Isolation and characterization 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 crops. Optimisation of root architecture provides a viable strategy to improve nutrient and water uptake efficiency and maintain crop productivity under water-limiting, 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 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. Segregation analysis in F_2 populations suggest that the *arn1* phenotype is controlled by multiple genes while the *arn2* phenotype fits a 3:1 segregation ratio characteristic of 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, 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 (Kuhlmann and Barraclough 1987) 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, 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 the increased soil water exploration at depth 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 3 to 6 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 lack of identified 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 genomes (A, B and D). High sequence similarity in the coding region of

these genomes results in high levels of genetic redundancy that mask the phenotypic effects of underlying natural variation for many traits, including RSA phenotypes (Uauy et al. 2017). Also, the “out-of-sight” nature and extreme phenotypic plasticity of roots under native field conditions makes root phenotyping difficult, cumbersome and time-consuming.

The use of induced variation has proven useful to uncover novel phenotypes and dissect genetic pathways underlying complex phenotypes in plants (Parry et al. 2009). Our current understanding of the genetic determinants regulating root development in many cereals have almost entirely stemmed from the isolation and characterization of mutants defective in one or more RSA traits (Coudert et al. 2010; Hochholdinger et al. 2018; Marcon et al. 2013). Genetic variation for seminal root number in modern wheat germplasm has been defined to broad QTL which makes their genetic dissection difficult and their use in breeding limited. Induced variation present in mutant population represents an alternative strategy to identify variation that can be exploited to improve RSA. However, mutation analyses have not hitherto been exploited for studying the genetic architecture of root development in wheat, most likely due to the genetic redundancy often seen in polyploids (Krasileva et al. 2017; Uauy et al. 2017). The recent development of an in-silico platform for the rapid identification of mutations in 1,200 mutants of the UK hexaploid wheat cultivar Cadenza now makes large-scale reverse and forward genetic investigation of traits more feasible (Krasileva et al. 2017). Progress has also been made on the root phenomics front, with the development of fast, low-cost, and flexible two-dimensional root phenotyping pipelines with sufficient throughput for phenotyping large populations (Selvara et al. 2013; Atkinson et al. 2015)

Taking advantage of these new developments, we conducted a forward genetic screen for variation in seminal root number using a subset of the exome-sequenced mutant population in the Cadenza background. From this work, we describe the isolation and characterization of mutants with decreased and increased numbers of seminal roots and examine the genetic basis of this variation.

MATERIALS AND METHODS

Mutant Population

A subset of 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 (663 mutants). 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 root phenotyping

Primary Screen: To phenotype seminal root traits in Cadenza mutant population, we developed a two-dimensional (2D) root phenotyping platform based on the protocol described by Atkinson et al. (2015) with some modification to increase the throughput from 360 to 1,800 plants per run. In brief, lines were first 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 and 3.35 mesh and the 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 30 120 cm x 27 cm x 36 cm boxes (Really Useful Product, West Yorkshire, UK) with 60 pouches per box. The sides of the box were covered in black plastic back plastic paper and sticky back cover film to block out light from the roots of the developing seedling. The bottom of each box was filled with 10 L of half-strength Hoagland's growth solution containing: $NH_4H_2PO_4$, 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 the 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. Seedlings grew for seven days before the roots were imaged. After 7 days of growth, pouches were taken out of the phenotyping box and placed on a copy stand. The black plastic back covering the germination paper was gently pulled back to reveal the root structure and images of the roots were taken with a Nixon D3400 DSLR Camera fitted to the copy stand. Phenotyping of the 663 mutant lines 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 seeds were selected and placed onto moist filter paper in a 90mm 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 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 stood in the stand provided with the germination pouches in a reservoir of full strength Hoagland's 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 plants grew 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 10 plants per line and 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 and Caesium image processing software before

being processed in RootNav – a software for semi-automated quantification of root architecture (Pound et al. 2013). Captured root architectures were imported into RootNav viewer database for measurement of RSA traits using standard RootNav functions.

Statistical analysis

All statistical analyses were performed in R 3.4.2 (R Core Team 2018) and Minitab 17 statistical software. Statistically significant root architectural difference in the primary screening experiment was determined by ANOVA using a Dunnett's comparison within each phenotyping batch with the Cadenza plants in each batch used as controls. Adjusted probability values of < 0.05 were considered statistically significant. Statistically significant root architectural difference in the validation experiment and M_6 and F_1 phenotyping was based on student t-test comparison of individual spike/line to Cadenza control.

RESULTS

Identifying induced variation for seminal root number in hexaploid wheat

We developed a rapid root phenotyping platform suitable for large scale phenotyping of the mutant population at a throughput of 1,800 seedlings per run (Figure 1). Using this platform, we performed a screen for variation in seminal root number using 663 seed-size stratified (small, medium and large) exome-sequenced M_5 mutants from the Cadenza TILLING population. Cadenza mainly display five seminal roots (4.9 ± 0.05) including a primary seminal root SR_1, as well as first and second pairs of seminal root, hereafter referred to as $SR_{2,3}$ and $SR_{4,5}$ respectively (Figure 2A). We observed variation in seminal root number relative to Cadenza with seminal root number ranging from 1 to 7 in individual plants and a modal root number of 5 across the 663 mutants. The mean seminal root number per mutant (n>=4 plants per mutant) ranged from 2.9 to 5.9 (Figure 2B). Consistent with Cadenza seminal root architecture, 84% 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 4.3, respectively, and total root length of 1464.4 mm and 968.4 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 genotype (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 per genotype of 2.9 to 4.1 and modal seminal root number between 3 and 4.

To further assess these lines, we phenotyped a subset (391 mutants) of the primary population in a secondary screen. This included 31 of the 52 mutants with a significantly different seminal root number to Cadenza (four higher and 29 lower root count). Details for each individual mutant line phenotyped in both primary and secondary screens is 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 all 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 the four 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, we observed the altered seminal root number phenotype in the three additional spikes, including a higher root number and seven lower root number mutants. 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. We subsequently named these eight mutants as *altered root number* mutants: *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. Six other lines 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 the timing when the *arn* phenotype was first visible and to detect the seminal root type (SR_1, first pair or second pair) defective in these mutants (Figure 4). Cadenza showed fully emerged SR_1, at 1dpg, while 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 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, particularly SR_5. Contrary to this, *arn1* shows a faster rate of seminal root development relative to Cadenza with SR_1 and the first and second pair of seminal roots emerged by 3 dpg and an extra sixth root emerged at 7 dpg. All plants for *arn3* were indistinguishable from Cadenza, whereas *arn2* showed a strong dormancy phenotype and was not included in this experiment.

Genetic characterization 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 5). F_1 progenies of *arn1* x

Cadenza all had five seminal roots, similar to Cadenza (*P* = 0.08; Figure 5), 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* and *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 2.57 to 3.33 (Figure 5). The F_1 progenies of these mutants also showed a 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 3.16 and 3.89 (Figure 5). This suggests that the *arn2,* and *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. Neither M_6 or F_1 progenies of *arn3* showed significantly different seminal root number to Cadenza, further highlighting the instability of the *arn3* phenotype.

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 238 F_2 progenies 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) was 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 an important 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 phenotype (Taramino et al. 2007; Suzuki et al. 2015; von Behrens et al. 2011; Xu et al. 2015). Despite the buffering effect expected from 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 for the presence of mutations in candidate genes from other species. For example, the higher seminal root number phenotypes of *arn1* was similar to the phenotype of maize *bige1* (Suzuki et al. 2015) mutants. Examination of mutations in Cadenza0900 (*arn1*), however, did not identify any mis-sense or non-sense mutations in the coding sequences of the three wheat homoeologs of *BIGE1.* 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(s) underlying *arn1* might also be responsible for variation in the presence of 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). Examination of these coding regions in the *arn2* to *arn8* mutants revealed that Cadenza1273 (*arn8*) 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 (IWGSC, 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 morphology and seminal root traits.

Seed-size stratification of lines during our study allowed an examination of the relationship between seed size and root architecture. We observed a positive effect of seed size on root number and length. However, seed size only accounts for a small proportion of total

variance in root number, suggesting that seed size *per se* is not a major determinant of root number. This is further highlighted by the fact that differently sized wild-type Cadenza seeds show similar root number averages. This is also supported by the report that variation in seminal root number between wheat species mainly emanates from factors in the embryo rather than difference in seed morphology. In contrast, seed size account for 82% of the total variance in root length in the mutant population consistent with the rationale that bigger seeds have more nutrient reserve to support faster root elongation. Although informative, the qualitative measure of seed size (large, medium and small) adopted in this study does not allow a quantitative modelling of seed size effect on the root trait. We propose that a finer calibration and partitioning of the grain size measurement into constituent parameters (width, length, height) and tissue (embryo and endosperm) components will allow for a finer understanding of the effects of these seed size components on root architecture.

Genetic control of seminal root development

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

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

All the *arn* mutants develop SR_1 and $SR_{2,3}$ but show defects in the development of $SR_{4,5}$ as in *arn2* to *arn8*, or develop an extra root (SR_6) as in *arn1*. We could not recover mutant lines defective in either SR_1 nor $SR_{2,3}$. This likely suggests that the developments of the different seminal root types are under distinct genetic control, with SR_1 and $SR_{2,3}$ being more conserved than $SR_{4,5}$ and SR_6. This notion is supported by reports that $SR_{4,5}$ show only negligible contribution to water uptake and does not confer any beneficial fitness under well-watered conditions (Golan et al. 2018). It is however possible that $SR_{4,5}$ and indeed SR_6 may contribute significantly to nutrient and water uptake under resource-limiting conditions where increase in root surface area maximises soil exploration. Detailed field physiological evaluation will be required to better understand the cost-benefit relationship of the altered seminal root phenotype of the *arn* mutants and evaluate the potential of these mutations to improve resource uptake efficiency in plants.

Future outlook

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

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Figure Legends

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

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

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

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

Figure 5: Genetic mode of action of the *arn* mutants. Seminal root number distribution in M_6 and F_1 progenies of the *arn* mutants and Cadenza. The number of the seeds phenotyped for 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
	Cadenza1085		5.90 (0.06)	Het	3/4
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

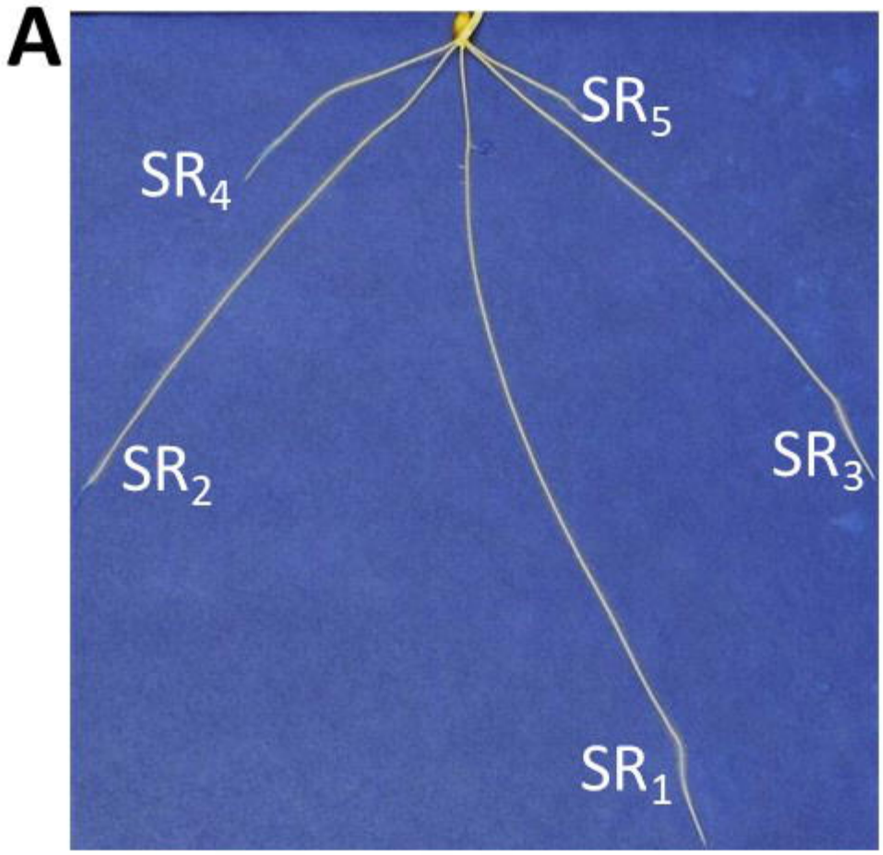
*Mean is calculated from pooled root number counts of all four spike except for lines with heterozygous phenotype, where was calculated from pool of spikes showing significant difference

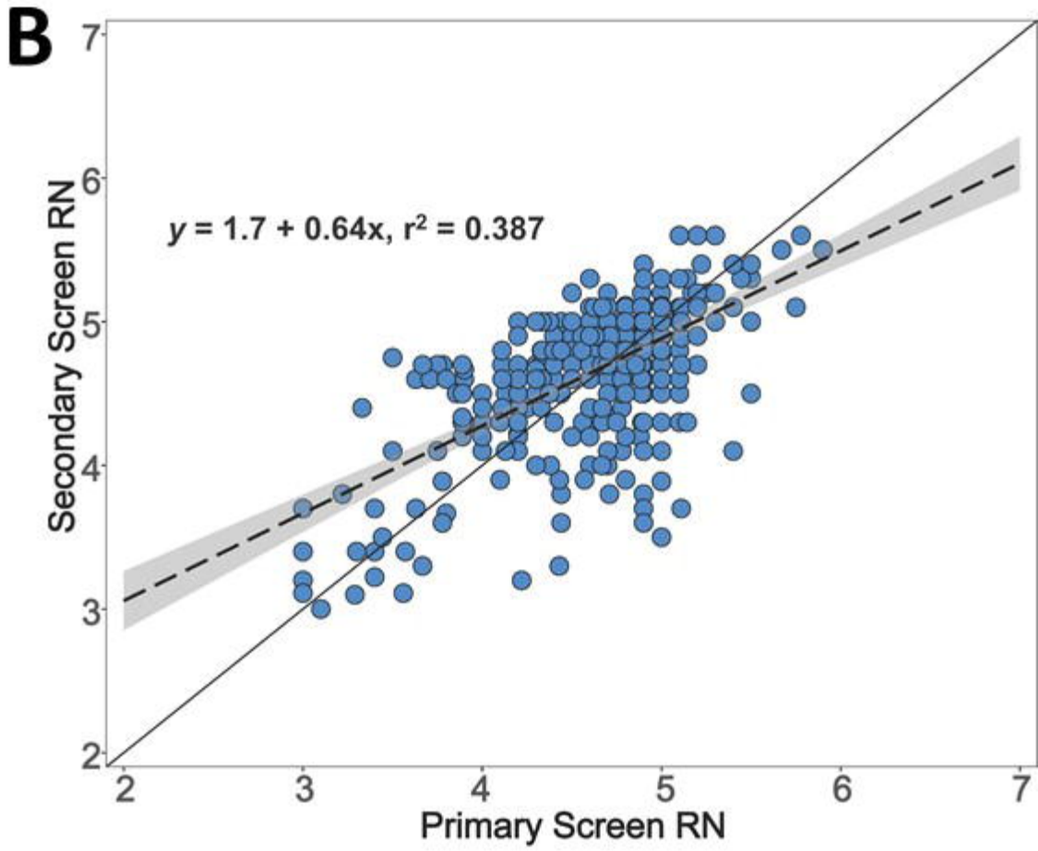
Figure S1: *arn* mutations show altered seminal root number phenotypes. Representative images of seminal root number phenotypes of the *arn* mutations as observed in the primary screen.

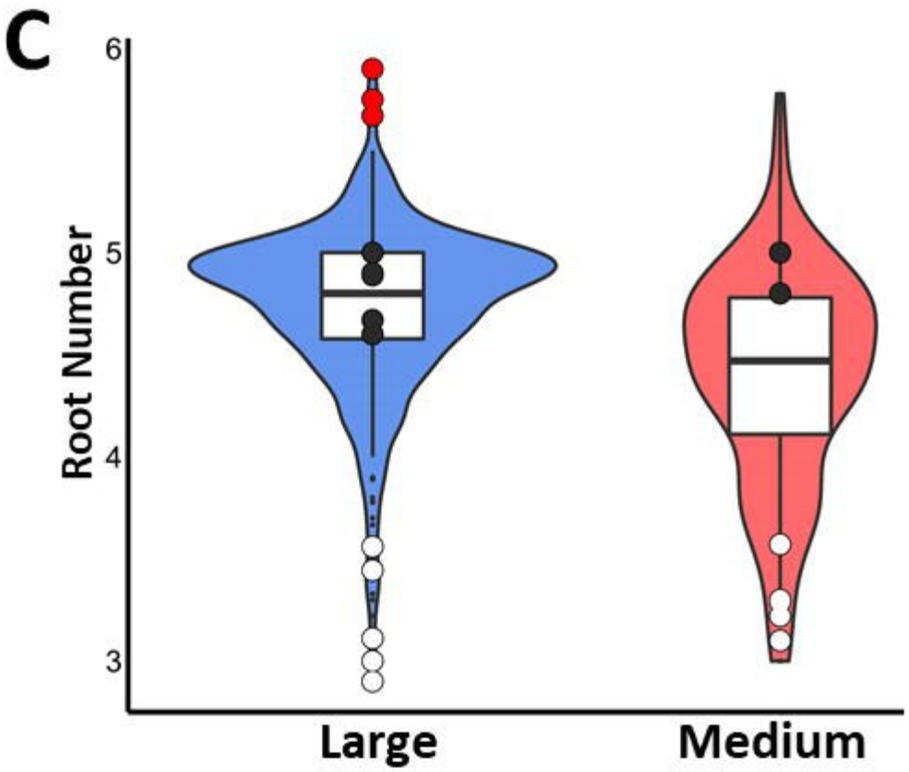
Table S1: Summary information of mutants with significantly different seminal root number to Cadenza in the primary screen.

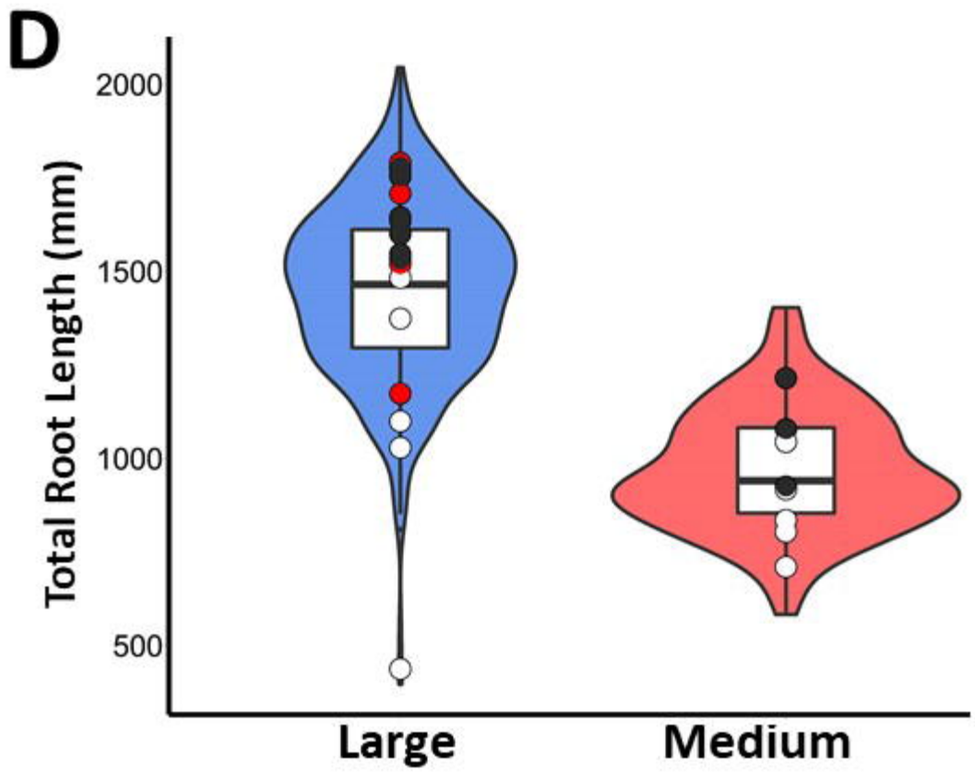
Table S2: Summary information on seminal root number of mutants phenotyped in both the primary and secondary screen.

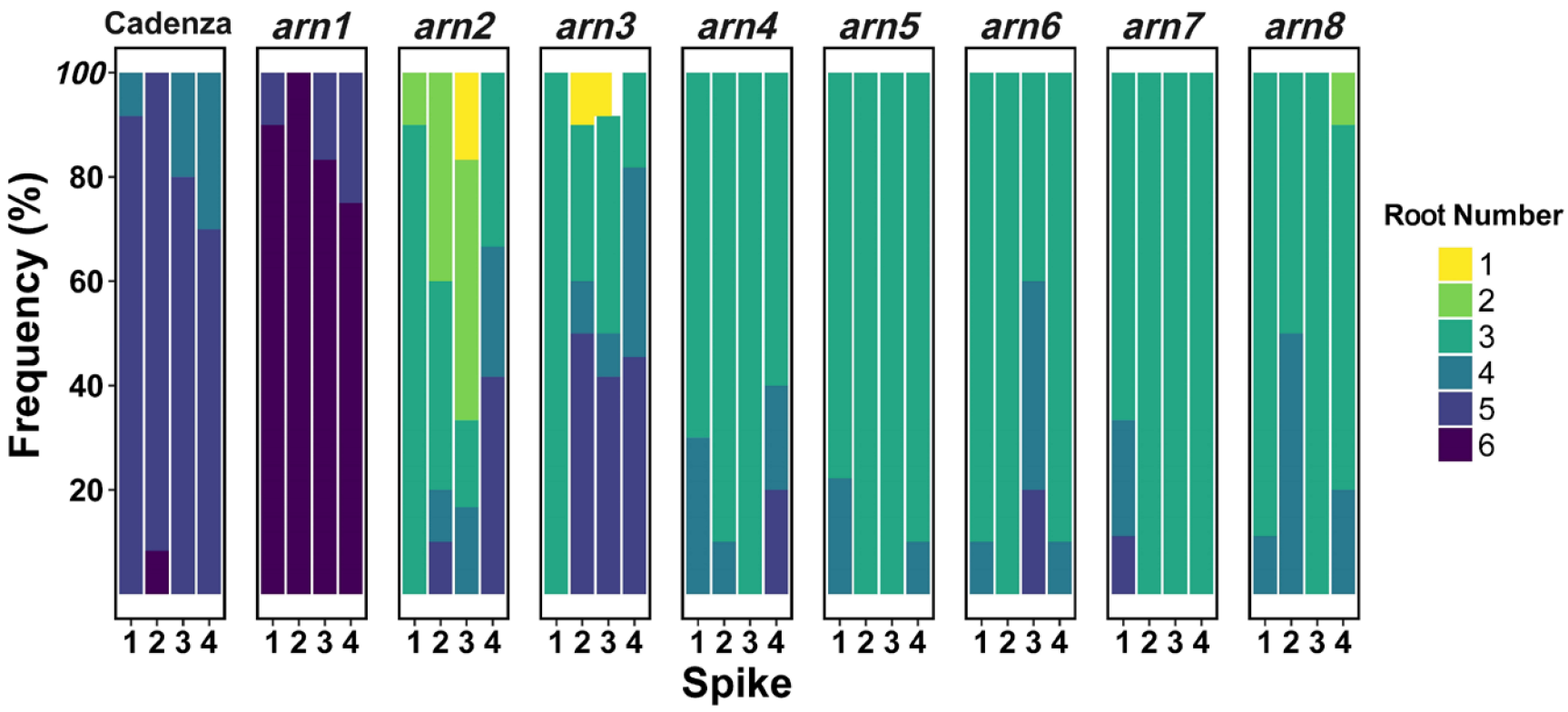
Table S3: Summary statistic of ANOVA of seminal root number phenotypes of M_6 and F_1 progenies of *arn* mutants to respective Cadenza control.

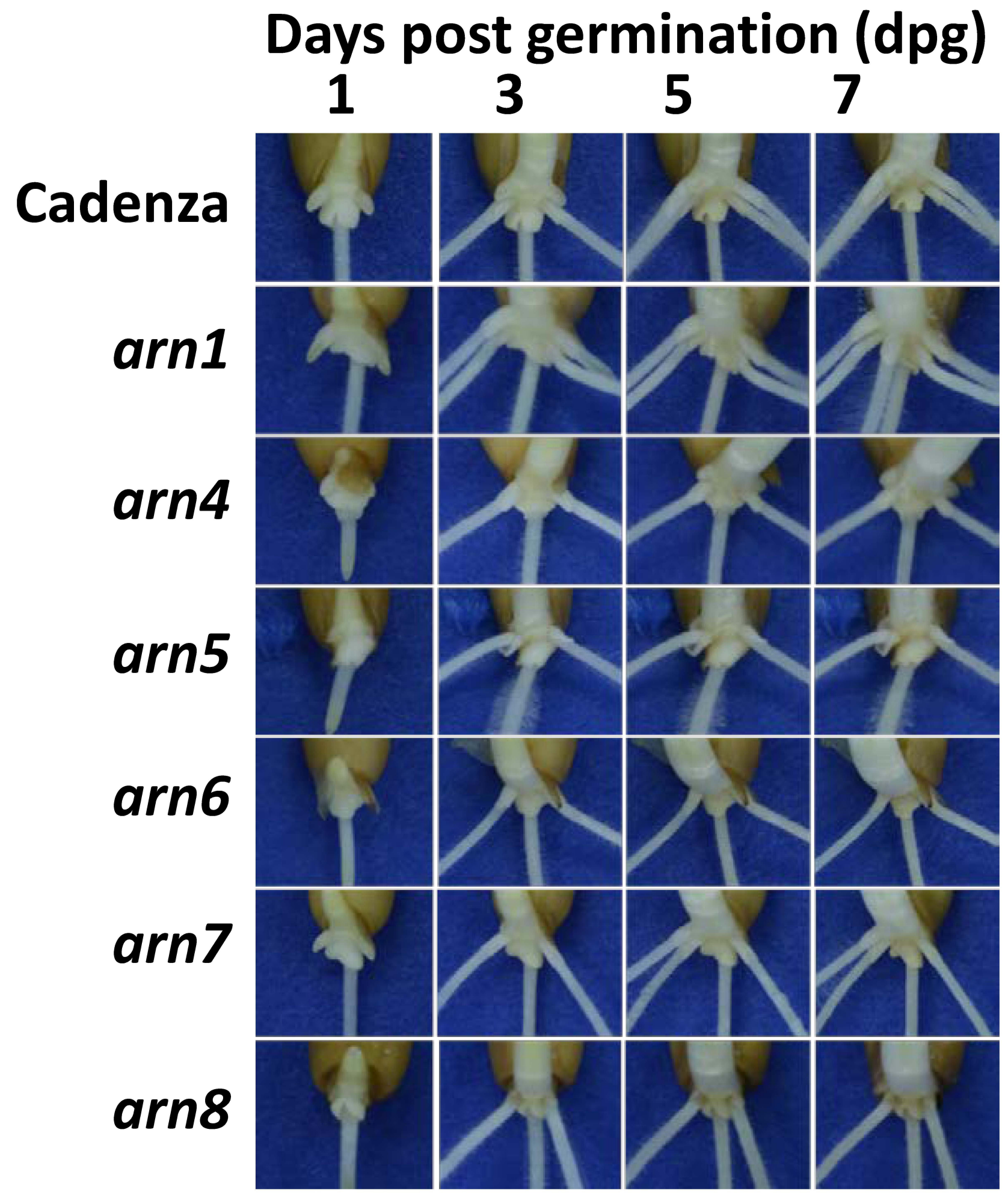

A
SR4
SR5
SR2
SR3
SR1

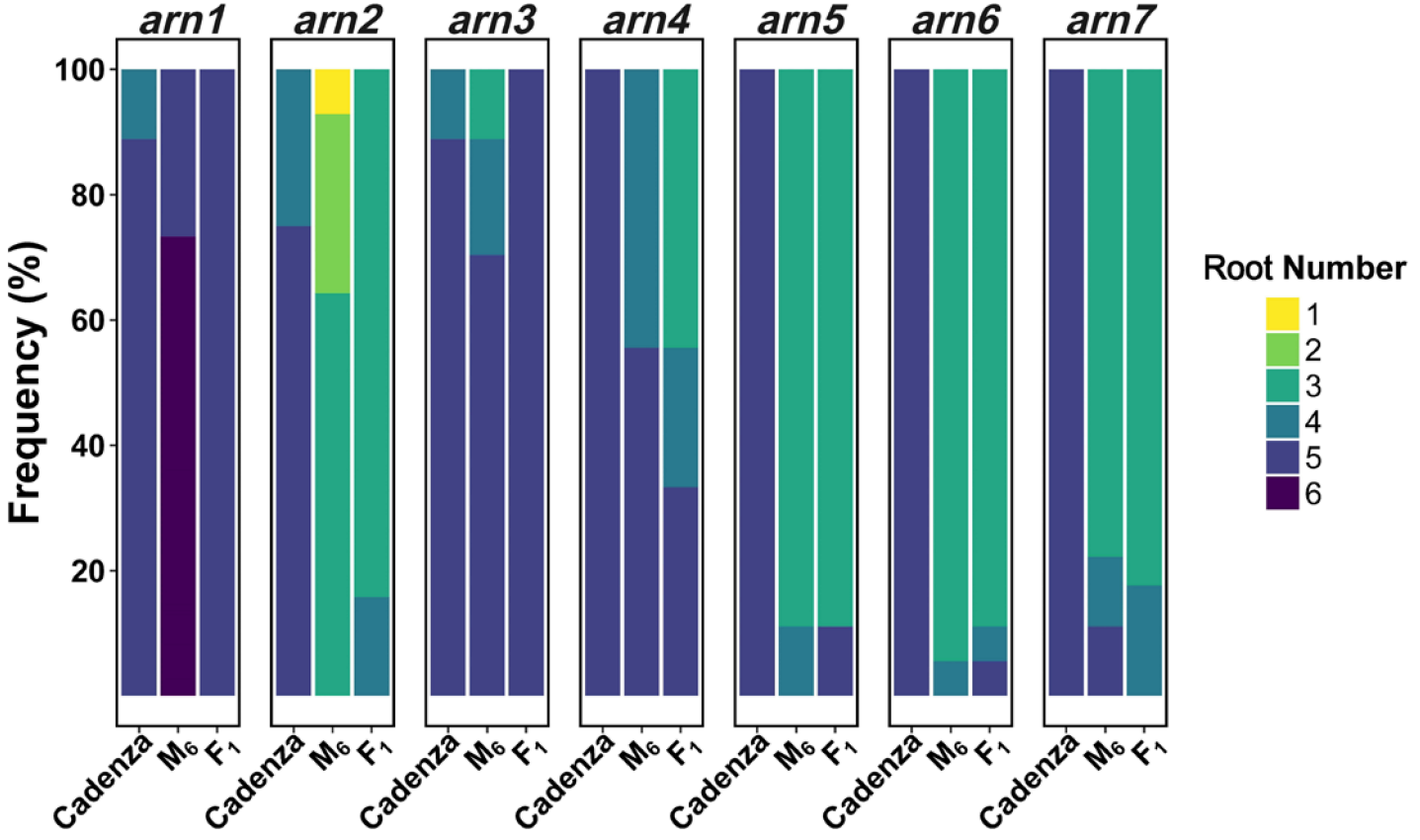

B
y = 1.7 + 0.64x, r2 = 0.387
Secondary Screen RN
Primary Screen RN
2
3
4
5
6
7


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


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