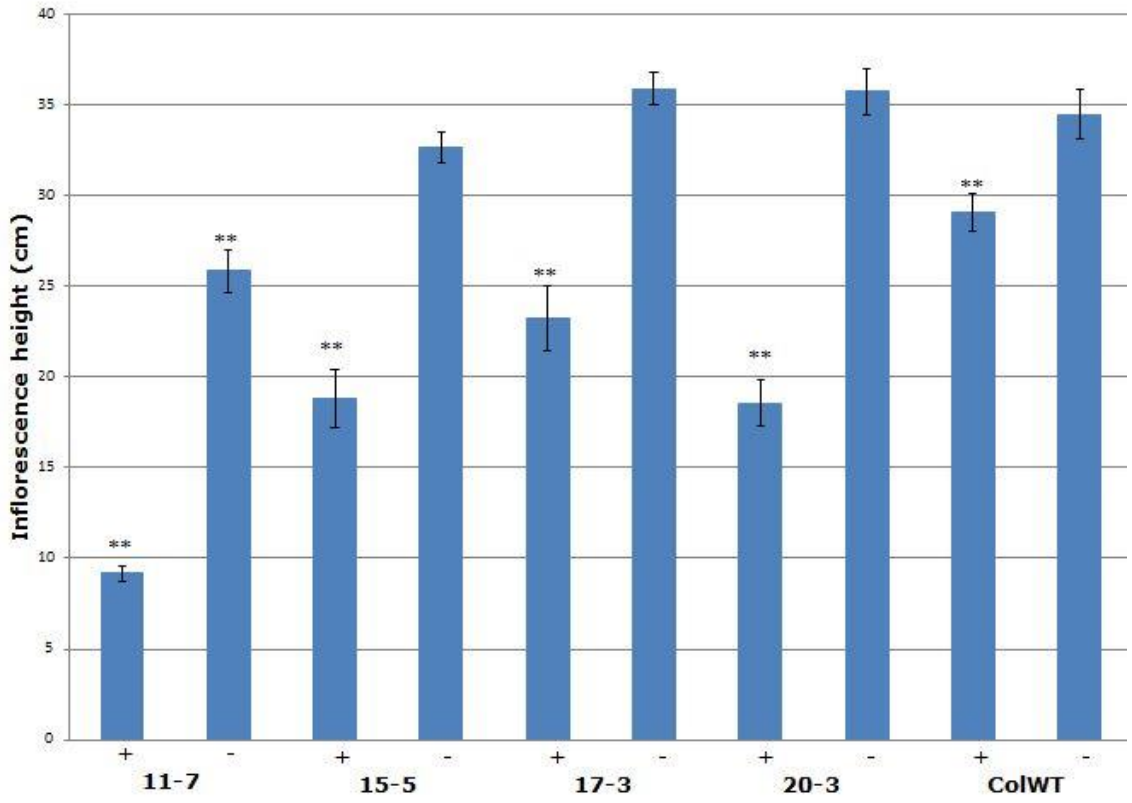


Figure 5.11. Effect of (+) DEX or (-) DEX control treatment post floral induction on the primary inflorescence height. Statistically significant changes compared within lines indicated with ** when $p<0.01$. Error bars represent standard error of the mean; n=15.

Silique abortion was measured after 2 weeks of treatment with (+) DEX or (-) DEX control. The number of aborted siliques is presented in **Fig. 5.12**. Transgenic plants treated with DEX had significantly (p<0.01) more aborted siliques than the (-) DEX controls

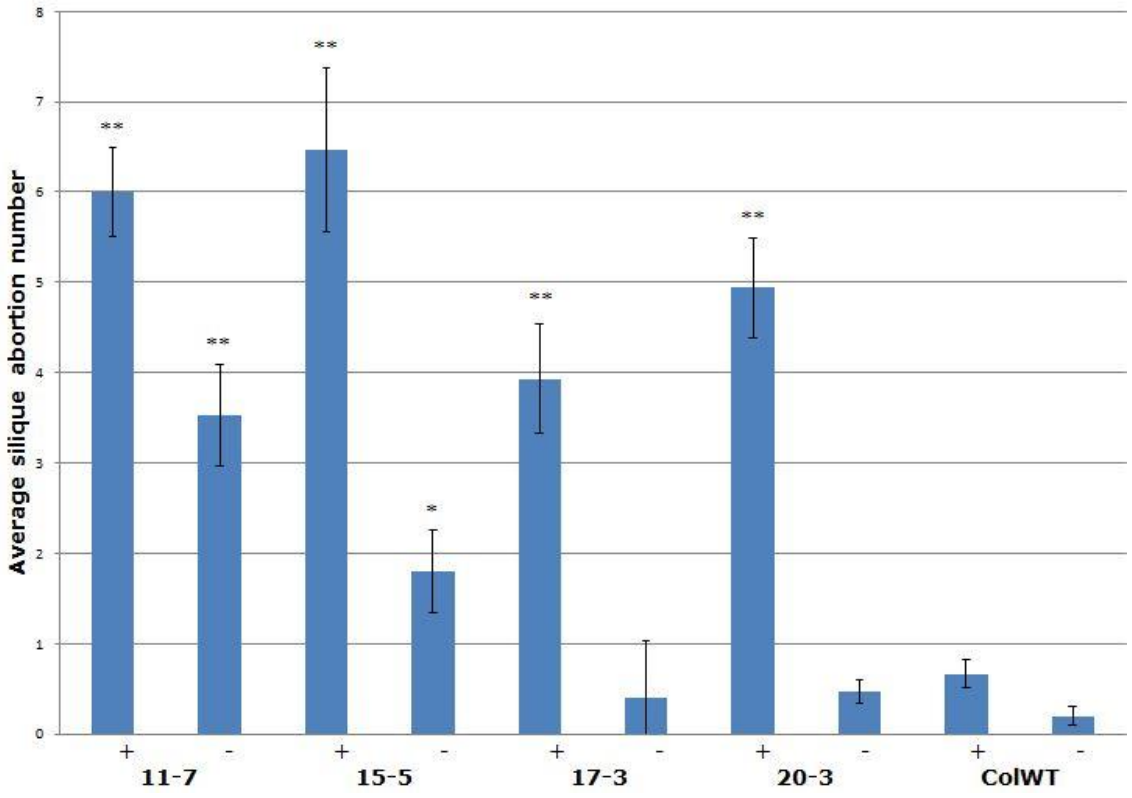
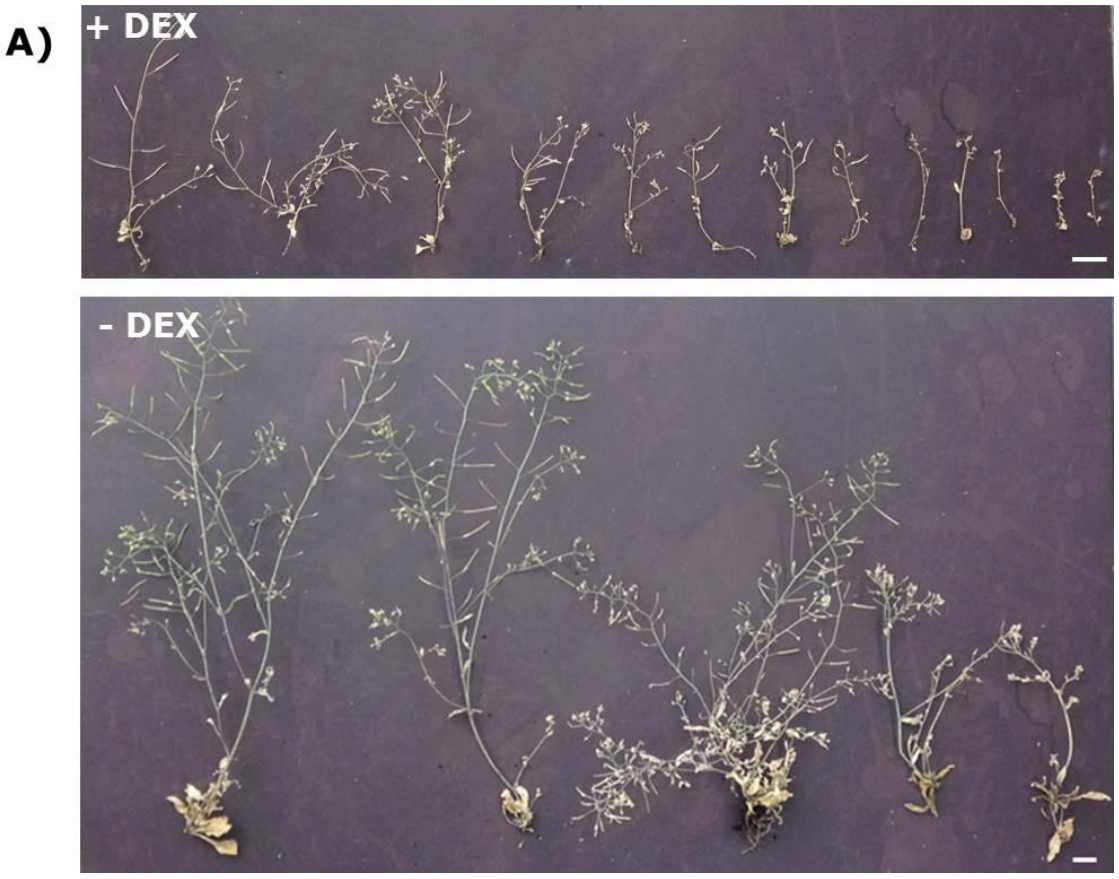


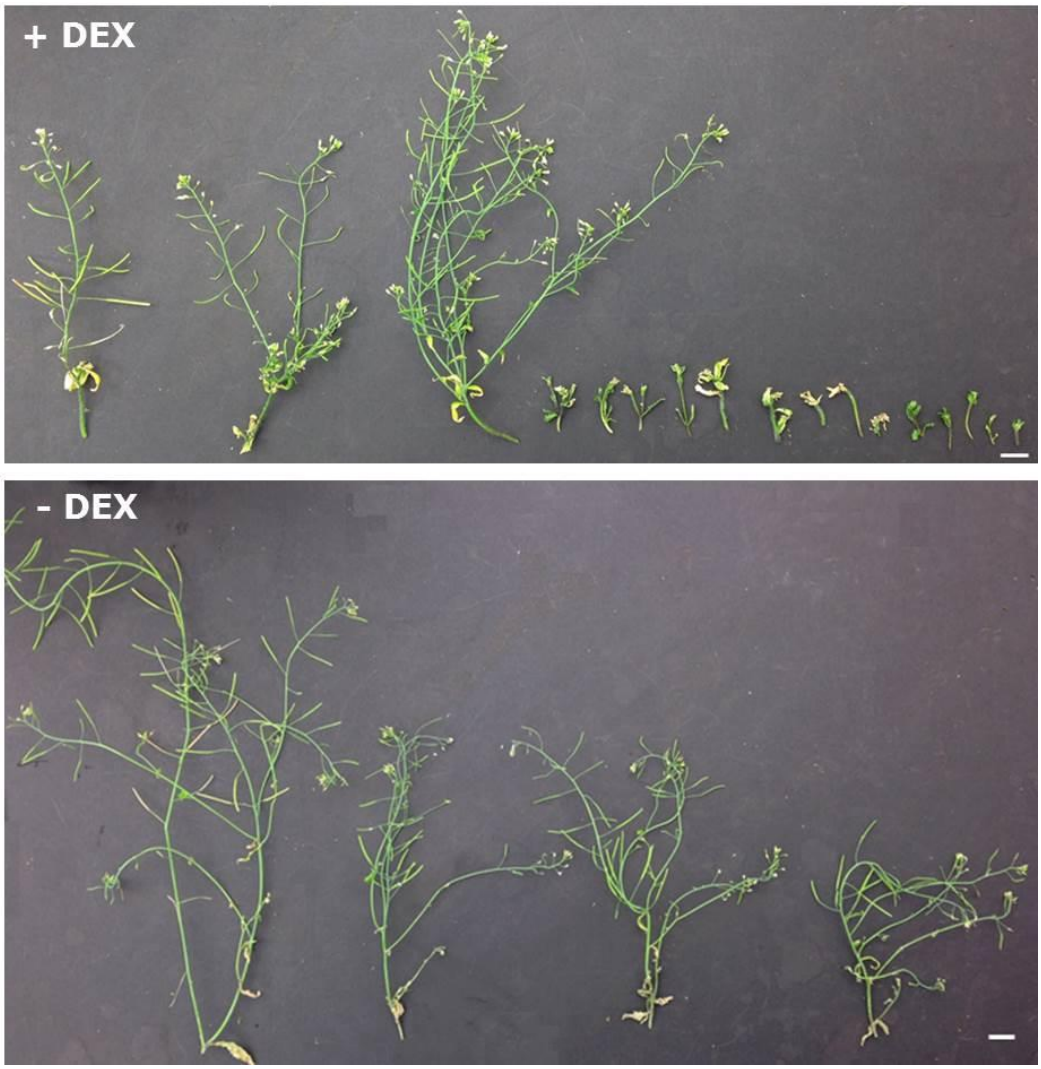
Figure 5.12. Effect of (+) DEX or (-) DEX treatment for 2 weeks on silique abortion. Statistically significant changes compared within lines and between controls indicated with * when p<0.05 and ** when p<0.01. Error bars represent standard error of the mean; n=15.

After 2 weeks of treatment with (+) DEX or (-) DEX control, plants were dissected out (**Fig. 5.13**) and basal branch number was recorded. DEX treated transgenic lines had significantly (p<0.01) more basal branches than the (-) DEX controls (**Fig. 5.14**). No significant difference was observed between (+) DEX treated ColWT and the (-) DEX control.

When all (-) DEX controls were compared, *Pro$_{35S}$:ARR22* line 11-7 was significantly different to ColWT (p<0.01).

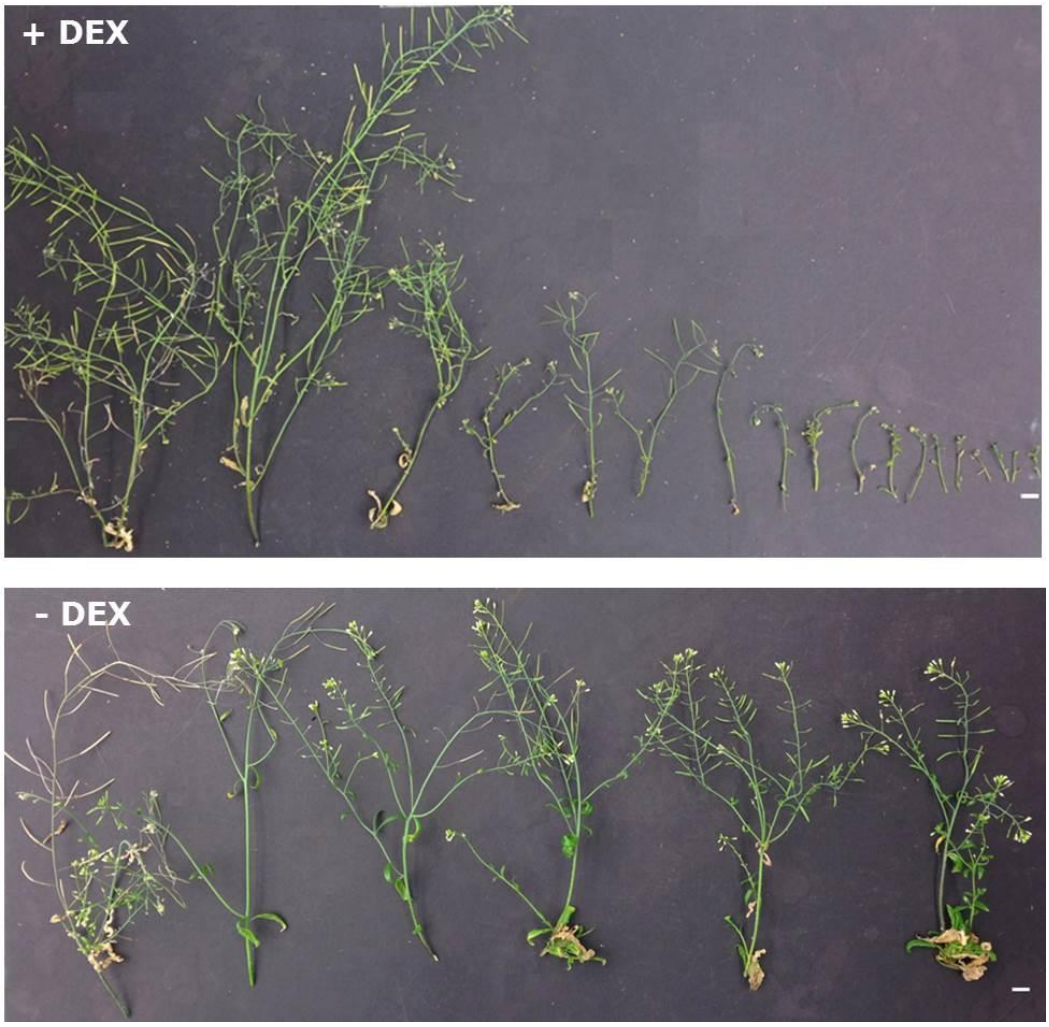


B)



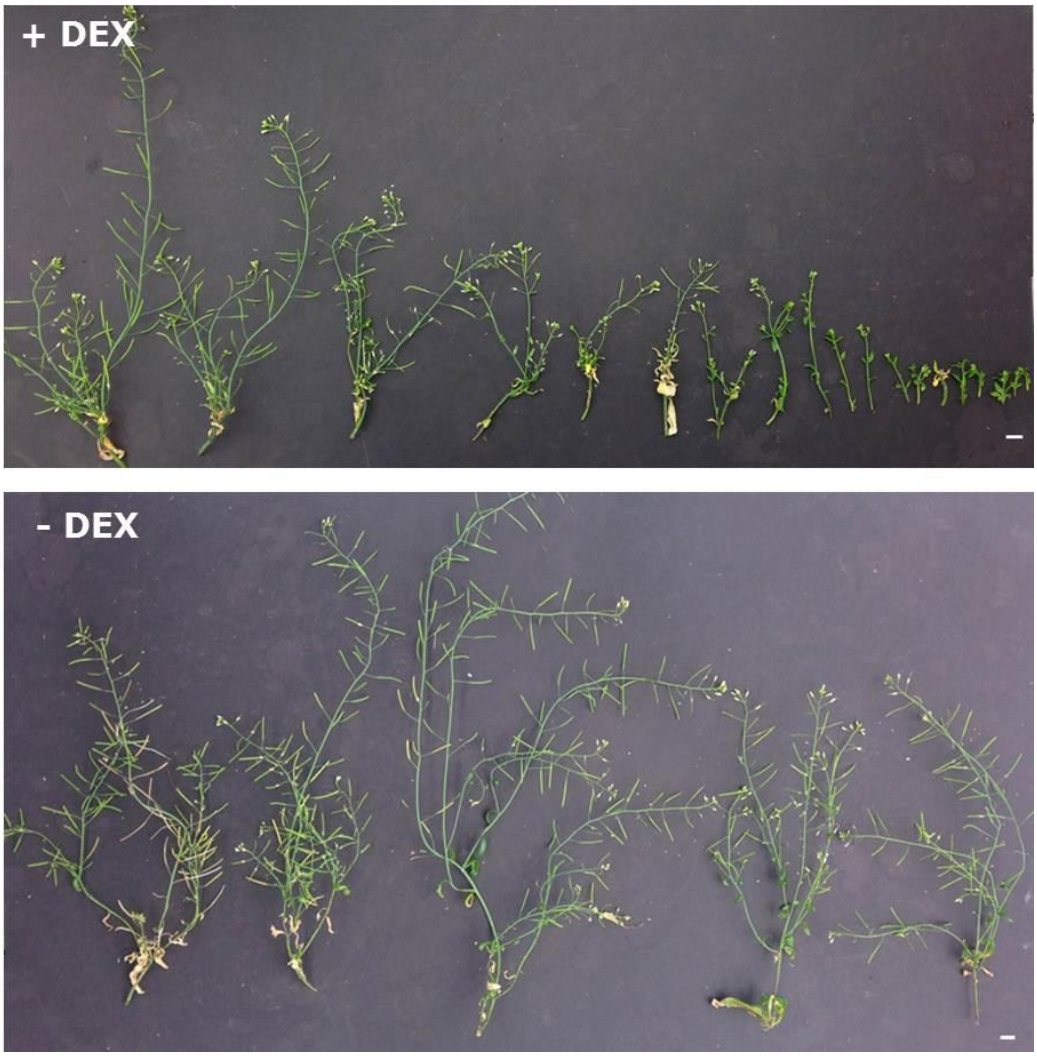
2390

C)



2391

D)



2392

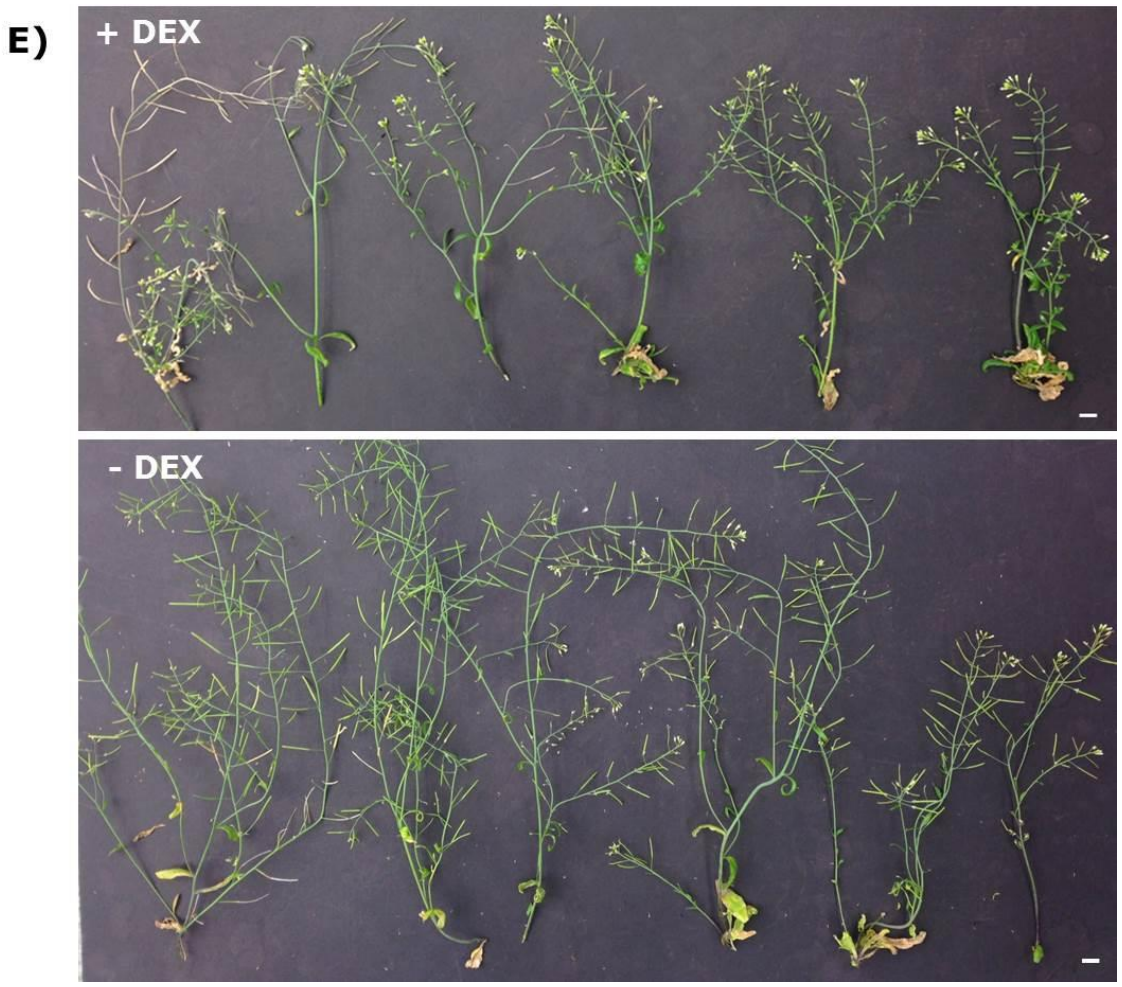


Figure 5.13. Effect of (+) DEX or (-) DEX control treatments for 2 weeks post floral induction on axillary branch phenotype in (**A**) *Pro$_{35S}$:ARR22* line 11-7 (**B**) *Pro$_{35S}$:ARR22 line 15-5 (**C**) Pro$_{35S}$:ARR22^{D74N} line 17-3 (**D**) Pro$_{35S}$:ARR22^{D74N} line 20-3 and (**E**) ColWT plants. Bar = 1 cm.*

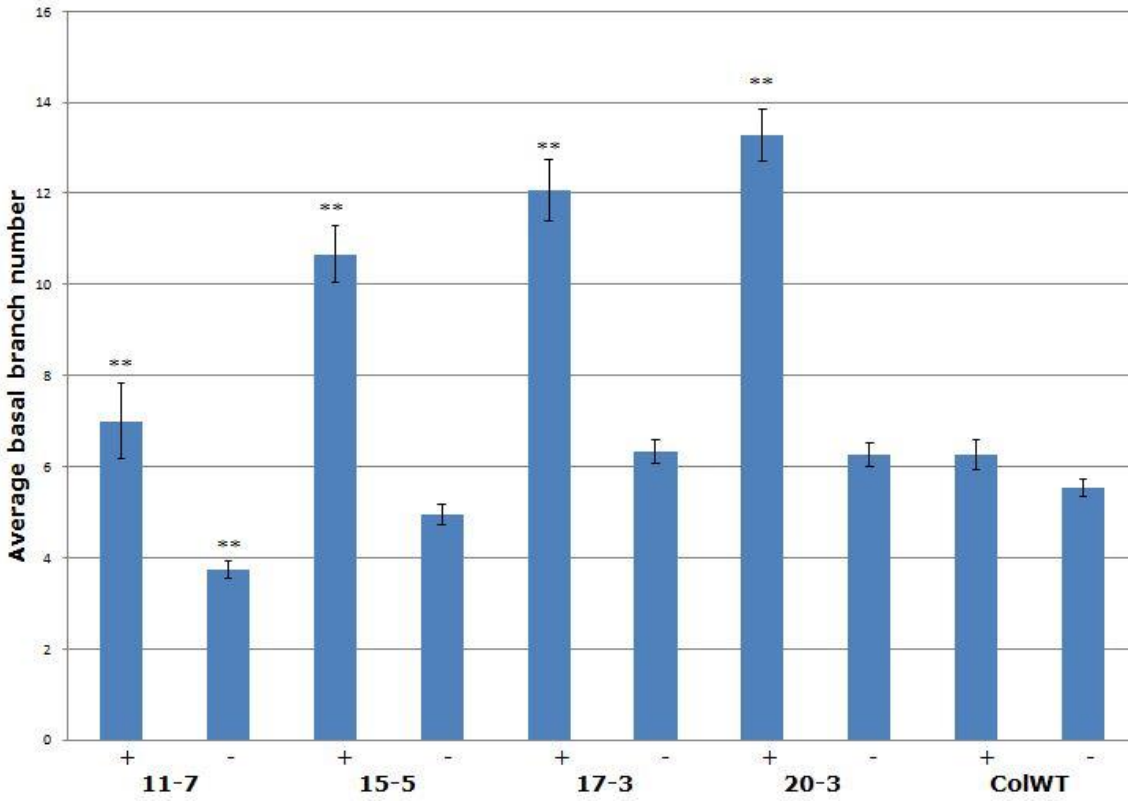


Figure 5.14. Effect of (+) DEX or (-) DEX control treatments for 2 weeks post floral induction on basal branch number. Statistically significant changes indicated with ** when $p<0.01$. Error bars represent standard error of the mean; n=15.

5.4.1 Post floral GUS analysis

Histochemical analysis was performed on open flowers from plants that had been sprayed with (+) DEX or (-) DEX control every day for 1 week from floral induction. GUS activity was prominent in the petals and sepals of DEX treated *Pro$_{35S}$:ARR22* lines 11-7 and 15-5 (**Fig. 5.15**). A small amount of staining was observed in the petals and sepals of *Pro$_{35S}$:ARR22^{D74N} line 17-3 and 20-3.*

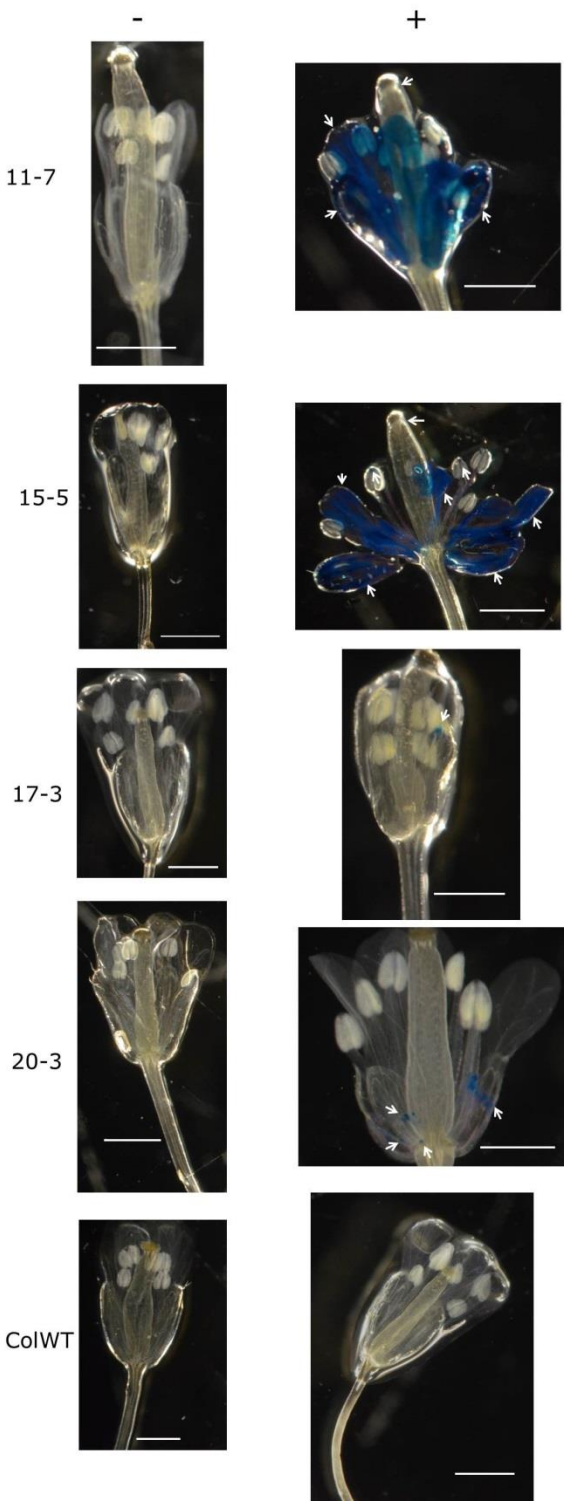


Figure 5.15. Histochemical localisation of DEX-induced *ARR22* gene expression in *Arabidopsis* flowers from plants treated with (+) DEX or (-) DEX control for 1 week post floral induction. White arrows indicate localisation of GUS activity. Bar = 1 cm.

5.5 GUS analysis of DEX induced *ARR22* expression in specific tissues

In an attempt to observe whether *ARR22* could be upregulated in specific tissues DEX was applied to open flowers and siliques and a subsequent GUS analysis performed. (+) DEX or (-) DEX control was applied to flowers attached to the plant and incubated for 24 hours. **Fig. 5.16** shows intense blue staining in Pro_{35S}*:ARR22* lines 11-7 and 15-5 that had been treated with (+) DEX. GUS expression was visualised in the pedicel, petals and stigma. Very little staining was observed in Pro_{35S}*:ARR22*^{D74N} line 17-3 and was absent in line 20-3. No GUS expression was visible in (-) DEX controls or ColWT.

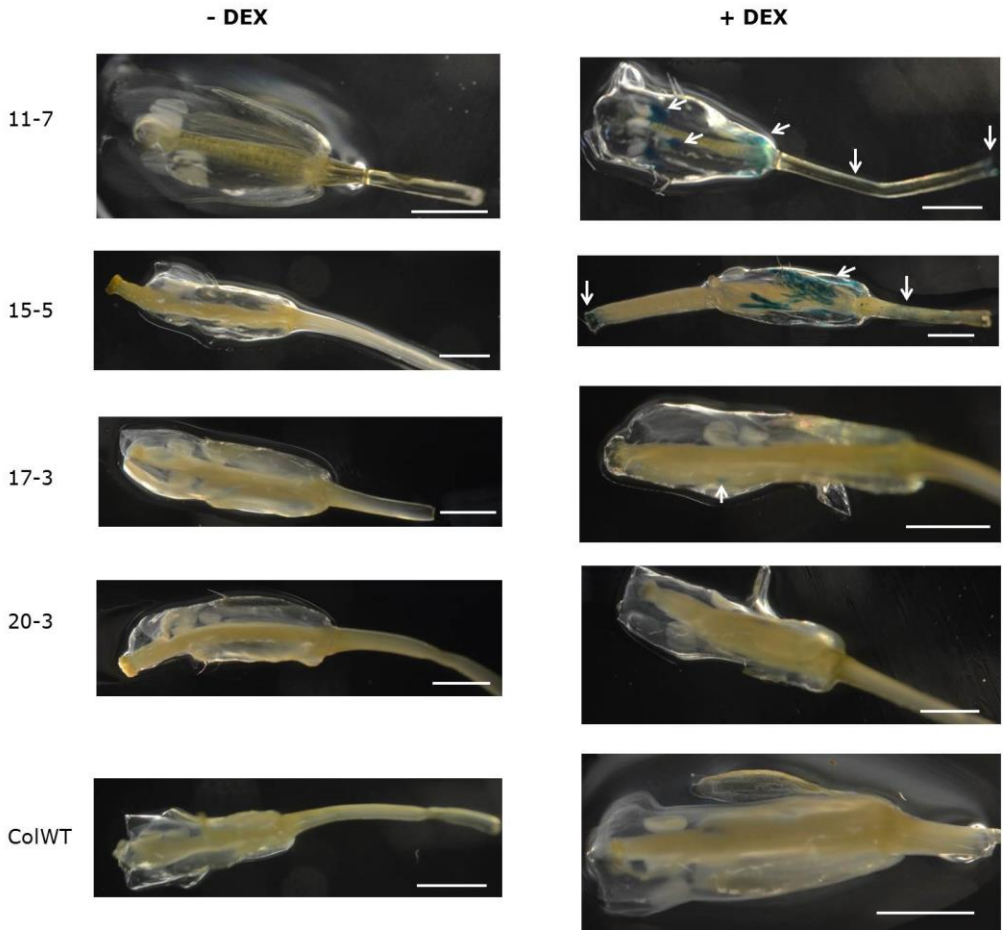


Figure 5.16. Histochemical localisation of *ARR22* gene expression in *Arabidopsis* flowers incubated with (+) DEX or (-) DEX control for 24 hours. White arrows indicate localisation of GUS activity. Bar = 1 cm.

To analyse the expression of DEX induced *ARR22* expression in pods, elongating siliques (4 – 8 DAF) were excised from the plant and the pedicle placed in (+) DEX or (-) DEX control for 48 hours. In *Pro$_{35S}$:ARR22* lines 11-7 and 15-5 and *Pro$_{35S}$:ARR22^{D74N}* line 17-3 treated with DEX GUS expression was visualised in the pedicel and adjacent to the abscission zone (**Fig. 5.17**). Some staining was observed in the silique wall in lines 15-5 and 17-3. A small amount of

GUS expression was visualised in the style of 15-5. No expression was detected in Pro_{35S}:$ARR22^{D74N}$ line 20-3 or ColWT.

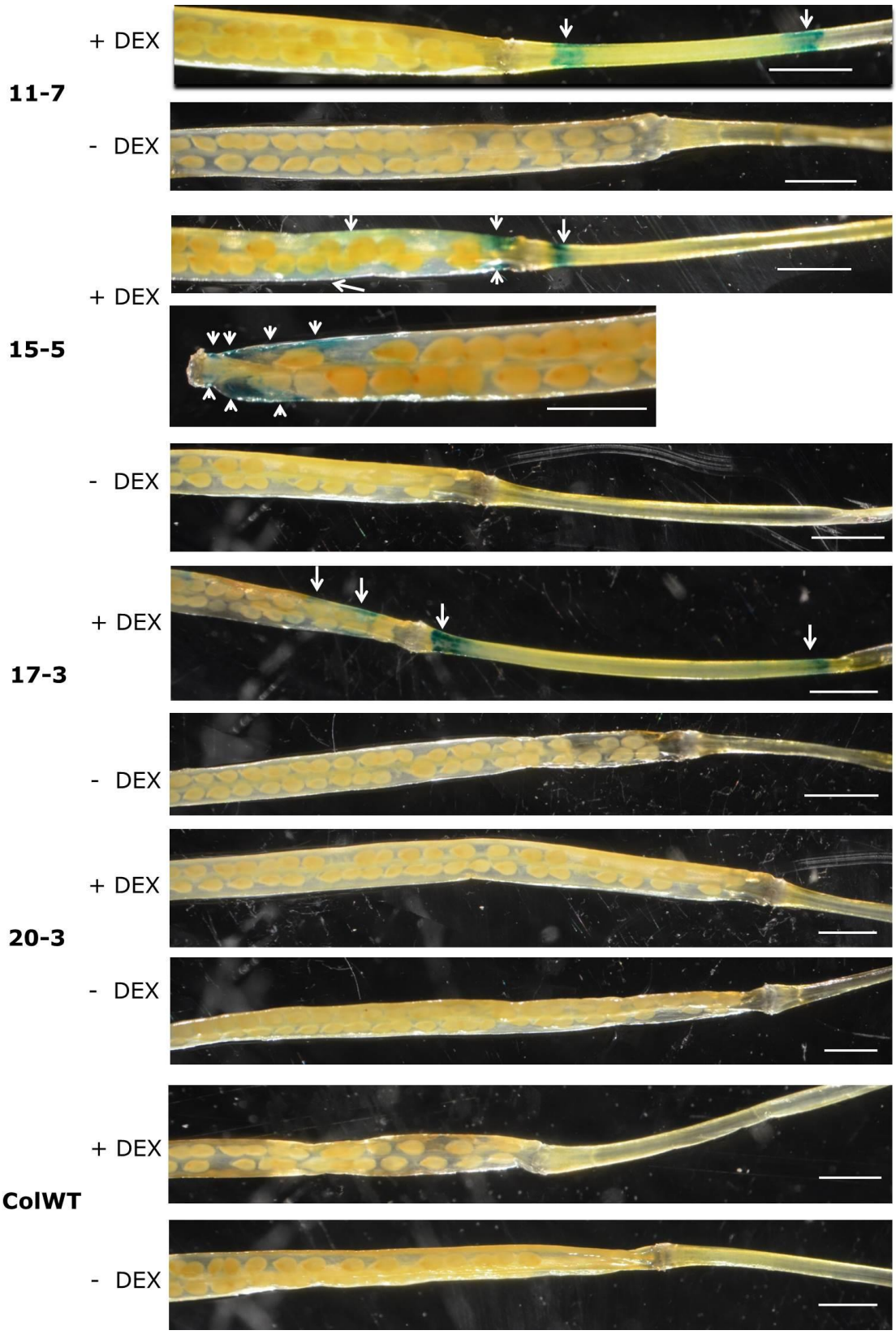


Figure 5.17. Histochemical localisation of *ARR22* gene expression in excised *Arabidopsis* siliques incubated with (+) DEX or (-) DEX control for 48 hours. White arrows indicate localisation of GUS activity. Bar = 1 cm.

5.6 Discussion

Previous characterisation studies of *ARR22* gene function has utilised methods that include ectopic overexpression and gene silencing (Kiba et al., 2004; Gattolin et al., 2008; Horak et al., 2008). Overexpression of *ARR22* driven by a 35S promoter resulted in severely dwarfed plants while analysis of T-DNA insertion plants did not yield a detectable phenotype (Gattolin et al., 2008; Horak et al., 2008). An additional study has employed a DEX-inducible system to ectopically overexpress *ARR22* (Kang et al., 2013). It was shown that overexpression of *ARR22* in a DEX-induced manner resulted in an increased tolerance to drought and freezing in 3 and 4 week old plants (Kang et al., 2013). Moreover, mutation of the predicted phospho-accepting Asp residue at amino acid 74 to Asn in ARR22 leads to the abolishment of the aforementioned observations. This thus suggested that this site is crucial for ARR22 protein function during stress response (Kang et al., 2013). General phenotype and plant growth and development were not examined by Kang et al. (2013) nor were they previously quantified in overexpressing lines. Hence this study primarily focussed on characterising the physiological effects of DEX-induced *ARR22* overexpression.

5.6.1 DEX-induced ARR22 overexpression severely compromises growth and development

The DEX-inducible system provides a novel way in which to overexpress *ARR22* at specific developmental time points. As such, experiments were carried out in plants overexpressing *ARR22* in a DEX-induced manner from germination to flowering and in plants post-floral induction. In all transgenic lines that were treated with DEX from germination to flowering, a significant reduction in rosette area, leaf size and root development was observed which is consistent with previous observations (Kiba et al., 2004; Gattolin et al., 2008). It has been previously suggested by Kiba et a. (2004) that the impaired root growth in *ARR22* overexpressing plants resembles that of the phenotype in wooden leg (*wol*) mutants which have a loss-of-function in the cytokinin receptor AHK4 (Mahonen et al., 2000). In the double *ahk2-1 ahk3-1* and triple *ahk2-1 ahk3-1 ahk4-1* cytokinin receptor mutants smaller leaves and rosette sizes reminiscent to the observations seen in this study have also been described (Nishimura et al., 2004). On dissection of the two transgenic lines 11-7 and 15-5, that do not harbour the phospho-accepting amino acid mutation, a high number of leaves was uncovered. Moreover a bushy phenotype was noted after 3 weeks of DEX treatment which is comparable to the *amp1* mutant in which levels of cytokinin biosynthesis are elevated (Chaudhury et al., 1993; Nogué et al., 2000). However within these same lines there was evidence of necrotic lesions and leaf senescence. Chlorophyll content has been seen

to be considerably lower in histidine kinase and *cre1* mutants (Riefler et al., 2006). Horak et al. (2008) did not uncover a direct interaction of ARR22 with histidine kinases but this observation suggests that the cytokinin pathway is 'interfered' with when traces of ARR22 are present. It appears that upregulation of ARR22 may hence lead to an increase in cytokinin while at the same time instigating a downregulation in the expression of the hormone's sensing receptors via an unknown mechanism. However quantification of cytokinin in the DEX treated lines is required to ascertain whether this hypothesis is correct.

Although DEX treated *Pro_{35S}:$ARR22^{D74N}$ lines 17-3 and 20-3 did not exhibit the bushy phenotype and had fewer leaves than Pro_{35S}:ARR22 lines* 11-7 and 15-5, similar phenotypes were observed post-floral induction. Stunted growth (reduced height), an increased basal branch number, reduced flower emergence/ number and high rates of pod abortion were observed in all DEX treated plants. Again these phenotypes resemble cytokinin receptor mutants (Nishimura et al., 2004; Riefler et al., 2006). As comparable effects were observed across all lines, the supposition that the amino acid mutation would have a prominent role in attenuating the overexpression effects is not supported in this study. Small variations in phenotype across the lines, for example the serrated leaves observation in line 20-3, are therefore possibly a result of transgene position effects.

Kang et al. (2013) showed that modification of the predicted phosphorylation site at amino acid 74 in ARR22 lead to the abolition of the stress resistance response. This therefore suggests that this site is crucial for protein function. However in this study the lines harbouring the mutation of amino acid 74 from Asp to Asn were as effective in altering plant phenotype when *ARR22* overexpression was induced. This implies that ARR22 may in fact be acting through another mechanism or interacting with other targets in non-stressed conditions. This hypothesis is supported by studies examining the phosphorylation sites in type-A ARR5 and ARR7 (To et al., 2007; Leibfreid et al., 2005). Introduction of ARR5^{D87E} into the *arr3,4,5,6* quadruple mutant partially rescued plant phenotype and overexpression of ARR7^{D85E} induces meristem arrest (To et al., 2007; Leibfried et al., 2005) suggesting that these proteins are still functional without phosphorylation.

A notable observation was the expression of the transgene in the (-) DEX controls with a small amount of protein also detected in one of the lines. This could be due to genomic contamination. An alternative explanation is leaky expression which is sometimes seen in chemically regulated expression systems (Padidam 2003). Kang et al. (2013) did also detect expression of *ARR22* in control plants, however they also showed expression in ColWT controls which was not detected in this study nor has *ARR22* gene expression been witnessed in leaves in previous studies (Gattolin et al., 2006; Horak et al., 2008). Expression

of the transgene in (-) DEX controls could also account for the physiology differences observed. Interestingly, a small level of protein was detected in ColWT plants. Potentially the *ARR22* gene may be expressed at a very low level in leaves and is hence undetected in expression studies. As previously discussed in Chapter 4 gene expression and protein levels do not always correlate which could account for the detected protein. Furthermore differences in plant height and leaf number were observed in DEX treated ColWT plants. Whether this was an effect caused by spraying, the DEX itself or the DMSO used in the DEX treatment is unclear however Nethery and Hurtt (1967) have reported decreased height in plants exposed to DMSO.

5.6.2 Reproductive consequences of DEX-induced ARR22 expression

In previous *ARR22* overexpression studies it was found that transgenic plants were sterile and few flowers and siliques developed (Kiba et al., 2004; Gattolin et al., 2008). This study showed that pods did develop however there were a large number of aborted siliques. While histochemical staining of flowers revealed that DEX-induced expression was concentrated in petals and sepals, a very small amount of staining was observed on the stigma and in the style. Since the stigma facilitates pollen tube growth (Edlund et al., 2004) it is possible that this is disrupted when ARR22 is present. Alternatively, it has been demonstrated that a number of type-B response regulators are also

expressed in reproductive tissues (Lohrmann et al., 2001; Tajima et al., 2004). Overexpression of *ARR20* resulted in sterile siliques (Tajima et al., 2004) and potentially suggests a disturbance in cytokinin signalling.

As reproductive organs did not fully develop in previous studies on ARR22 it was impossible to monitor the effect of overexpression on seed development. GUS expression analysis carried out here aimed to overexpress *ARR22* specifically in the silique. Overexpression was detected at precise locations, notably adjacent to the abscission zone and pedicel.

Overexpression was not achieved throughout the whole silique which could be due to closure of the plasmodesmata which blocks cell-to-cell transport (van Doorn et al., 2003; Xu et al., 2012) and may have reduced uptake of the DEX solution. GUS staining was additionally not fully observed in whole rosettes or throughout flowers. Kang et al. (2013) showed intense staining of plants although these were grown on agar supplemented with DEX and spraying may therefore not represent an effective means on inducing overexpression.

5.7 Conclusions

This study is the first time that *ARR22* has been overexpressed at different and specific developmental stages to observe physiological effects using a unique DEX-inducible system. It has previously been

hypothesised that *ARR22* interacts with cytokinin signalling (Kiba et al., 2004; Horak et al., 2008). Certainly, the plant growth and developmental defects seen here as a result of overexpression support this hypothesis. Measuring cytokinin levels in vegetative and reproductive organs during overexpression will aid in confirming this hypothesis.

How ARR22 precisely disturbs the cytokinin pathway is unknown and further in depth studies are required to unearth the underlying molecular mechanisms. The high level of silique abortion in overexpressing plants suggests that *ARR22* may also be implicated in either pollen or ovule growth and development. Full analysis of the gene expression programme that facilitates fertilisation while overexpressing *ARR22* in flowers may shed light on its possible involvement.

It has been proposed that the Asp residue located at amino acid 74 in ARR22 is essential for phosphatase activity and consequently protein function during stress response (Kang et al., 2013). Phenotypes of the DEX treated transgenic lines with a mutation (D74N) at this site were comparable to those without and hence it is difficult to determine whether this site is essential for plant growth and development. Comparing the effects of mechanical wounding on seed development in all *ARR22* transgenic lines will be particularly useful for determining the

role and function of the phosphorylation site while inducing *ARR22* during this time may aid in elucidating its mechanism of action and.

In the current study siliques were detached to specifically overexpress *ARR22* in pods which did not prove to be an effective means of execution. Fine tuning of the DEX-inducible system in terms of application of treatment *in planta* will assist in further studies that will elucidate the exact role of *ARR22* in plant growth and development, particularly in the development of seeds.

Chapter 6:
General Discussion

In *Arabidopsis* there are 24 ARRs that are split into three groups known as type-A, type-B and type-C (Kiba et al., 2004). *ARR22* is a novel type-C RR in *Arabidopsis* that, unlike other *ARR*s, is not transcriptionally regulated by hormones such as cytokinin and ethylene (Kiba et al., 2004; Gattolin et al., 2006; Horak et al., 2008). However it has been confirmed that *ARR22* has a role within a plant phosphorelay system (Horak et al., 2008). Previous analyses have shown that *ARR22* is expressed in flowers and siliques and is hypothesised to be post-transcriptionally up-regulated in response to mechanical wounding at the seed:funiculus junction (Gattolin et al., 2006). Furthermore at 90 mins post-wounding the gene expression of SSPs is down-regulated while the gene expression of protease genes is up-regulated suggesting that *ARR22* may have a role in assimilate partitioning when a seed is damaged (Naomab, 2008).

B. napus is the third largest global source of vegetable oil and is hence an economically important crop. Understanding the underlying molecular mechanisms and networks that govern seed oil quality and yield are therefore imperative for genetic improvement. *SAC29* was identified as the putative orthologue of *ARR22* in *B. napus* (Whitelaw et al., 1999). During this study *in silico* analysis revealed the existence of 83 putative RRs in *B. napus* that were named *BnRR*s (see **section 3.2; Chapter 3**). A small subset of type-A and type-B *BnRR*s were further analysed via RT-PCR analysis which revealed expression during early

seed stages (see **section 4.2; Chapter 4**), an observation not previously detected in *Arabidopsis*. As expression was detected at discrete stages it has hence been predicted that these *BnRR*s may be implicated in seed embryogenesis. Further characterisation, for example through seed specific up-regulation of these genes, is required to fully understand what role they may play.

Four putative *ARR22* orthologues in *B. napus* were identified (*BnRR76 – BnRR79)* and share 81.25% amino acid similarity with *ARR22*. *B. napus* is an allotetraploid (AACC genome) crop formed from the hybridization of *B. rapa* and *B. oleracea* and as such it was revealed that *BnRR76* and *BnRR77* originated from *B. rapa* while *BnRR78* and *BnRR79* originated from *B. oleracea*. Examination of their genomic structures showed the presence of two introns, comparable to those in *ARR22*, located within the 5' UTR and ORF. However further sequence analysis discovered distinctive differences in nucleic and amino acid sequences (see **section 3.5.3; Chapter 3**). Specifically *BnRR76* and *BnRR78*, originating from *B. rapa* and *B. oleracea* respectively, possess an additional five amino acids as a result of fifteen supplementary nucleotides within the coding region. It was hypothesised that the addition, or lack, of this extra sequence could alter gene function or expression.

RT-PCR analysis was hence carried out to investigate this, as well as to determine whether *BnRR76 – BnRR79* produce four splice variants as a

result of the presence of introns in the same way as *ARR22* (Gattolin et al., 2006). The present study (see **section 4.3; Chapter 4**) detected expression in *B. napus* siliques and flowers and also in buds. Furthermore it was revealed that *BnRR76 – BnRR79* operate in a different manner to *ARR22* and distinct patterns in spatial and temporal expression for *B. rapa* and *B. oleracea* transcripts were observed. This observation is comparable to previous studies that have demonstrated that transcripts from different genomes are differentially expressed in polyploids such as wheat (Shitsukawa et al., 2007). Intron retention also occurred in the present system, specifically in *BnRR77* and *BnRR79* transcripts. It was previously suggested that the intron located in the 5' UTR of *ARR22* is required for mRNA stability (Gattolin et al., 2006) however it was retention of the intron within the ORF that occurred in *B. napus* and consequently does not universally support this hypothesis across the Brassicaceae. Interestingly intron retention occurred throughout seed maturation and hence demonstrates a development specific example of alternative splicing that may allow these transcripts to modulate seed filling. Potentially this mechanism allows either the blocking of mRNA translation or the production of different protein isoforms that may provide a variety of functions via modulation of protein-protein interactions.

Wounding of the seed has been proposed to post-transcriptionally up-regulate *ARR22* (Gattolin et al., 2006). It was additionally suggested

that wounding could in fact promote ARR22 protein expression without eliciting a change in transcript level as studies have shown that mRNA and protein expression levels are not always parallel (Gygi et al., 1999; Gfeller et al., 2011). An antibody was designed and produced using an amino acid sequence present in ARR22 and its putative *B. napus* orthologues to explore this hypothesis in *B. napus* seeds. This study did not show an alteration in the splicing profile but rather observed an up-regulation in gene and protein expression (see **section 4.5.1 and section 4.6; Chapter 4**). In a Western blot analysis the expression of BnRR76 – BnRR79 protein in 20 DAF seeds was up-regulated 60 mins post-wounding which was subsequently decreased at 120 mins. In contrast, the gene expression level at 60 mins was comparable to the control however an up-regulation was induced at 120 mins. This evidence suggests that *BnRR76 – BnRR79* protein is in fact produced to generate a rapid response, presumably to activate defence signalling pathways or modify plant metabolism.

The gene expression of a small number of major SSPs and protease genes was additionally examined in response to wounding up to 120 mins post-wounding. No down-regulation in SSP gene expression or up-regulation in cysteine protease gene expression were detected in *B. napus* seeds (see **section 4.5.2; Chapter 4**) and hence the results of the present study did not correspond to that of the microarray analysis in *Arabidopsis* (Naomab, 2008) in which a number of gene expression

changes were detected 90 mins post-wounding. It could be that, as *B. napus* is larger than *Arabidopsis*, 120 mins does not present a sufficient time in which to detect changes and as such it may take hours for a response to be elicited.

It has been proposed that *ARR22* may interfere with cytokinin signalling as plants ectopically overexpressing *ARR22* resemble cytokinin receptor mutants (Kiba et al., 2004; Horak et al., 2008). During this study a unique DEX-inducible system (Kang et al., 2013) was employed to explore the effects of overexpressing *ARR22* at defined developmental stages. Severe phenotypic effects were observed when *ARR22* overexpression was induced pre- and post-floral induction and these indeed resembled cytokinin receptor mutants (see **Chapter 5**). Furthermore a high rate of pod abortion was noted which further supports the hypothesis that *ARR22* may interfere with events associated with ovule fertilisation.

This study also explored the role and importance of the predicted phospho-accepting site at amino acid 74 in ARR22. It had been reported that mutation of this site from an Asp to an Asn residue prohibits an enhanced response to dehydration and freezing stress tolerance, thus indicating that this site may be crucial for protein function (Kang et al., 2013). As no phenotypic differences were observed in the DEX-induced $ARR22^{D74N}$ lines when compared to the unmodified lines it is

hypothesised that ARR22 may be interacting with other components or pathways in non-stressed conditions. One aim was to specifically induce overexpression in siliques to monitor seed development; however this study failed to successfully overexpress *ARR22* throughout whole siliques. Fine tuning of the application of DEX will allow full developmental and phenotypic effects of *ARR22* overexpression to be analysed and the role and function of the predicted phosphorylation site to be investigated during seed development.

FUTURE WORK

- ***Exploring the wound response in B. napus* seeds**

  As 120 mins post-wounding may not provide a sufficient time point to monitor SSP and protease expression changes in *B. napus*, longer time points *in planta* should be analysed. It may also be necessary to excise siliques as previously carried out in *Arabidopsis* to rule out excision as a wound response initiator.

- ***Exploring BnRR76 – BnRR79 response to abiotic stress***

  This study focussed on mechanical damage to seeds. As it has been reported that *ARR22* is involved in drought tolerance (Kang et al., 2013) this should be explored in *B. napus*. Specifically, gene and protein expression should be analysed in seeds and vegetative tissues in response to a variety of stresses including drought and temperature. Stressing plants at specific developmental stages, particularly during seed filling, and analysing *BnRR76 – BnRR79* gene and protein expression could also be coupled with monitoring the expression of SSPs and protease genes.

- ***DEX-inducible overexpression of ARR22***

  A highly important avenue is the monitoring of *Arabidopsis* seed development during the induced overexpression of *ARR22* in terms of seed phenotype, contents, gene expression and hormone

levels. A mechanism of inducing overexpression specifically in siliques would be favoured which may involve injecting DEX into the pedicle or silique however this may induce a wound response. Alternatively overexpressing *ARR22* post-floral induction may be explored using a hydroponics system to control the exact volume of DEX applied. Additional studies may also explore the use of a silique specific promoter in a DEX-inducible system to ensure precise overexpression in siliques and seeds.

- ***Co-Immunoprecipitation***

  One of the major outstanding questions is what ARR22 and putative *B. napus* orthologues BnRR76 – BnRR79 bind to. Elucidating the signalling network and downstream components is critical for fully understanding the role of *ARR22* and its putative *Brassica* orthologues. A possible technique that may aid in clarifying this is co-immunoprecipitation which could utilise the designed *ARR22* and *BnRR76 – BnRR79* antibody to pull out protein complexes in both *Arabidopsis* and *B. napus*.

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APPENDIX I

List of primers

Gene	Primer Name	Sequence (5' - 3')
Bra040204	SAC29_For5’ (F1)	CAGCAAAATTCATGTAAAAGATGC
Bra040204	SAC29_For (F2) ORF	GGGGGTATTTCACAGACAGC
	SAC29_Rev	TCCATCAAGCATCCATGAGTT
BnaA05g33120D BnaC03g33640D BnaC05g47370D BnaA03gXXXXXD	Nested_For	CAATTCACAATCTTCTTTAGAATCCA
	Nested_Rev	TGAAGTCACCCCAACAATCA
Bra040204 BnaA05g33120D	Brapa_Mismatch_For	GACAGCAAATAACGGC
Bol001327 BnaC05g47370D	Bol_Mismatch_For	GACAGCGAATAACGGT
BnaA05g33120D BnaC05g47370D	ExtraAAspan_For	CGAGAAAATAAAGAAGAAACTAAACG
BnaA05g33120D	BnA05_For	AAATCGAAGATGGCAACAAA
	BnaA05_Rev	AAGTCACCCCAACAATCATTGAC
BnaA03g19150D BnaC03g22790D	Bnapus_ARR16_For	GAATGCGATTAGAGCATTGGA
	Bnapus_ARR16_Rev	TGAGCTCCACTAGCTAAACA
BnaAnng25110D BnaA10g23650D BnaC02g01700D BnaC09g48380D	Bnapus_ARR21_For	TCAGCTTGTTTGATGATCTTGG
	Bnapus_ARR21_Rev	CGGATTCAAGAACGACCAGT
BnaA04g14760D BnaC04g56320D	Bnapus_ARR12_For	TGTTGACATGCCTGATATGGA
	Bnapus_ARR12_Rev	TCAGCTTCTCAACATTCATCAGA
BnaA04g02540D BnaA09g35830D BnaC04g55620D BnaC08g27330D	Bnapus_ARR17_For	ATGGGATCAGAGCATTGGAG
	Bnapus_ARR17_Rev	GCTTCTGCAGTTTAAGAGATGACA
FJ529184.1	Bnapus UBQ10.1_For	TAAAAACTTTCTCTCAATTCTCTCT
	Bnapus UBQ10.1_Rev	TTGTCAATGGTGTCGGAGCTT
DQ209288	B.napusCys_For	CAGCTGAAAACGTCGGTGTA
	B.napusCys_Rev	TCTTCCCCATCTCCATCTTG

J02798	B.napusNapA_For B.napusNapA_Rev	CTTCTCACCAATGCCTCCAT TTTAACCGCTTTGGATGCTC
AY208880	SeedSpecific_For SeedSpecific_Rev	ACTCTAATGGTCATCACATTGGT ATCTAAGACTTTGCGAGCGT
X59294.1	Cruciferin_For Cruciferin_R	GCTCGGCTCTCATCTCTTCT TCAGTGTTTCAACCAAGCGG
AT4G05320	ArabidopsisUBQ10_For ArabidopsisUBQ10_Rev	TAAAAACTTTCTCTCAATTCTCTCT TTGTCAATGGTGTCGGAGCTT
AT3G04280	GattolinARR22_For GattolinARR22_Rev	TGATGCAATGCCTACCTTCTTAG ATTAATGAGCTCTCATCCATCAAGCATCG
AT3G04280	KangARR22DEX_For KangARR22DEX_Rev	GAGAAAACCAAGTCGATAGAAGTGA CAAGCATCGAAGAGGTGGCTAATG
Universal primers	M13_For M13_Rev	GTAAAAACGACGGCCAG CAGGAAACAGCTATGAC

APPENDIX II

Full genomic alignment

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BnRR77      ------------------------------------------------------------
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BnRR79      ------------------------------------------------------------
ARR22       atattagtttgttata-caactcacttagaataatgtagattacatttcagccaaattca
BnRR78      ------------------------------------------------------------
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BrRR39      ----------taagacaactcagttgcaacgatgcagattacattttaggaaagttca
BnRR76      ------------------------------------------------------------


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BnRR77      ------------------------------------------------------------
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BnRR79      ------------------------------------------------------------
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BnRR78      ------------------------------------------------------------
BoRR38      tgtaagaaagatattttgcattgtggtgtgaaaatatgcctctttcacttttt-caact
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BnRR76      ------------------------------------------------------------


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BnRR76      ------------------------------------------------------------


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BnRR76      ------------------------------------------------------------------


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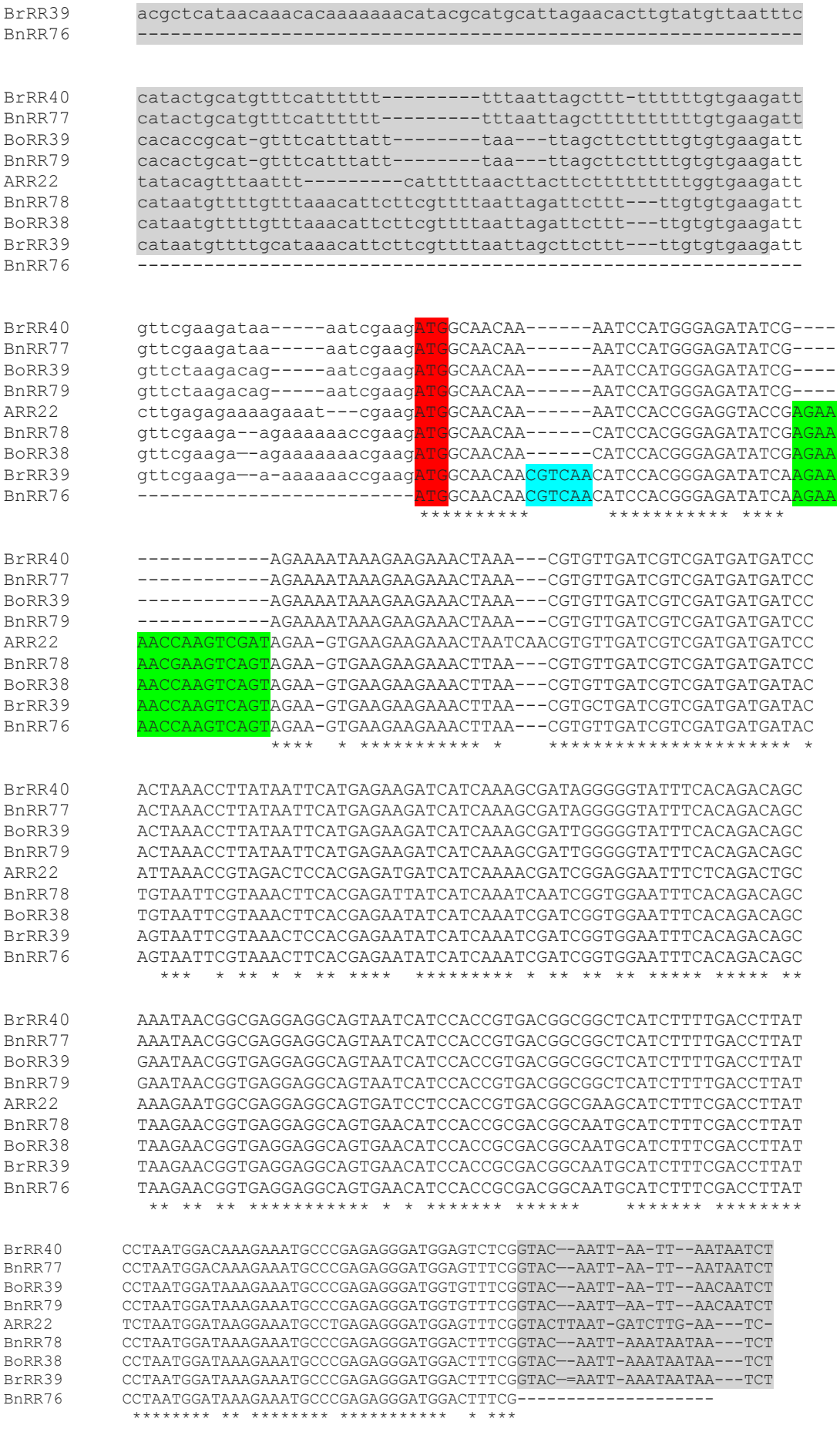
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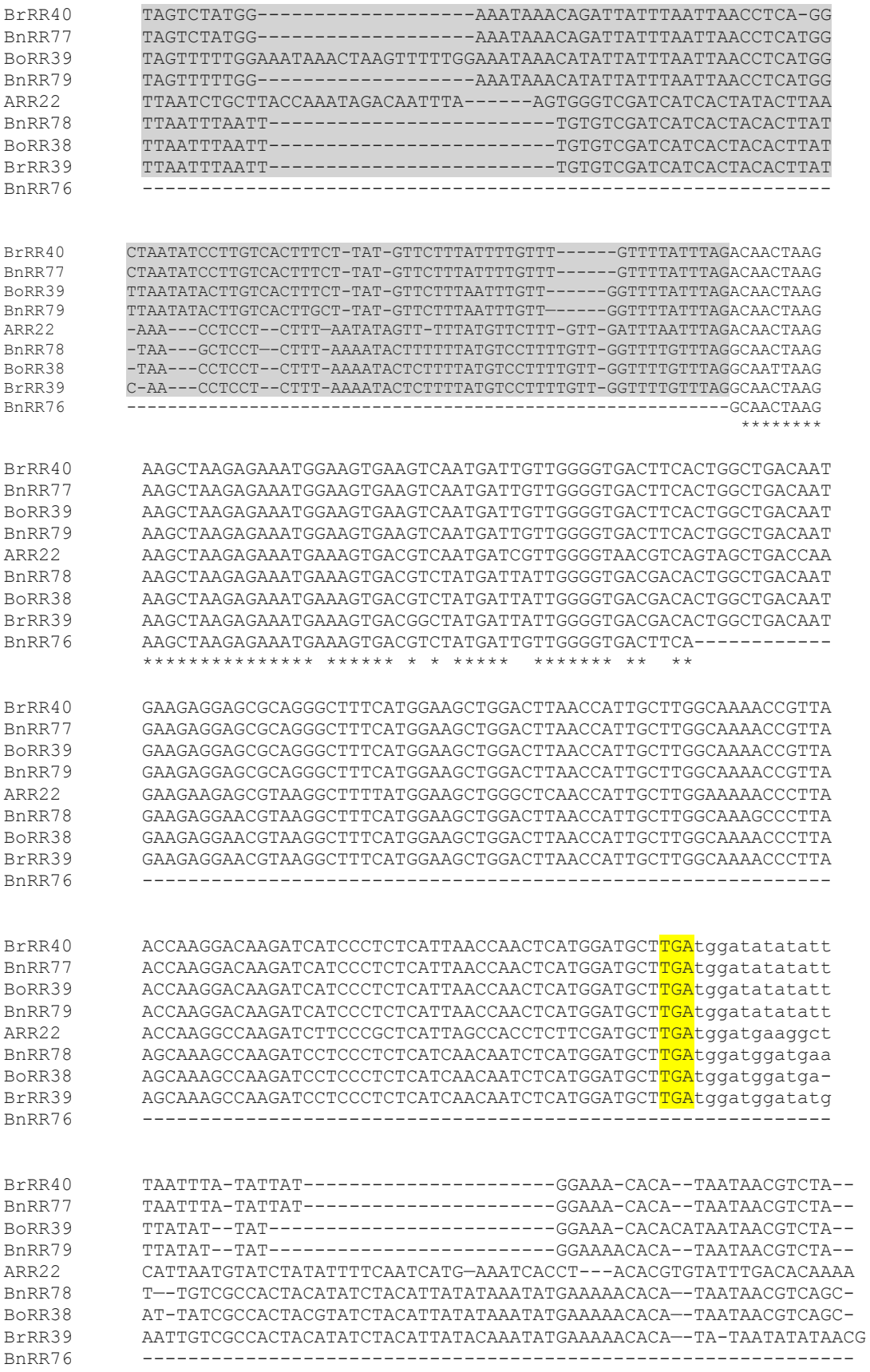
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BrRR39 AAGCTAAGAGAAATGAAAGTGACGGCTATGATTATTGGGGTGACGACACTGGCTGACAAT
BnRR76 AAGCTAAGAGAAATGAAAGTGACGTCTATGATTGTTGGGGTGACTTCA----------
********** ****** * * ***** ******* ** **
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BnRR77 GAAGAGGAGCGCAGGGCTTTCATGGAAGCTGGACTTAACCATTGCTTGGCAAAACCGTTA
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BnRR78 GAAGAGGAACGTAAGGCTTTCATGGAAGCTGGACTTAACCATTGCTTGGCAAAGCCCTTA
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BnRR76 ----------
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ARR22 ACCAAGGCCAAGATCTTCCCGCTCATTAGCCACCTCTTCGATGCTTGAtggatgaaggct
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BoRR38 AGCAAAGCCAAGATCCTCCCTCTCATCAACAATCTCATGGATGCTTGAtggatggatga-
BrRR39 AGCAAAGCCAAGATCCTCCCTCTCATCAACAATCTCATGGATGCTTGAtggatggatatg
BnRR76 ----------
BrRR40 TAATTTA-TATTAT----------GGAAA-CACA--TAATAACGTCTA--
BnRR77 TAATTTA-TATTAT----------GGAAA-CACA--TAATAACGTCTA--
BoRR39 TTATAT--TAT----------GGAAA-CACACATAATAACGTCTA--
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BnRR78 T--TGTCGCCACTACATATCTACATTATATAAATATGAAAAACACA--TAATAACGTCAGC-
BoRR38 AT-TATCGCCACTACGTATCTACATTATATAAATATGAAAAACACA--TAATAACGTCAGC-
BrRR39 AATTGTCGCCACTACATATCTACATTATACAAATATGAAAAACACA--TA-TAATATATAACG
BnRR76 ----------

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BrRR40      -----------AGTGTGTATGTATGCATAGATACTTGCATGTGTGTGTTTTAGAATTTAG
BnRR77      -----------AGTGTGTATGTATGCATAGATACTTGCATGTGTGTGTTTTAGAATTTAG
BoRR39      -----------AGTGTGTATGTATGCATAGATACTTGCATGTGTGTGTTTTAGAATTTAG
BnRR79      -----------AGTGTGTATGTATGCATAGATACTTGCATGTGTGTGTTTTAGAATTTAG
ARR22       ATCTGCATTTGTT--------GTGATATAGGGTTTCTCA----------------TATCT
BnRR78      TTATACACCTGTGTGTGTAT--GCATATATCTATCTGCATGTGTGTGTTTTAGGGTTGTT
BoRR38      TTATACACCTGTGTGTGTATGCATAGATATCTATCTGCATGTGTGTGTTTTAGGGTTGTT
BrRR39      TCATACACCTGTGTGTGTATGCATAGATATCTATCCGCATGTGTGTTT-TTAGGGTTGTT
BnRR76      ------------------------------------------------------------


BrRR40      GGT----------TCTTTATCGTCCGTGATATA--TAATCATGTAACCTGTTGCTTTAAGCT
BnRR77      GGT----------TCTTTATCGTCCGTGATATA--TAATCATGTAAGCTGTTGCTTCAAGCT
BoRR39      GGT----------TCTTTATCGTCCGTGATATA--TAATCATGTAAGTTGTTGCTTTAAGCT
BnRR79      GGT----------TCTTTATCGTCCGTGATATA--TAATCATGTAAGTTGTTGCTTTAAGCT
ARR22       ATGTTTGATT--TATTTTCTTATC-GTCCGAGGTAAAATCATGCAAGTCATTTCTTTTGGCT
BnRR78      ATGTTTGATTTTTATCGTGCGTGGCGTGATATACA--ATCATGTAAGTCATTACTTT-GGCT
BoRR38      ATGTTTGATTTTTATTGTGCGTGGCGTGATATACG--ATCATGCAAGTCGTTACTTTTGGCT
BrRR39      ATGTTTGATTTTTATTGTGCGTGGCGTGATATACA--GTCATGTAAGTCGTTACTTTTGGCT
BnRR76      ------------------------------------------------------------


BrRR40      TATAAAATATTAAAATAAGGGTTT----------------------
BnRR77      TATAAAATATTAAAATAAGGGTTTCCTC------------------
BoRR39      TATAAAATATTTAAATAAGGGTTTCCT-------------------
BnRR79      TATAAAATATTTAAATAAGGGTTTCCTCTACCAGAAAAAAAAA--
ARR22       AATAAAATATTAAAATAAGGTTTTCTCTT----------------
BnRR78      TATAAAATAAT-GAATAAGATTT-CTTATGATCAGATGCATTC--
BoRR38      TATAAAATAAT-GAATAAGATTT-GTTATGA--------------
BrRR39      TATAAAATAAT-GAATAAGATTT----------------------
BnRR76      ---------------------------------------------
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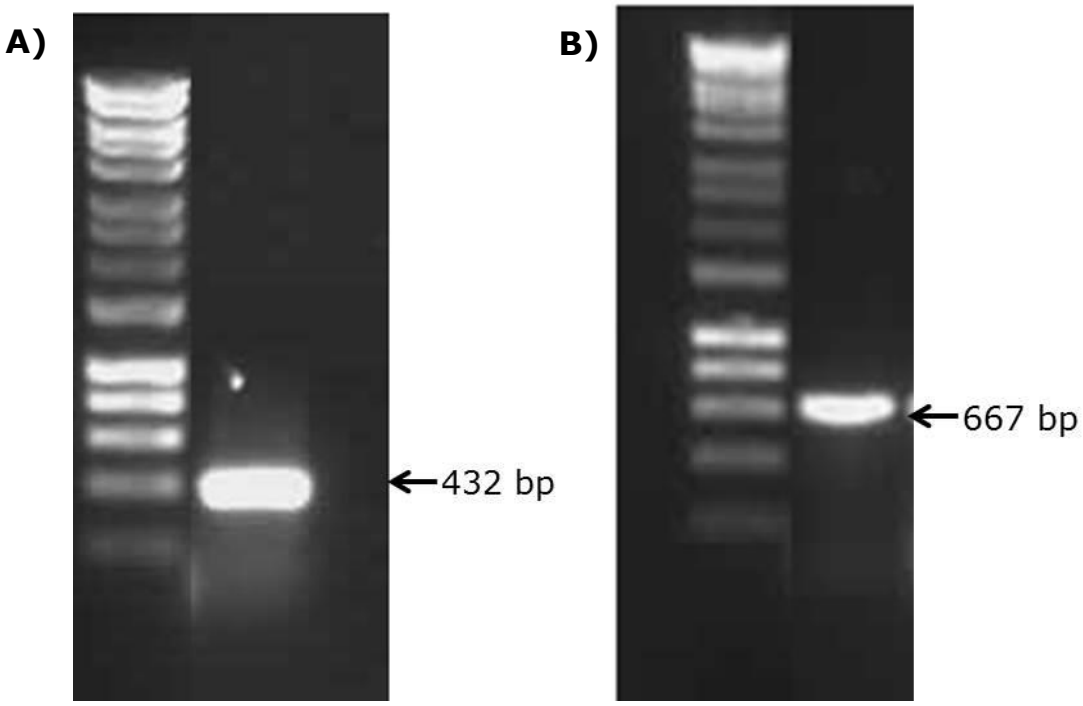
APPENDIX III



(**A**) PCR using forward primer designed in ORF of *BrRR40* to amplify transcript in *B.oleracea* genomic DNA for cloning and sequencing. (**B**) PCR confirming transformed *E.coli* colony with inserted *B.oleracea* gDNA. Genomic DNA was extracted from leaf tissue in kale.

APPENDIX 4

PIPS Reflective Statement Template

Note to examiners:

This statement is included as an appendix to the thesis in order that the thesis accurately captures the PhD training experienced by the candidate as a BBSRC Doctoral Training Partnership student.

The Professional Internship for PhD Students is a compulsory 3-month placement which must be undertaken by DTP students. It is usually centred on a specific project and must not be related to the PhD project. This reflective statement is designed to capture the skills development which has taken place during the student's placement and the impact on their career plans it has had.

PIPS Reflective Statement

Between April and July 2015 I carried out my placement as a Campaigns Intern at Sense About Science, a charitable trust that is focused on promoting public understanding of science and evidence. During the 3-months I carried out a huge variety of tasks but I was primarily focussed on part management of the Plant Science and Energy Panels. These are two online resources comprised of a board of scientists that cover the width and breadth of plant sciences and the energy and climate sciences. They allow the public to put their concerns and curiosities, often driven by topics they have seen within the media or online, to an expert in that subject. My role was to advertise the panels via social media channels and writing blogs for learned societies to drum up questions as well as conversing with researchers to put together a lay response. I also helped put together and oversee two live online Q&As which addressed the much debated topic of fracking and the threat to potatoes. They were two exciting one hour fast paced sessions that required co-operation from the whole office.

I was additionally heavily involved with the Voice of Young Science (VoYS) campaign which is essentially a community of early career researchers (PhD and Post-Doc) that play an active role in public debate on scientific issues. As part of this I was involved in 'asking for evidence' behind certain topics such as food science/ nutrition and allergies and successfully put together a number of engaging webpages. In addition to the office work I regularly represented Sense About Science at events such as Parliamentary Links Day, Delivering the UK AgriTech Strategy, Cheltenham Science Festival and the Soil Association's Glyphosate Briefing.

In terms of skills acquisition I found the internship to be highly profitable and an invaluable opportunity which has certainly energised my CV. In just 3-months I very rapidly developed my communicative skills, particularly in writing, which has both provided a lifelong ability as well as undoubtedly aiding in the completion of my thesis. Moreover I cultivated expertise in creativity, organisation and networking. This internship truly opened my eyes to the world that lies outside of academia and has directed me to pursue a career to build upon my existing abilities in communication and engagement. I honestly believe that all PhD students should undertake a placement to obtain the skills that you would not necessarily acquire from the lab.