Isolation and characterisation of mutants with altered seminal root numbers in hexaploid wheat

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ABSTRACT

The root is the main channel for water and nutrient uptake in plants. Optimisation of root architecture provides a viable strategy to improve nutrient and water uptake efficiency and maintain crop productivity under water-limiting and nutrient-poor conditions. We know little, however, about the genetic control of root development in wheat, a crop supplying 20% of global calorie and protein intake. To improve our understanding of the genetic control of root development in wheat, we conducted a high-throughput screen for variation in seminal root number using an exome-sequenced hexaploid wheat mutant population. The screen identified eight independent mutants with homozygous and stably inherited **a**ltered seminal **r**oot **n**umber (*arn*) phenotypes, referred to as *arn1 to arn8*. One mutant, *arn1*, displays a recessive extra seminal root number phenotype, while six mutants (*arn2*, *arn4 to arn8*) show dominant lower seminal root number phenotypes. We show that the lower seminal root number phenotype of *arn2, arn4 to arn8* originates from defects in the formation and activation of seminal root primordia. Segregation analysis in F_2 populations suggest that the *arn1* phenotype is controlled by multiple genes whereas the *arn2* phenotype fits a 3:1 mutant:wild-type segregation ratio characteristic of dominant single gene action. This work highlights the potential to use the sequenced wheat mutant population as a forward genetic resource to uncover novel variation in agronomic traits, such as seminal root architecture. Characterisation of the mutants and identification of the genes defining this variation should aid our understanding of root development in wheat.

INTRODUCTION

The 1960's Green Revolution demonstrated the impact that changes to plant architecture in major crops like wheat and rice can have on increasing food production (Hedden 2003). While the Green Revolution focused on improving shoot architecture, it did not optimise root architecture, in part because selection was primarily for performance under management regimes involving high rates of fertiliser application (Lynch 2007). In addition to providing anchorage, the root is the main channel for water and nutrient uptake in crops and serves as an interface for symbiotic interaction with the soil microbiome. Roots are therefore often considered as the hidden and neglected other-half of plant architecture and have not been a direct target for selection during early wheat domestication and in modern wheat breeding programmes (Waines and Ehdaie 2007). In many environments, water-availability is the main factor defining crop rotations and performance. Projections on future climate forecast more variable weather events relating to the timings and intensity of precipitations which could negatively affect food security (Cattivelli et al. 2008). Optimising root system architecture (RSA) for improved nutrient and water uptake under these uncertain scenarios provides a rational approach to help achieve future food and nutrition security.

The wheat root system is comprised of two main root types, seminal (embryonic) and nodal (post-embryonic) roots, that develop at different times and perform important yet different functions. The seminal root system develops from the root primordia in the embryo of a germinating wheat seed and is comprised of a primary root that emerges first followed by two pairs of secondary seminal roots that emerge sequentially. As the first root type that emerges, seminal roots are entirely responsible for nutrient and water uptake in seedlings. Seminal roots are therefore important for seedling vigour and early plant establishment which determines competitiveness against weeds. Nodal roots on the other hand are shoot-borne

and develop soon after tillering to provide anchorage and support resource uptake especially during the reproductive stage of wheat growth.

Seminal roots may remain functionally active through to the reproductive stage and may grow up to 2 m in length (Sanguineti et al. 2007; Manschadi et al. 2013). They have been shown to have similar nutrient uptake efficiency as nodal roots in wheat and contribute to yield potential especially under conditions of low soil moisture where nodal roots may not grow (Weaver and Zink 1945; Sanguineti et al. 2007; Sebastian et al. 2016). Given their importance, seminal root traits, such as angle and number, have been linked to adaptive responses under water limiting conditions (Manschadi et al. 2008; Cane et al. 2014; Golan et al. 2018). Steep seminal root angle has been associated with increased soil water exploration at depth which is beneficial in drought condition where topsoil moisture is depleted (Richard et al. 2015; Olivares-Villegas et al. 2007; Manschadi et al. 2008).

Genetic variation for seminal root number exists among wheat genotypes. Typically, most wheat cultivars develop between three to six seminal roots (Araki and Iijima 2001). A few quantitative trait loci (QTL) have been identified to underlie variation in seminal root number in wheat germplasm (Atkinson et al. 2015; Maccaferri et al. 2016; Ren et al. 2012; Sanguineti et al. 2007; Ma et al. 2017; Iannucci et al. 2017). However, unlike other cereals (e.g. rice, maize) only one gene controlling root system architecture (RSA), *VRN1* (Voss-Fels et al. 2018), has been identified in wheat.

This delay in identifying genetic loci controlling root traits is most likely due to a series of factors, which make genetic analyses in wheat difficult. Bread wheat is a hexaploid plant with a relatively large (15 Mb) and repeat-rich (>85%) genome comprised of three homoeologous sub-genomes (A, B and D). High sequence similarity in the coding region of these sub-genomes results in high levels of genetic redundancy that mask the phenotypic effects of underlying natural variation for many traits, including RSA traits (Uauy et al. 2017). Also, the

112 "out-of-sight" nature and extreme phenotypic plasticity of roots under native field conditions
113 makes root phenotyping difficult, cumbersome and time-consuming.
114 The use of induced variation has proven useful to uncover novel phenotypes and dissect
115 genetic pathways underlying complex phenotypes in plants (Parry et al. 2009). Our current
116 understanding of the genetic determinants regulating root development in many cereals have
117 almost entirely stemmed from the isolation and characterisation of mutants defective in one
118 or more RSA traits (Reviewed in Coudert et al. 2010; Hochholdinger et al. 2018; Marcon et
119 al. 2013). Genetic variation for seminal root number in modern wheat germplasm has been
120 defined to broad QTL which makes their genetic dissection difficult and their use in breeding
121 limited. Exploiting induced variation present in mutant populations represents an alternative
122 strategy to identify variation that can be used to improve RSA.
123 Mutation analyses, however, have not hitherto been exploited for studying the genetic
124 architecture of root development in wheat, most likely due to the genetic redundancy often
125 seen in polyploids (Krasileva et al. 2017; Uauy et al. 2017). The recent development of an *in-*
126 *silico* platform for the rapid identification of mutations in 1,200 mutants of the UK hexaploid
127 wheat cultivar Cadenza now makes large-scale reverse and forward genetic investigation of
128 traits more feasible in wheat (Krasileva et al. 2017). Progress has also been made on the
129 root phenomics front, with the development of fast, low-cost, and flexible two-dimensional
130 (2D) root phenotyping pipelines with sufficient throughput for phenotyping large populations
131 (Selvara et al. 2013; Atkinson et al. 2015)
132 Taking advantage of these new developments, we conducted a forward genetic screen for
133 variation in seminal root number using a subset of the exome-sequenced mutant population
134 in the Cadenza background. From this work, we describe the isolation and morphological,
135 anatomical and genetic characterisation of mutants with decreased and increased numbers
136 of seminal roots.

MATERIALS AND METHODS

Mutant Population

The hexaploid wheat TILLING population previously developed by ethyl methanesulfonate (EMS) treatment of a UK bread wheat cultivar - Cadenza (Krasileva et al. 2017; Rakszegi et al. 2010) was used for this study. Part of this population, 663 mutants, was used in this study. All the mutants used show greater than 90% field germination rate. To ensure homogeneity of phenotype and reduce the masking effect of segregating background mutations, single spikes harvested from field-grown M_4 plants were individually threshed and derived M_5 seeds were used for the forward screen.

High-throughput seminal root phenotyping

Primary Screen: To phenotype seminal root traits of the Cadenza mutant population, we developed a 2D root phenotyping platform based on the protocol described by Atkinson et al. (2015) with some modifications to increase the throughput from 360 to 1,800 seedlings per run. In brief: Lines were first size-stratified as having either large, medium or small-sized seeds by sieving the seeds through two sets of calibrated graduated sieves with 2.8 mm and 3.35 mm mesh sizes. Large-sized seeds were collected above the 3.35 mm sieve, medium-sized seeds collected between the 2.8 mm and 3.35 mm mesh, and small-sized seeds were collected below the 2.8 mm sieve. Seeds (15 – 20 per line) were surface sterilized by rinsing in 5% (v/v) Sodium Hypochlorite (Sigma Aldrich, UK) for 10 mins and were rinsed with water three times before being imbibed in 1.75 mL of water for 5 days at 4°C to ensure uniform germination. Germinated seeds (with seed coat ruptured) were placed crease facing down into individual growth pouches made from a sheet of germination paper (21.5 cm x 28 cm; Anchor Paper Company, St Paul, MN, USA) clipped to a black polythene sheet (22 cm x 28

cm, 75 µm thickness, Cransford Polythene LTD, Suffolk, UK) using an acrylic rod and 18 mm fold clip (Figure 1). The growth pouches were suspended in an upright position in plastic storage boxes (120 cm x 27 cm x 36 cm, Really Useful Product, West Yorkshire, UK) with 60 pouches per box. The sides of the box were covered in black plastic sticky back cover film to block out light from the roots of the developing seedlings. The bottom of each box was filled with 10 L of half-strength Hoaglands growth solution containing (Hoagland and Arnon 1950): $NH_4H_2PO_4$, 0.6 g; $Ca(NO_3)_2$, 3.3 g; $MgSO_4$, 1.2 g; KNO_3, 1.0 g; H_3BO_3, 14.3 mg; Cu_2SO_4, 0.4 mg; $MnCl_2(H_2O)_4$, 9.1 mg; MoO_3, 0.1 mg; $ZnSO_4$, 1.1 mg; $KHCO_3$, 2.0 g, Ferric Tartrate, 2.8 g. The base of each pouch was suspended in the growth solution to supply nutrients to the developing seedling through capillary action. A randomised complete block design was adopted with each line replicated 10 times across different boxes (block). The phenotyping boxes were placed in a controlled environment room under long day conditions with 16h light (250–400 mmol) at 20°C, 8h darkness at 15°C and at 70% relative humidity. After 7 days of growth, pouches were taken out of the phenotyping box; placed on a copy stand and the black plastic sheet covering the germination paper was gently pulled back to reveal the roots. Images of the roots were taken with a Nixon D3400 DSLR Camera fitted to the copy stand. Phenotyping of the mutant population was split over five experiments. The same phenotyping set-up was used for the validation experiments (phenotyping of three additional spikes) and to characterise M_6 and F_1 progenies.

Secondary Screen: A secondary screen was carried out using a subset of the lines used in the primary screen. From each line, 10 visually uniform M_5 seeds were selected and placed onto moist filter paper in a 90 mm round petri dish. Petri dishes were wrapped in aluminium foil to exclude light and placed at 4°C for 2 days, seeds were placed crease side down into individual CYG seed germination pouches (Mega-International, Minnesota, USA) with the

bottom removed to allow wicking of growth solution from a reservoir of media. Pouches were wrapped in aluminium foil in batches of 5 to exclude light from the roots and were placed upright in in a reservoir of full strength Hoaglands No 2 growth solution ($NH_4H_2PO_4$, 115.03 mg; $Ca(NO_3)_2$, 656.4 mg; $MgSO_4$, 240.76 mg; KNO_3, 606.6 mg; H_3BO_3, 2.86 mg; Cu_2SO_4, 0.08 mg; $MnCl_2(H_2O)_4$, 1.81 mg; MoO_3, 0.016 mg; $ZnSO_4$, 0.22 mg; Ferric Tartrate, 5 mg. per Litre). Pouches were placed in long day conditions (as above) and were allowed to grow for 5 days before roots were imaged. For imaging, pouches were placed onto a copy stand; the front of the pouch carefully removed, and the root system imaged using a Sony Cybershot DSC-RX100. Plants were screened in rounds of twenty lines with a total of 200 plants per round.

Image analysis: High-resolution images captured from the phenotyping were pre-processed (rotated, cropped and compressed) using ImageJ (https://imagej.nih.gov/index.html) and Caesium image compressor before being processed in RootNav (Pound et al. 2013). Captured root architectures were imported into RootNav viewer database for measurement of RSA traits using standard RootNav functions.

Anatomical characterisation of seminal root primordia

Embryos from mature dry grains were fixed in FAA solution (10% formaldehyde, 5% acetic acid, 50% ethanol, and 35% distilled water by volume) overnight and dehydrated at room temperature in a graded ethanol series (30 min each, in 50%, 70%,90%, 95%, and 100% ethanol). Then, embryos were cleared in xylene, embedded in paraffin and sectioned (5 µM) using a microtome (Leica Biosystems, Germany). Cross sections were de-paraffinized with histoclear, rehydrated and stained with Harris Hematoxylin. A stereo microscope (SZX16, Olympus, Tokyo, Japan) was used for imaging.

211 **Statistical analysis**

212 All statistical analyses were performed in R 3.5.1 (R Core Team 2018) and Minitab 17
213 statistical software. Statistically significant root architectural difference in the primary
214 screening experiment was determined by ANOVA using a Dunnett's comparison within each
215 phenotyping batch with the Cadenza plants in each batch used as controls. Adjusted
216 probability values of ≤ 0.05 were considered statistically significant. Statistically significant
217 root architectural difference in the validation experiment as well as in the M_6 and F_1
218 phenotyping experiments were based on Student's t-test comparison of individual spike/line
219 to the Cadenza control.

220

221 Seeds of mutant lines reported in this study can be ordered through the SeedStor site at
222 www.seedstor.ac.uk. Supplemental materials are available at *FigShare*. Table S1 contains
223 information on mutants with significantly different seminal root number phenotypes to
224 Cadenza. Table S3 contains information on all the mutants phenotyped in both the primary
225 and seconday screen. Table S3 contains information on summary ANOVA statistics of
226 seminal root number comparison in the M_6 and F_1 generation.

227

228

229 **RESULTS**

230 **Identifying induced variation for seminal root number in hexaploid wheat**

231 We developed a rapid root phenotyping platform suitable for large-scale phenotyping at a
232 throughput of 1,800 seedling per run (Figure 1). Using this platform, we performed a screen
233 for variation in seminal root number using 663 seed-size stratified (small, medium and large)
234 M_5 mutants from the exome-sequenced Cadenza TILLING population. Cadenza mainly

displayed five seminal roots (4.9 ± 0.05) including a primary seminal root (SR_1), as well as first ($SR_{2,3}$) and second ($SR_{4,5}$) pairs of seminal root (Figure 2A). We observed variation in seminal root number in the mutant population, with seminal root number ranging from 1 to 7 in individual plants and mean seminal root number per mutant ($n \geq 4$ plants per mutant) ranging from 2.9 to 5.9 (Figure 2B). Consistent with Cadenza seminal root architecture, 82% of the mutants phenotyped had a modal seminal root number of 5.

Seed size groups showed significant difference in seminal root number ($P < 0.0001$) and total root length ($P < 0.0001$) with mutants with large and medium-sized grains having an average seminal root number of 4.7 and 4.4, respectively, and total root length of 483.2 mm and 322.8 mm, respectively (Figure 2C-D). We observed a significant positive correlation ($R^2 = 0.31$, $P < 0.0001$) between the number of seminal roots and the total root length in the population.

Dunnett's multiple comparison identified 52 mutants with significantly different number of seminal roots relative to the Cadenza control within each of the three seed size groups (Table S2). Five of these mutants had significantly higher number of seminal root with mean seminal root number ranging from 5.7 to 5.9 and modal seminal root number of 6 per mutant (Table S1). The higher seminal root phenotype is mainly driven by the development of an extra root, hereafter referred to as SR_6. The remaining 47 mutants showed significantly lower number of seminal roots with mean seminal root number permutant of 2.9 to 4.1 and modal seminal root number between 3 and 5.

To further assess these lines, we phenotyped a subset (398 mutants) of the primary population in a secondary screen. This included 33 of the 52 mutants with significantly different seminal root number to Cadenza (four higher and 29 lower root count mutants). Details of each individual mutant line phenotyped in both the primary and secondary screens are presented in Table S2. We observed a significant positive correlation between seminal

root number measurements in the primary and secondary screen (R^2 = 0.39; *P* <0.001; Figure 2B). The heritability estimate of the seminal root measurement across the two screens was 0.77 suggesting a strong heritable genetic effect in the determination of seminal root number in the mutant population. We confirmed the phenotypes of three of the four higher root number mutants identified in the primary screen; these lines displayed mean seminal root number of 5.1 to 5.5 in the secondary screen. However, only 20 of the 29 lower root number mutants displayed fewer number of seminal roots (less than 4 roots) than Cadenza in the secondary screen. We subsequently selected all three higher and 20 lower root number mutants with consistent phenotype in both screens for further phenotypic validation.

altered root number (_arn_) mutants show stable homozygous seminal root number phenotypes

To validate the selected mutants, we phenotyped seeds from three additional M_5 spikes. These M_5 spikes originate from successive seed bulking of multiple M_3 and M_4 plants. Selecting three separate spikes increases the probability of phenotyping plants with independent background mutations thereby providing robust biological replications to examine the stability of the mutation effects and segregation patterns (homozygous or heterozygous). As controls, we also phenotyped additional spikes for two mutant lines that did not show any significant seminal root number difference in the primary and secondary screens.

For eight of the selected mutants, including a higher root number and seven lower root number mutants, we observed the altered seminal root number phenotype in the three additional spikes (Figure S1). This suggests that the phenotypes of these mutants are robust, consistent, and controlled by mutations that were homozygous in the original single M_2 plant

from which the population was derived. We subsequently named these eight mutants as ***a**ltered **r**oot number* **m**utants (*arn*): *arn1* for the higher root count mutant and *arn2* to *arn8* for the lower root count mutants (Table 1, Figure 3). It is noteworthy that the *arn* mutants show varying degree of phenotypic penetrance (percentage of plants displaying a phenotype) with *arn3* having the least penetrant phenotype and was therefore not characterised further. Six other mutants show alteration in seminal root number, but only in two of the three additional spikes examined (Table 1). These most likely represent mutations that were heterozygous in the initial single M_2 plant and that have segregated in the subsequent generations.

We further characterized the *arn* mutants from 1 to 7 days post germination (dpg) to examine when the *arn* phenotype was first visible and to identify the seminal root type (primary, first pair or second pair) defective in these mutants (Figure 4). Cadenza showed fully emerged primary root (SR_1) at 1 dpg, while the first ($SR_{2,3}$) and second pairs ($SR_{4,5}$) of seminal roots emerged at 3 and 5 dpg, respectively. Similar to Cadenza, *arn4* to *arn8* developed the primary and first pair of seminal roots at 1 dpg and 3 dpg, respectively, but are defective in the development of the second pair of seminal roots. Contrary to this, *arn1* shows a faster rate of seminal root development relative to Cadenza with the primary, first and second pair of seminal roots emerged by 3 dpg and an extra sixth root emerged at 7 dpg. *arn2* showed a strong dormancy phenotype and was not included in this experiment.

Lower root number *arn* mutants involve defects in primordia development and growth

Seminal roots emerge from root primordia that form in the embryo of a developing seed. Differential activation of root primordia of $SR_{4,5}$ has been shown to underlie variation in seminal root number in young seedlings (Golan et al., 2018). We therefore examined if the seminal root phenotypes of the lower root number *arn* mutants (*arn2, arn4 to arn8*) originate from altered root primordia activity or rather defects in primordia development. Cadenza (WT)

consistently develop five fully formed root primordia (Figure 5). All the lower root number *arn* mutants examined showed altered root primordia development compared to Cadenza, with the SR_4 or $SR_{4,5}$ primordia either absent or reduced in size (Figure 5). In addition, primordia activity was altered in the mutants, as all mutants had greater number of primordia compared to the number of roots recorded in the seedlings. Notably, *arn5* to *arn8* also have significantly smaller embryo compared to Cadenza (Figure S2).

Genetic characterisation of the *arn* phenotypes

To understand the transgenerational stability and mode of inheritance of the *arn* mutants, we characterized M_6 and F_1 progenies derived from crosses of the *arn* mutants to Cadenza. Like the M_5 phenotype, M_6 progenies of *arn1* showed significantly (P <0.0001) increased number of seminal roots compared to Cadenza with an average root number of 5.73 and more than 73% of the plants having six roots (Figure 6). F_1 progenies of *arn1* x Cadenza all had five seminal roots, similar to Cadenza (P = 0.19; Figure 6), suggesting that the *arn1* phenotype originates from a recessive mutation or is caused by a combination of loci segregating independently.

The M_6 plants of *arn2, arn4* to *arn7* all showed a significantly lower number of seminal roots (P <0.05) compared to Cadenza with mean root number ranging from of 2.6 to 4.6 (Figure 6). The F_1 progenies of these mutants also showed significantly (P <0.01) lower number of seminal roots compared to Cadenza, similar to their M_5 parents, with an average root number of between 3.2 and 3.9 (Figure 6). This suggests that the *arn2, arn4* to *arn7* phenotypes are caused by dominant mutations. Unlike its M_5 parent, the *arn2* x Cadenza F_1 did not show any reduced germination, suggesting that the dormancy phenotype of *arn2* segregates independently of its altered root number.

We further characterized F_2 progenies of the higher root number mutant, *arn1,* and the lower root number mutant, *arn2*. We used the chi-square test-statistic to test the goodness of fit of the inheritance pattern of *arn1* and *arn2* phenotypes to those consistent with segregation of single recessive and single dominant traits, respectively. The phenotype of *arn1* F_2 progenies (238) was not consistent with the expected 3:1 Cadenza:*arn1* phenotype segregation ratio of a single recessive gene (χ^2 = 52.71, *P* <0.0001), suggesting that multiple genes may be responsible for *arn1* phenotype. In contrast, the segregation pattern of the *arn2* F_2 population (51 plants) is consistent with the 3:1 *arn2*:Cadenza segregation ratio expected of a single dominant gene (χ^2 = 0.53, *P* = 0.4669), suggesting that the *arn2* phenotype is caused by a single dominant gene.

DISCUSSION

Mutant analyses have played a key role in the identification of genes controlling key stages of root development. For instance, all eight genes identified to control root architecture in maize were identified via mutant analyses (Hochholdinger et al. 2018; Marcon et al. 2013), including *RTCS, RTCL, RUM1* and *BIGE1* with seminal root phenotypes (Taramino et al. 2007; Suzuki et al. 2015; von Behrens et al. 2011; Xu et al. 2015). Despite the buffering effect of genetic redundancy that often mask single homoeolog mutations in polyploid wheat, our study highlights the usefulness of forward screens to identify heritable variation for root development traits in wheat.

arn mutants are useful for characterising known and novel RSA genes in wheat

The use of a sequenced mutant population in this study provided the opportunity to examine the presence of mutations in candidate genes from other species. For example, the higher seminal root number phenotype of *arn1* was similar to the phenotype of maize *bige1* (Suzuki et al. 2015) mutants. However, *in-silico* examination of mutations on the EnsemblPlant database show that *arn1* (Cadenza0900) does not harbour any mis-sense or non-sense mutations in the coding sequences of the three wheat homoeologs of *BIGE1* - *TraesCS4A02G350200, TraesCS5B02G522900 and TraesCS5D02G521600.* It is also noteworthy that the extra SR_6 root phenotype of *arn1* bears some similarity to the sixth root phenotype reported in some tetraploid wheat varieties (Sanguineti et al. 2007), suggesting that the gene underlying *arn1* might also be responsible for variation in the development of the sixth seminal root in natural populations.

Similarly, the lower seminal root number phenotypes of *arn2* to *arn8* are similar to the phenotypes of maize *rtcs* and *rtcl* mutations (Taramino et al. 2007), and their orthologous rice mutants (Liu et al. 2005; Inukai et al. 2005). *In-silico* examination of these coding regions in the *arn2* to *arn8* mutants revealed that *arn8* (Cadenza1273) contains a functional mutation in *TraesCS4D01G312800,* one of the three wheat homoeologs of *RTCS, RTCL* and *ARL1/CRL1*. *arn8* harbours a G765A mutation in *TraesCS4D01G312800* producing a premature termination codon which results in a truncated 265 amino acid (aa) protein instead of the 289 aa native protein. Further molecular and genetic characterisation will be required to test if the G765A mutation in Cadenza1273 is responsible for the *arn8* phenotype. However, this exemplifies the power of combining the sequenced mutant information with known candidate genes and now a fully annotated wheat genome (Appels et al. 2018). *arn2* to *arn7* do not contain any functional EMS mutations in the three wheat homoeologs of *RTCS, RTCL* and *ARL1/CRL1* and therefore might represent new variation controlling seminal root development in cereals. It is important to note that these *in-silico* investigations

are restricted to mutations in the coding region of the wheat genome and we cannot rule out that mutations in promoter regions of these candidate genes might be responsible for some of the *arn* mutants identified.

Relationship between grain size and seminal root traits

Size stratification of seeds in our study allowed an examination of the relationship between grain size and root architecture. We observed a positive effect of grain size on root length and number. Grain size account for 72% of the total variance in root length in the mutant population consistent with the rationale that bigger grains have more carbohydrate reserve to support faster root elongation. We also noticed a weak but positive effect of grain size on the number of seminal roots developed. Grain size effect on seedling traits, including seminal root traits, can be attributed to constituent components – embryo and/or endosperm (Bremner et al. 1963; Meyer 1976). Interestingly, four of the lower root number mutants (*arn5* – *arn8*) have smaller embryo compare to Cadenza. However, it is premature to conclude without further genetic analyses that the small embryo of these mutants directly affects their seminal root number phenotypes. More so, grain size only accounts for a small proportion (9%) of the total variance in root number in our study, suggesting that seed size per se is not a major determinant of root number. This is further underlined by the fact that differently sized wild-type Cadenza seeds show similar root number averages. In support of this, a recent report suggests that variation in seminal root number between wheat species mainly originates from genetic factors in the embryo rather than broad differences in seed morphology (Golan et al. 2018).

403 Although informative, the qualitative stratification of grain size (large, medium and small)
404 adopted in this study does not allow a quantitative modelling of grain size effect on root traits.
405 We propose that a finer calibration and partitioning of grain size measurement into
406 constituent parameters (width, length, height) and tissue (embryo and endosperm)
407 components (Brinton and Uauy 2018) will allow for a finer understanding of the effects of
408 these seed size components on root architecture.

409

410 **Developmental and genetic characterisation of seminal root development**

411 Natural variation in wheat seminal root number have been shown to originate from defects in
412 root primordia development in the embryo and/or differential activation of developed
413 primordia to form fully emerged seminal roots (Golan et al. 2018). Our anatomical
414 characterisation of intact non-imbibed seeds of the lower root number mutants (*arn2, arn4 to*
415 *arn8*) highlight a tendency for these mutants to develop less than five root primordia, whereas
416 wild-type Cadenza plants consistently develop five root primordia. In addition, these mutants
417 form even fewer number of seminal roots than the root primordia they developed, suggesting
418 that some primordia in these mutants might be dormant/inactive or arrested soon after
419 activation. Together these results highlight both primordia development and activation as two
420 important development check points in the formation of seminal root in wheat.

421 All the *arn* mutants develop SR_1 and $SR_{2,3}$ but show defects in the development of $SR_{4,5}$ as in
422 (*arn2* – *arn8*), or develop an extra root (SR_6) as in *arn1*. We could not recover mutants
423 defective in either SR_1 nor $SR_{2,3}$. This likely suggests that the developments of the different
424 seminal root types are under distinct genetic control, with SR_1 and $SR_{2,3}$ being more
425 conserved than $SR_{4,5}$ and SR_6. This notion is supported by reports that $SR_{4,5}$ show only
426 negligible contribution to water uptake and does not confer any beneficial fitness under well-

427 watered conditions (Golan et al. 2018). It is however possible that $SR_{4,5}$ and indeed SR_6 may
428 contribute significantly to nutrient and water uptake under resource-limiting conditions where
429 increase in root surface area maximises soil exploration. Detailed field physiological
430 evaluation will be required to better understand the cost-benefit relationship of the altered
431 seminal root phenotype of the *arn* mutants and evaluate the potential of these mutations to
432 improve resource uptake efficiency in plants.

433 Despite the high plasticity associated with root traits, we obtained a high heritability estimate
434 for seminal root number measurements across different experiments, similar to estimates
435 reported in other studies (Maccaferri et al. 2016; Ma et al. 2017). While these high heritability
436 may be due to the controlled hydroponic environment used in these experiments (Figure 1), it
437 nonetheless demonstrates that seminal root number is a stable phenotype under strong
438 genetic control and can be targeted for selection to improve RSA in wheat breeding
439 programmes. There is also evidence to suggest that seminal root number phenotypes
440 observed in hydrophonic conditions are transferrable to soil conditions (Richard et al. 2015)
441 and might therefore be useful under field conditions.

442 Most of the lower root number mutations (*arn2, arn4* to *arn7*) isolated in this study show a
443 dominant mode of action and for *arn2* we further documented this by the 3:1 segregation
444 ratios in the *arn2* F_2 populations. The dominant nature of these phenotypes makes it
445 impossible to test the allelism of these mutations and as such we cannot rule out the
446 possibility that these mutations are allelic and are controlled by the same gene. Unlike the
447 lower root number mutations, *arn1* shows a recessive, multigenic phenotype that might point
448 to high redundancy or multiple layers of gene regulation against the development of higher
449 (more than five) numbers of seminal roots in wheat. More detailed genetic characterisation
450 and mapping will be required to better dissect the genetic control of these traits.

451

452 **Future outlook**

453 Our work provides a complementary approach to the study of natural variation in dissecting
454 the genetic control of seminal root development in wheat. The isolation of these mutants
455 represents an important first step in identifying the genetic determinants controlling wheat
456 seminal root development and growth. These will be followed by extensive genetic
457 characterisation to map these mutations to defined chromosomal positions, examine
458 interactions between the alleles and identify the causal gene(s) underlying the *arn*
459 phenotypes.

460

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Figure Legends

570 Figure 1: Root phenotyping set-up: (A) Growth pouch. (B) Phenotyping box containing growth
571 pouch and nutrient solution at the bottom. (C) Root phenotyping in controlled environment
572 room. (D) Camera mounted on copy stand for root imaging.

573

574 Figure 2: Variation in seminal root number in the Cadenza wheat mutant population. (A)
575 Seminal root architecture of a Cadenza seedling showing the primary root (SR_1) and the first
576 ($SR_{2,3}$) and second pairs of seminal roots ($SR_{4,5}$). (B) Correlation of the seminal root number
577 phenotypes observed in the primary and secondary screens. Only lines phenotyped in both
578 screens are shown. The regression line from the two experiments (dotted diagonal line) is
579 compared to a perfect correlation (solid line) between the experiments. (C-D) Distribution of
580 the seminal root number (C) and total root length measurements (D) phenotypes observed in
581 the primary screen across large and medium seed size groups. The top, bottom, and mid-line
582 of the insert boxes represent the 75^{th} percentile, 25^{th} percentile and median of the
583 distribution, respectively, while coloured dots represent the data points for wild-type Cadenza
584 (black) and validated higher (red) and lower (white) root number mutants presented in Table
585 1.

586

587 Figure 3: *arn* mutants show homozygous seminal root number phenotypes. Seminal root
588 number distribution in the *arn* mutants and Cadenza (WT) across four spikes phenotyped in
589 the primary screen (spike 1) and validation experiments (spikes 2 to 4). The number of the
590 seeds phenotyped from each spike ranged from four to ten.

591

592 Figure 4: *arn* mutants show altered $SR_{4,5}$ and SR_6 seminal root types phenotypes. Temporal
593 characterization of the seminal root development of the *arn* mutants from 1 to 7 days post
594 germination (dpg).

595

596 Figure 5**:** Number of seminal root primordia in *arn* mutants. Longitudinal cross sections of *arn*
597 mutants prior to seed imbibition. The seminal root primordia of Cadenza are marked by
598 asterisks.

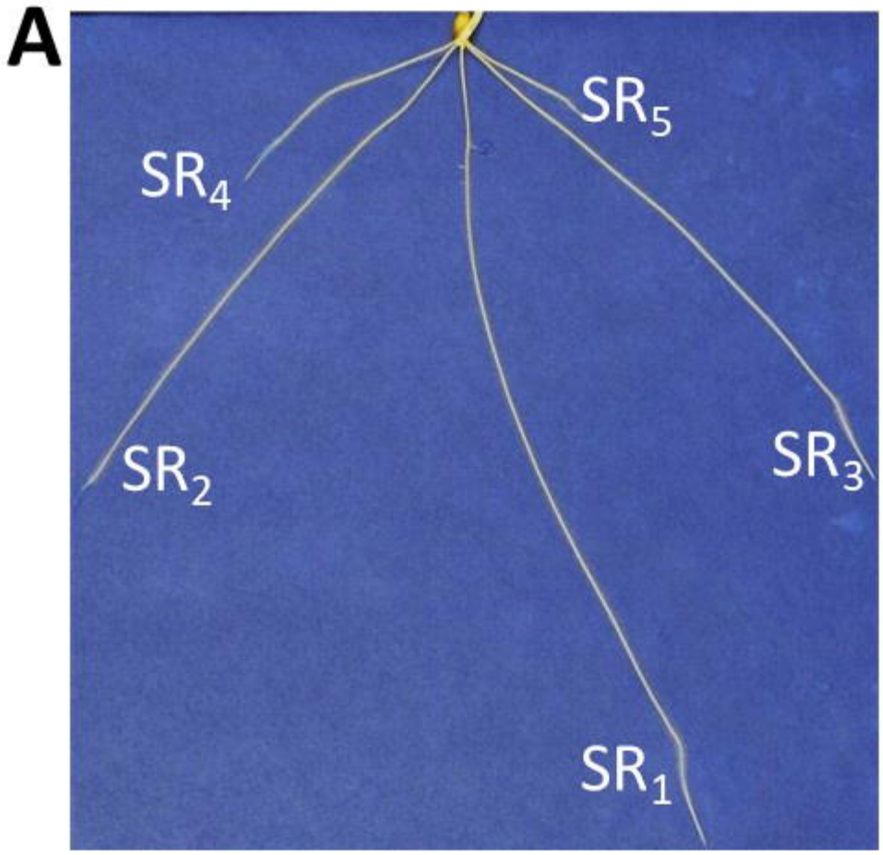
599

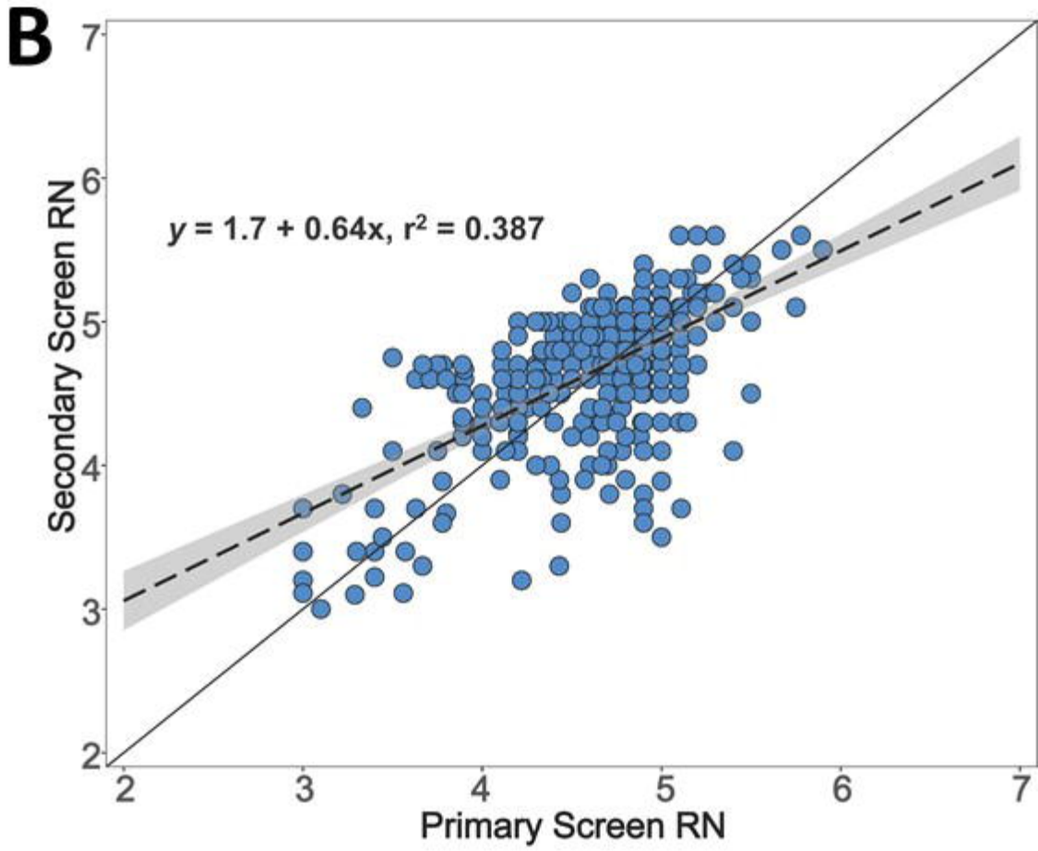
600 Figure 6: Genetic mode of action of the *arn* mutants. Seminal root number distribution in M_6
601 and F_1 progenies of the *arn* mutants and Cadenza. The number of the seeds phenotyped for
602 each line ranged from 9 to 27.

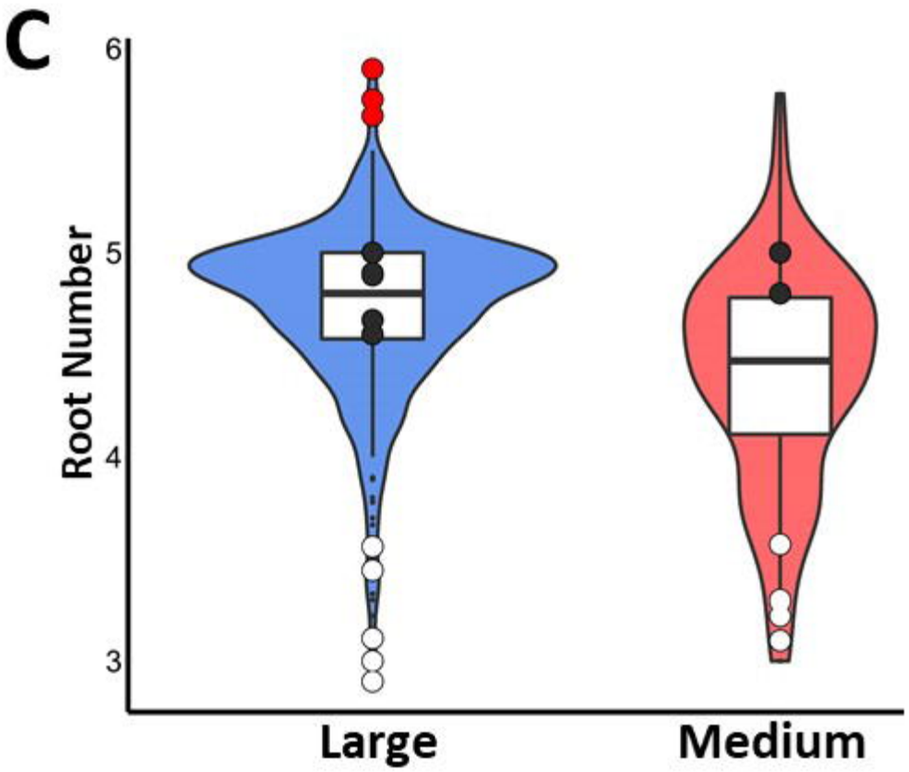
Table 1: Phenotypic summary of validated altered root number mutants with information on the mutation type and phenotype frequency in selected spikes.

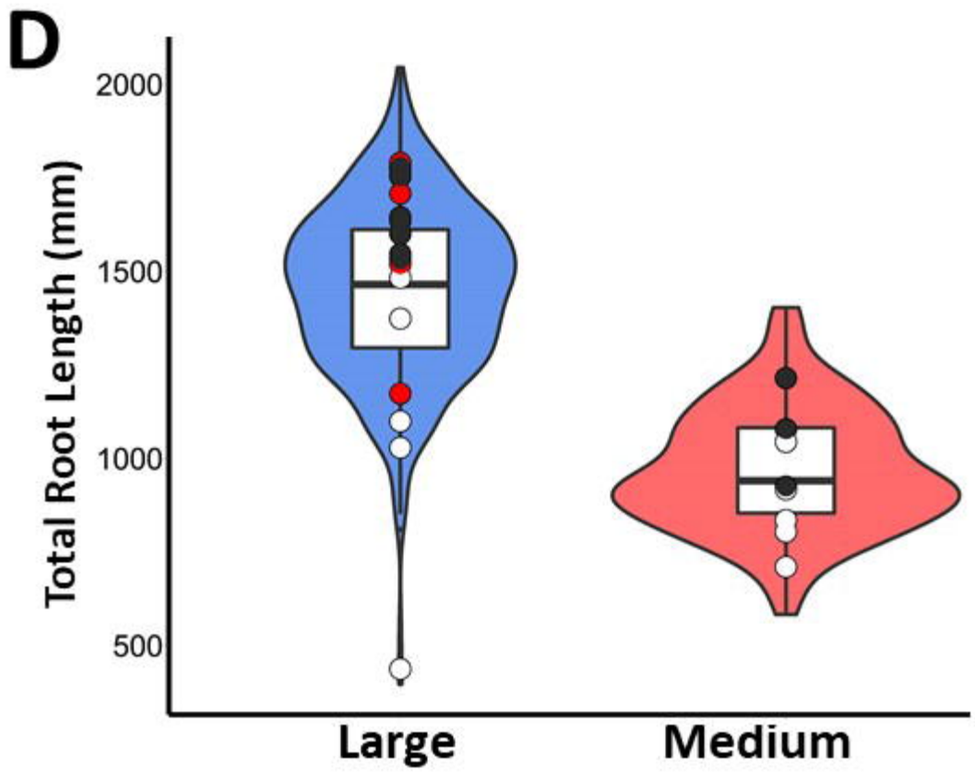
Type	Mutant	*arn* name	Mean (Sem)	Het/hom	Mut/Total
Wild-type	Cadenza		4.90 (0.05)	-	-
Higher Root Count Mutant	Cadenza0927		5.32 (0.09)	Het	3/4
	Cadenza0173		5.69 (0.08)	Het	3/4
	Cadenza0900	*arn1*	5.87 (0.05)	Hom	4/4
Lower Root Count Mutant	Cadenza0393	*arn5*	3.08 (0.04)	Hom	4/4
	Cadenza0818	*arn7*	3.11 (0.06)	Hom	4/4
	Cadenza0062	*arn2*	3.18 (0.17)	Hom	4/4
	Cadenza1273	*arn8*	3.18 (0.07)	Hom	4/4
	Cadenza0369	*arn4*	3.25 (0.09)	Hom	4/4
	Cadenza0465	*arn6*	3.25 (0.09)	Hom	4/4
	Cadenza0904		3.31 (0.13)	Het	3/4
	Cadenza0122		3.67 (0.16)	Het	3/4
	Cadenza0335	*arn3*	3.86 (0.19)	Hom	4/4
	Cadenza0167		3.88 (0.16)	Het	3/4

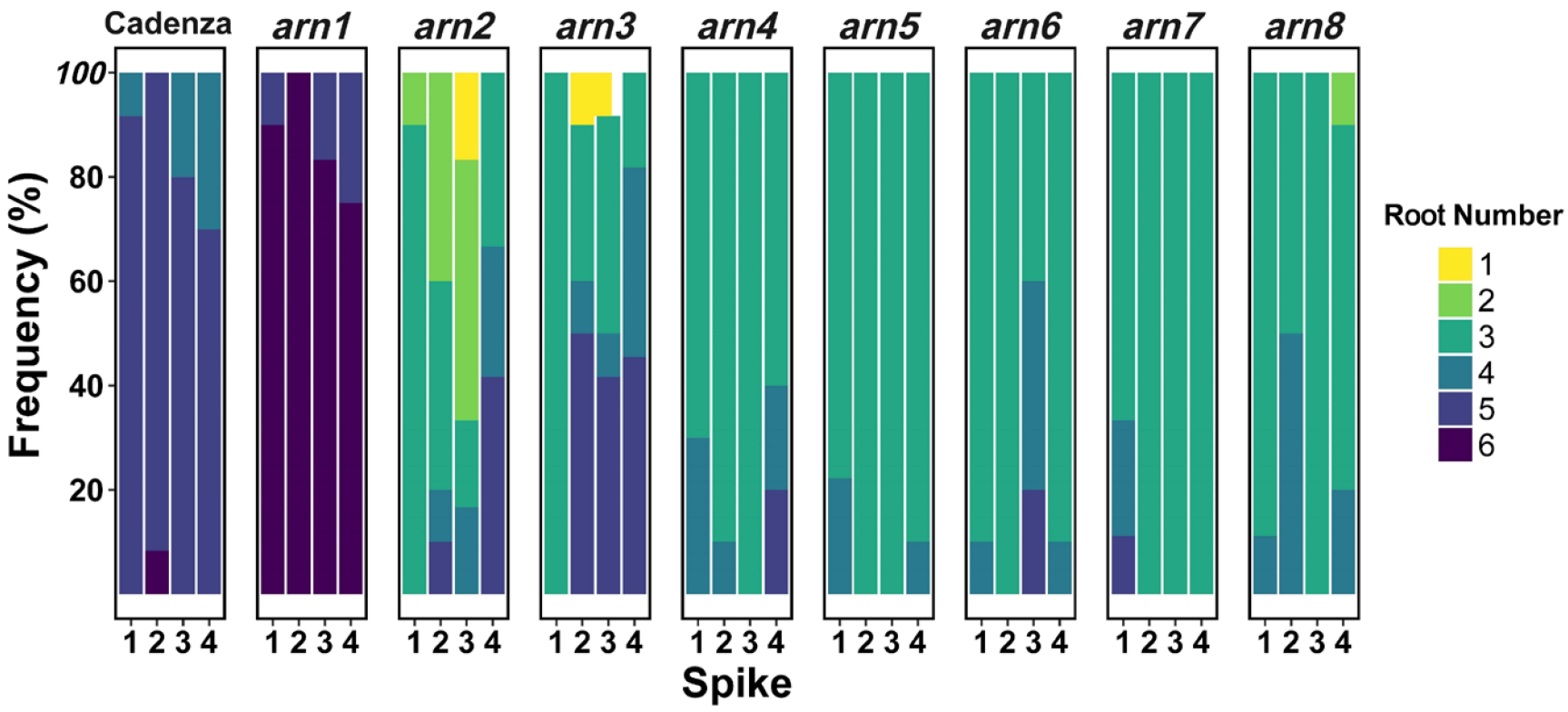
Mean is calculated from pooled root number counts of all four spikes except for lines with heterozygous phenotype, where was calculated from pool of spikes showing significant difference

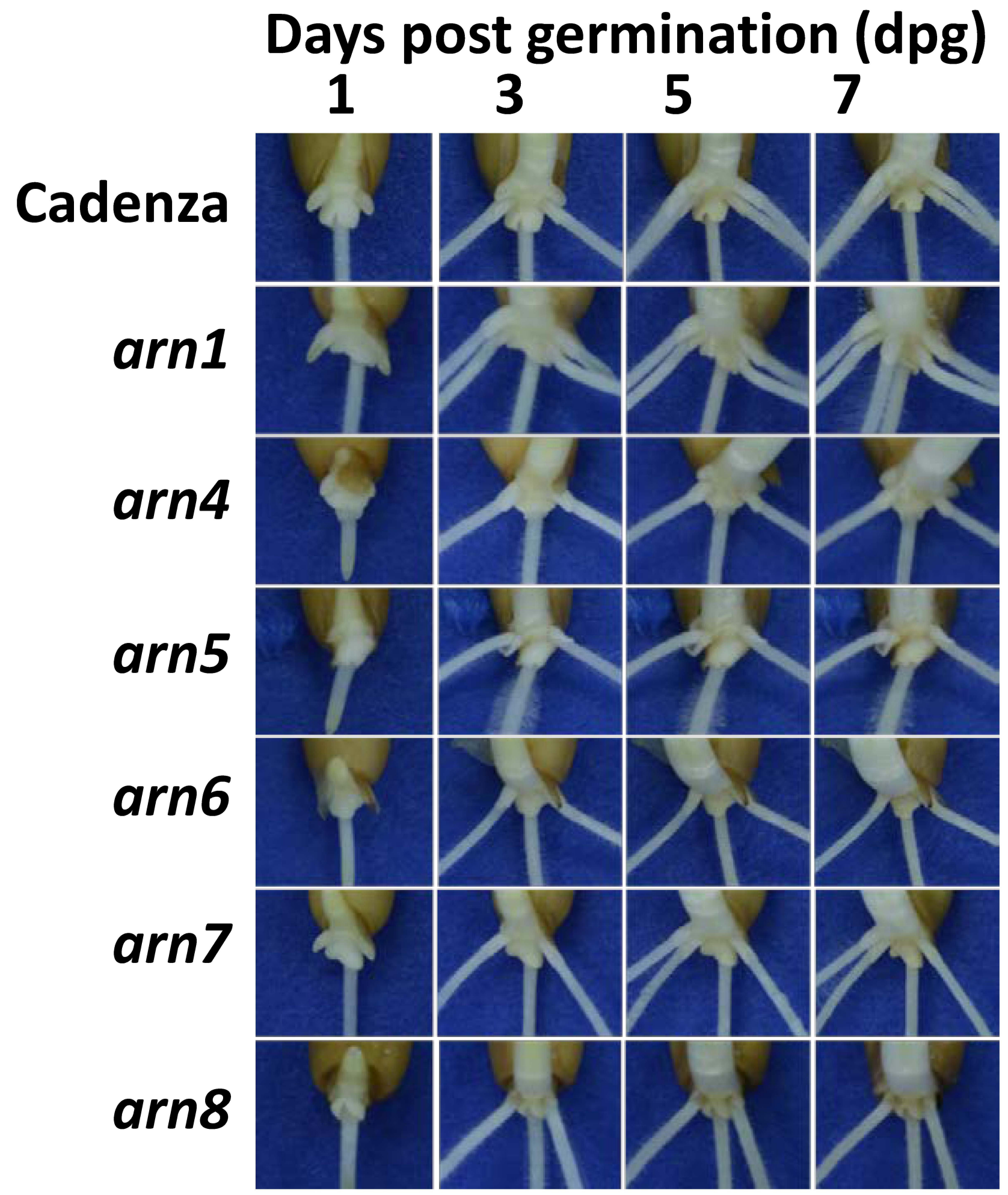

A
SR4
SR5
SR2
SR3
SR1

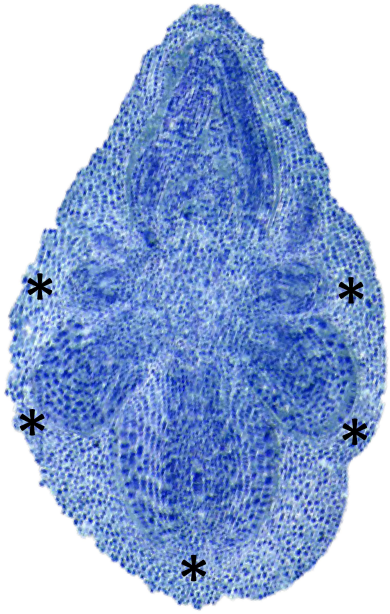

B
y = 1.7 + 0.64x, r2 = 0.387
Secondary Screen RN
Primary Screen RN
2
3
4
5
6
7


C
Root Number
3
4
5
6
Large
Medium

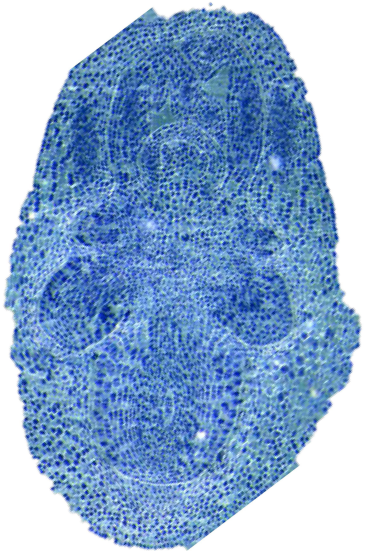

D
Total Root Length (mm)
500
1000
1500
2000
Large
Medium


Cadenza
arn1
arn2
arn3
arn4
arn5
arn6
arn7
arn8
Frequency (%)
100
80
60
40
20
1 2 3 4
Spike
Root Number
1
2
3
4
5
6

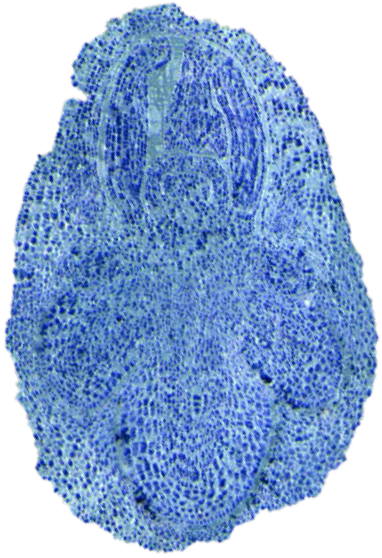

Days post germination (dpg)
1
3
5
7
Cadenza
arn1
arn4
arn5
arn6
arn7
arn8



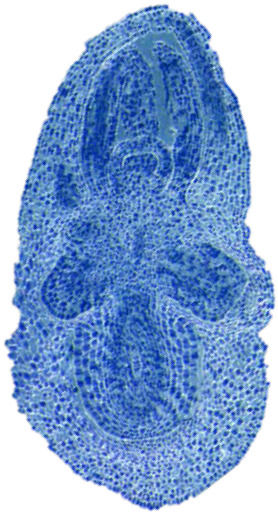
Cadenza



arn2

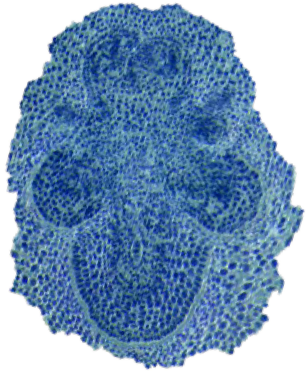


arn4

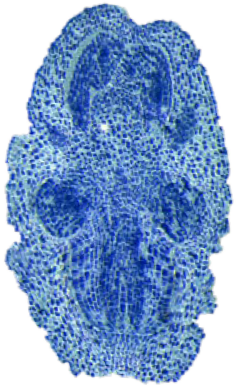


arn5

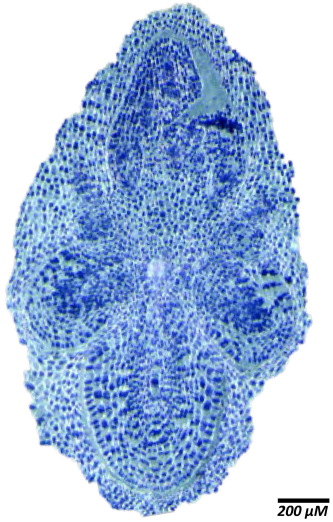
Line	Mean Primordia Count (StDev)
Cadenza	5.00 (-)
arn2	4.50 (0.27)
arn4	4.83 (0.16)
arn5	3.90 (0.25)
arn6	4.33 (0.33)
arn7	3.75 (0.48)
arn8	4.25 (0.31)



arn6

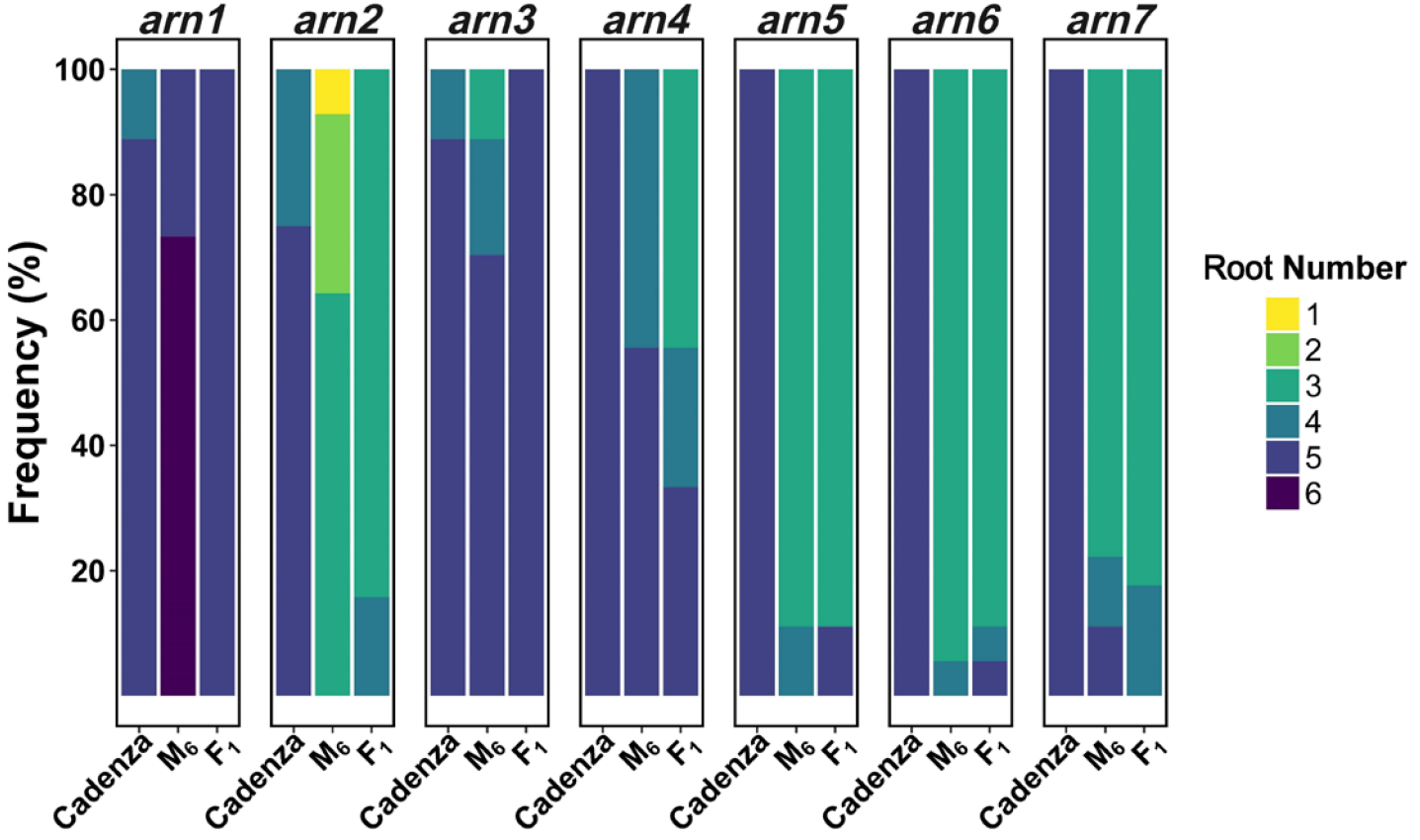


arn7



arn8

200 µM


arn1
arn2
arn3
arn4
arn5
arn6
arn7
Frequency (%)
100
80
60
40
20
Cadenza
M6
F1
Root Number
1
2
3
4
5
6