Control of adventitious root formation in the alpine perennial *Arabis alpina*

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Dedicated to my parents

for everything

Abstract

Successful adventitious root development ensures the efficient clonal propagation of alpine perennials under harsh environmental conditions, but the molecular basis of this process is not well understood. I used the alpine perennial *Arabis alpina* to explore natural variation of adventitious rooting and investigate the molecular basis of adventitious root development in alpine perennials. Plants of the *A. alpina* accessions, Pajares (Paj), Dorfertal (Dor), Totes Gebirge (Tot) and West Carpathians (Wca), and the *perpetual flowering 1-1* (*pep1-1*) mutant were scored after growth in a long day greenhouse. The occupancy of adventitious roots on the hypocotyl, main stem and axillary branches varied between genotypes. Especially, Wca plants produced adventitious roots on the main stem, which correlated with the higher expression of the *A. alpina* homolog of *GH3.3*. Exogenous auxin application by foliar spraying promoted adventitious root formation robustly in a genotype and age-dependent manner. I also applied auxin spray on vernalized Paj plants and scored the presence of adventitious roots on stems after plants were transferred in long day greenhouse. Adventitious roots developed from the vascular cambium cells specifically on younger internodes. High-throughput RNA sequencing revealed the differential regulation of auxin transporter genes in the internodes that produce adventitious roots compared to the ones that do not, indicating a key role for polar auxin transport during the induction of adventitious rooting after auxin spray. Auxin-responsive genes showed internode-specific transcript accumulation in response to auxin spray, which correlated with their rooting ability. In addition, transcripts of several meristem-associated genes were enhanced in the internodes that develop adventitious roots after auxin spray, indicating the establishment of root primordium during vernalization. Extended vernalization overcame the requirement to spray with synthetic auxin for the development of adventitious roots. After 21 weeks of vernalization, adventitious roots developed in young internodes and transcriptome profiling indicated the presence of initiator cells during vernalization and the involvement of auxin during the establishment of the initiator cells.

Zusammenfassung

Die erfolgreiche Ausbildung von Adventivwurzeln garantiert die effiziente klonale Vermehrung von alpinen mehrjährigen Pflanzen unter rauen Umweltbedingungen, die molekularen Grundlagen sind bisher jedoch kaum erforscht. In meiner Arbeit nutzte ich die alpine mehrjährige Arabis alpina um die natürliche Variation der adventiven Wurzelbildung zu erfassen und die molekularen Grundlagen der Adventivwurzel-Entwicklung in alpinen mehrjährigen zu erforschen. Die A. alpina Akzessionen Pajares (Paj), Dorfertal (Dor), Totes Gebirge (Tot) und West Carpathians (Wca), sowie die perpetual flowering 1-1 (pep1-1) Mutante wurden unter Langtag Gewächshausbedingungen bezüglich der adventiven Wurzelbildung phänotypisiert. Das Vorhandensein von Adventivwurzeln an Hypokotyl, Hauptspross und Seitentrieben variierte zwischen den Genotypen. Insbesondere bei Wca Pflanzen kam es zur Ausbildung von Adventivwurzeln am Hauptspross und dies korrelierte mit einer stärkeren Expression des A. alpina Homologes von GH3.3. Das Sprühen mit exogenem Auxin führte zu einer reproduzierbaren genotyp- und altersabhängigen Förderung der adventiven Wurzelbildung. Zusätzlich wurden vernalisierte Paj Pflanzen mit exogenem Auxin behandelt, in Langtag Gewächshausbedingungen transferiert und das Vorhandensein von Adventivwurzeln am Hauptspross ausgewertet. Ausschließlich bei jüngeren Internodien bildeten die Zellen des vaskulären Kambiums Adventivwurzeln aus. Eine Hochdurchsatz-Transkriptom-Sequenzierung enthüllte, dass Auxin-Transporter Gene zwischen wurzelschlagenden und wurzellosen Internodien differenziell reguliert waren. Dies deutet auf eine Schlüsselfunktion des polaren Auxintransports in der Induktion von Adventivwurzeln im Zusammenhang mit der Applikation von exogenem Auxin. „Auxin-responsive genes", die durch Auxin Signalwege aktiviert werden, zeigten ebenso eine Internodium-spezifische Akkumulation und dies korrelierte mit der Fähigkeit zur adventiven Wurzelbildung. Darüber hinaus waren die Transkripte mehrerer Meristem-assoziierter Gene in nach der Auxin-Behandlung wurzelschlagenden Internodien angereichert, was auf die Anlage von Wurzelprimordien währened der Vernalisierung hindeutet. Die Erfordernis der synthetischen Auxin-Gabe zur Ausbildung von Adventivwurzeln wurde mit einer verlängerten Vernalisierung überwunden. Nach 21 Wochen Vernalisierung entwickelten sich Adventivwurzeln in jungen Internodien. Eine Transkriptomanalyse deutete auf die Anwesenheit von Initiatorzellen und eine Beteiligung von Auxin in der Anlage jener Zellen während der Vernalisierung hin.

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1. INTRODUCTION

1.1. The importance of clonal propagation in the perennial life cycle

In the extreme arctic and sub-arctic environments, harsh and unpredictable climates can affect the fitness of plants following the loss of juvenile plants or uncertainty in successful flowering, fruiting and germination [1–3]. In such environments, a life style following slow clonal growth mixed with the sexual reproduction during permitting seasons ensures increased fitness [1,4]. Clonal propagation involves the production of 'clonal off-springs' from plant organs like stem, root and leaves [5]. While sexual reproduction can create opportunities to produce constructive genomic changes and cause speciation leading to rapid adaptation to changing environment, clonal propagation helps adaptations in severe habitats where sexual reproduction would be unsuccessful.

Alpine perennial plants benefit from clonal growth due to the reduction of time required for clonal offspring to achieve reproductive competence during the limited growth season [6,7]. Perennial plants follow both sexual and clonal propagation to control their fitness in different environmental conditions [8–12]. Unsurprisingly, as the altitude increases and the environment threatens to become tougher, the frequency of annuals decreases [13]. Annuals, a rarity in the cold arctic region representing a mere 12% of the alpine population, have evolved to grow and produce seeds in these regions during the short vegetative periods [14]. Agriculturally, clonal propagation helps fix important genotypes and desirable characteristics in crop plants such as potatoes, sweet potatoes, cassava, yam, and sugar cane to name a few, and at the same time provides an undemanding and unchallenging technique to propagate selective genotypes by circumventing the germination and juvenile phases.

1.2. Adventitious roots as a means of clonal propagation

Adventitious root formation is a widely exploited step and a key limiting factor during clonal propagation of important perennial crop and horticultural plants. Several crop specific clonal propagation techniques like cutting, grafting, layering, offset, suckering and tissue culture are used by breeding industries. One of the most studied artificial techniques for clonal propagation by far includes induction of adventitious roots on the stem cuttings of Eucalyptus, grapes, Petunia,

Populus and tea [15–19]. Agriculturally, a successful adventitious root formation ensures an effective vegetative propagation of perennial crops.

Adventitious roots, as the name suggests, are roots formed accidentally or at unusual anatomical positions. In nature, adventitious roots are post-embryonic aerial borne roots originating on shoot tissues and organs, unlike the post-embryonic lateral roots on the primary root system. Adventitious roots function in nutrient and water uptake, provide mechanical support, as well as promote clonal propagation [20]. Though adventitious roots are a widely exploited feature in agriculture, especially in horticulture, the molecular mechanisms regulating their development remain uncharacterised.

1.3. Adventitious root development

Although adventitious and lateral roots have similar structure, their origin and development differ, with adventitious root development being plastic and thus unpredictable [20]. Lateral roots originate from pericycle cells, whereas adventitious roots originate from different tissues depending on the induction protocol [21–23]. In Arabidopsis, adventitious roots can initiate from hypocotyl pericycle cells adjacent to the xylem pole, as well as the vascular cambium and surrounding tissues in de-rooted hypocotyls of older seedlings and stem cuttings [24–28]. In the woody perennial Poplar, adventitious roots originate from the phloem-cambium junction, whereas interfascicular cambium cells are activated during adventitious root formation in apple and ray cells represent the origin of adventitious roots in raspberry and white pines [21,22,29,30]. Therefore, the origin of ARs in plants seems to be complicated based on the fact that different tissue associated with the vascular bundle may require diverse stimulus to become the progenitors of adventitious roots.

Adventitious root formation can be divided into three stages: induction, initiation and expression [31]. During induction, few shoot based cells are stimulated to redifferentiate into root founder cells without undergoing any histological changes [32]. Auxin is known to stimulate the induction of adventitious root formation, the duration of which is species-dependent [16,32–34]. Active cell division followed by the establishment of meristematic cells characterised by dense cytoplasm and large nuclei take place during the initiation phase. The meristematic cells cluster together to form a root primordium meristem and subsequently elongate followed by the connection of the vascular

network during expression. The duration of these phases is regulated by the age of the tissue and environmental factors [33,35].

1.4. Regulation of adventitious root development

Adventitious root development is a complex process involving the dedifferentiation of non-root cells and their redifferentiation into root cells, controlled by endogenous factors such as hormones as well as exogenous factors such as nutritional status [20]. Auxin plays a key role in adventitious root formation but other phytohormones, including abscisic acid, brassinosteroids, cytokinin, ethylene, gibberellic acid, jasmonic acid, salicylic acid and strigolactones are also involved [20,36]. Transcriptomic studies have revealed the genes and networks responsible for adventitious rooting, and the multiple molecular processes involved, including hormone signalling, metabolism, microtubule modelling, and cell wall modification [18,37–43]. These studies focused on adventitious rooting in stem cuttings in the presence or absence of auxin, but did not address the regulation of competence factors required for adventitious root formation on intact plants.

1.4.1. Auxin

Auxin plays a central role in the development of both lateral and adventitious roots [20]. Shortly after the discovery of auxin, it was reported to promote adventitious roots in cuttings [44]. It was not long before auxin was used as a rooting agent in the agricultural industries [32,45]. Around 1995, several mutants, namely *aberrant lateral root formation1* (*alf1*), *hookless 3* (*hls3*), *rooty* (*rty*), and *superroot1* (*sur1*), isolated independently but allelic to each other, showed excessive adventitious root production [24,46–49]. Interestingly, the enhanced adventitious root production in these mutants was a result of increase in the endogenous indole-3-acetic acid (IAA) level [46]. Since the discovery of auxin as a rooting agent, genes that regulate auxin signalling have been discovered and their role in adventitious rooting has been characterized [26,50–54].

Auxin biosynthesis, regulated by several pathways, is a major regulator of auxin abundance. In *A. thaliana* seedlings, *superroot* mutants, *sur1* and *sur2*, and the *yucca* mutant overproduce auxin leading to spontaneous production of adventitious roots on the hypocotyl [24,55]. In rice, overexpression of a YUCCA homologue leads to an increase in crown root production [56]. YUCCA6 is responsible for maintaining constant active auxin levels during the establishment of

the adventitious roots [25]. Not just overproduction but underproduction affects adventitious root development. Mutations in *WEAK ETHYLENE INSENSITIVE2* (*WEI2*) and *WEI7* indirectly inhibit auxin biosynthesis thereby preventing adventitious root production [57]. The genes of the cytochrome P450 monooxygenase family regulate auxin biosynthesis as well as cell wall modifications, brassinosteroid biosynthesis, redox-related processes, jasmonic acid homeostasis, and anthocyanin accumulation [58]. One of the cytochromes involved in auxin biosynthesis (CYP83B1) has been shown to regulate adventitious root development [59]. Other cytochromes from CYP79B family are involved in auxin biosynthesis and the CYP87 family is involved in auxin signalling [58]. In addition to auxin, cytochromes regulate the biosynthesis and signalling of other phytohormones.

Auxin conjugation is a major part of auxin homeostasis regulating the storage and inactivation of auxin [60]. Several forms of conjugated auxin have been identified, including sugar and amino acid conjugates [61]. A few UDP glucosyltransferases (UGT) including UGT84B1, UGT74E2 and UGT74D1 prominently catalyse the addition of sugar moieties to auxin analogues, although related UGT proteins, such as UGT84B2, UGT75B1 and UGT75B2, have been identified with lower conjugation activities. UGT74B1 regulates glucosinolate biosynthesis, in turn regulating auxin homeostasis and is responsible for negatively affecting adventitious root production [62]. Members of auxin amido-synthetases, GH3 family, are responsible for conjugating auxin to amino acids such as alanine, aspartic acid, glumatic acid, leucine and tryptophan. Enhanced auxin conjugation by some of these GH3 proteins has been considered to reduce the ability of certain cultivars to produce adventitious roots efficiently [63]. Similar observations have been reported in sweet cherry with faster auxin conjugation preventing adventitious rooting formation in difficult-to-root cultivars [57]. Overall, regulation of auxin homeostasis plays a key role in adventitious root development since transient changes in auxin levels regulate the developmental phases of adventitious roots.

Regulators of auxin transport and accumulation have been found by transcriptomic and physiological analysis during auxin-induced adventitious rooting [18,34,64–66]. Microarray analysis of gene expression during adventitious rooting in petunia and *Pinus contorta* stem cuttings revealed an initial downregulation of auxin transporters genes followed by upregulation [18,64]. The homolog of auxin influx carrier *AUX1* is upregulated during adventitious rooting in carnation cuttings [66]. In

rice, *OsPIN1* is involved in the emergence of adventitious roots [65]. Mutation in *CROWN ROOTLESS 4* (*CRL4*) showed defective crown root formation due to impaired auxin transport [67]. Overexpression of PIN6 in *A. thaliana* promotes adventitious root formation [68]. ABCB19, an auxin efflux carrier, enhances localized auxin transport and accumulation, thereby inducing numerous auxin-responsive genes and leading to adventitious rooting in de-rooted *A. thaliana* hypocotyls [26]. The balance between the efflux activity of PIN1 and the influx activity of LAX3 is required for the establishment of adventitious roots in *A. thaliana* [25]. Bearing in mind that auxin transport inhibitors such as Naphthylphthalamic acid (NPA) also inhibit adventitious rooting, polar auxin transport seems to be an important feature regulating adventitious root formation [23].

Auxin homeostasis in turn regulates auxin signalling during adventitious root development. Auxin levels regulate the destruction of auxin signalling inhibitors of *AUX/IAA* family by SCFTIR protein assembly and CULLIN-ASSOCIATED AND NEDDYLATION- DISSOCIATED 1 (CAND1) [69]. Mutants of homologs of CAND1 in rice leads to defects in crown root emergence [70]. Mutations in the COP9 signalosome (CSN) subunits lead to inefficient degradation of *AUX/IAA* proteins thereby, supressing adventitious root formation in *A. thaliana* [71]. A gain-of-function mutation of SOLITARY-ROOT/IAA14 gene of *A. thaliana* blocks the inhibitory effect of chromate on adventitious root development [72]. The degradation of *AUX/IAA* genes leads to the de-repression of transcription factors of *AUXIN RESPONSE FACTOR* family. In *A. thaliana*, balance between ARF6, ARF8 and ARF17 is important for modulating the initiation of adventitious roots [51]. The positive regulators *ARF6* and *ARF8* are regulated by *miR167*, whereas *miR160* controls the activity of the negative regulator *ARF17*. In the absence of functional *miR160*, *ARF17* is overexpressed leading to inhibition of adventitious root production [50]. ARF7 and ARF19 also positively regulate adventitious root formation in *A. thaliana* [73]. The auxin responsive AP2/ERF transcription factor CRL5 promotes crown root initiation in rice [74]. Overall, auxin is the most studied hormone regulating adventitious root development playing a central role.

1.4.2. Other hormones

Numerous studies have reported the role of plant hormones other than auxin during adventitious root development. The auxin transcription factors ARF6, ARF8 and ARF17 regulate the expression of three *GRETCHEN HAGEN 3* genes, *GH3.3*, *GH3.5* and *GH3.6* [52]. These GH3

proteins affect the homeostasis of other hormones including jasmonic acid, in turn affecting adventitious root formation. Interestingly, while jasmonic acid promotes adventitious root formation in the Thin Cell Layer (TCL) in tobacco and petunia, it has an inhibitory role in the adventitious root development in *A. thaliana* [16,75].

Another stress related hormone, salicylic acid positively affects adventitious root formation. In *A. thaliana*, mutants defective in salicylic acid biosynthesis *eds5-1* and *eds5-2* produced fewer adventitious roots than the wild-type [52]. Exogenous salicylic acid application promotes adventitious root formation in mung bean as well [76]. Salicylic acid abundance increased during the establishment of adventitious root primordium, however was highly affected by exogenous auxin application in carnation cuttings [34].

Strigolactone, on the other hand, plays an inhibitory role during adventitious root development. In *A. thaliana* and pea, strigolactone deficient mutants and mutations in strigolactone signalling genes lead to enhanced adventitious rooting [77]. Strigolactone inhibits adventitious root development even in higher auxin abundance.

Ethylene generally promotes adventitious root development as shown in different species including maize, tomato, rice, petunia, apple, sunflower and mung bean [78–81]. Transcriptomic studies have suggested ethylene plays the role of a stimulator in petunia cuttings [18]. Ethylene aids the emergence of adventitious roots by inducing epidermal cell death [82]. In apple, ethylene is reported to play no role in adventitious root formation [83]. However, in some instances ethylene functions as a negative regulator of adventitious rooting suggesting that the response to ethylene might differ between genotypes and the developmental stage of the cutting [84]. In rice, during crown root initiation ethylene activity is stimulated by gibberellic acid and inhibited by abscisic acid [85]. Interestingly, gibberellic acid inhibits adventitious root formation in poplar cuttings [86]. Therefore, the function of ethylene and gibberellic acid vary during the development stages of the adventitious roots.

The crosstalk between auxin and abscisic acid has been addressed using mutants with lateral root formation phenotypes, but similar work has not been reported in the context of adventitious root development. Abscisic acid has been reported as a positive regulator of adventitious root development in *Vigna radiate* and *Hedera helix*, but as a negative regulator in rice and tomato

[85,87–89]. Another hormone that has been rarely reported to play a role in adventitious root development is brassinosteroid. Brassinosteroids might promote lateral root development in an auxin-dependent way [20]. Interestingly, the synthetic brassinosteroid analogue (22S,23S)-28-homobrassinolide promotes adventitious rooting in Norway spruce cuttings [90]. The role of abscisic acid and brassinosteroid during adventitious root development remains unclear.

High levels of cytokinin suppress adventitious root formation in *A. thaliana* and tomato suggesting cytokinin is a negative regulator [91]. Furthermore, trans-zeatin riboside, the transport and storage form of cytokinin, inhibits adventitious root development in cucumbers [92]. A Type-B Response Regulator (CRR) in Populus and a Type-A response regulator in rice have been implicated in adventitious root development [74,93]. Auxin affects cytokinin biosynthesis and transport during adventitious root development in pea and carnation cuttings [34,94]. Interestingly, certain cytokinin analogues at lower concentrations promote adventitious root formation in apple [95]. Cytokinin regulates auxin transporters PIN1 and LAX3 to regulate the establishment of adventitious roots in *A. thaliana* [25]. Overall, the role of cytokinin is determined by the developmental stage of adventitious root formation.

1.4.3. Low temperature

Although cold-storage is one of the protocols most relied in agriculture to promote adventitious root formation in cuttings, the molecular mechanisms that regulate adventitious root development are not well understood. One of the earlier studies on the effect of extended cold treatment on chest-nut cuttings indicated the establishment of rooting zones as the duration of cold exposure increased [96]. Furthermore, the extended cold exposure also improved the rooting efficiency of difficult-to-root species [97]. These results suggest the inactivation of inhibitors or the activation of promoters of adventitious rooting during optimal cold treatment. In some species, cold storage has also been shown to have an ecotype dependent inhibitory role in adventitious rooting [19,98–102].

Carnation cuttings are the most common horticultural crops propagated by stem cuttings exposed to cold storage. Polar auxin transport and its regulator AUX1 modulate adventitious root formation in the carnation cuttings [66]. Localised auxin response following polar auxin transport was responsible for efficient rooting in easy-to-root varieties, whereas enhanced auxin conjugation inhibited root formation in the difficult-to-root cultivars [63]. The homeostasis of abscisic acid and

salicylic acid during cold storage has also been reported to regulate adventitious root primordium formation in carnation cuttings [34]. Exogenous application of auxin promoted adventitious root formation on carnation cuttings during cold-storage, which was slightly inhibited in the presence of light during storage [103].

Interestingly, extended cold exposure inhibited both auxin transport and lateral root growth in vertically-grown *A. thaliana* seedlings [104]. Whereas, another study reported the promotion of lateral root initiation by CRF2 and CRF3 during cold exposure [105]. Amongst all the contradictory studies, it is difficult to assign a role for the effect of cold exposure on adventitious root formation. Though, it could be assumed that the effect of cold exposure on adventitious root formation is certainly environment and genotype dependent.

1.5. *A. alpina* clonally propagates in nature using adventitious roots

Recent studies have focused on the characterisation of the Brassicaceae member *A. alpina*, an arctic-alpine perennial, to understand the perennial life-history strategies in detail [106]. The majority of *A. alpina* are self-compatible species and reproduce sexually thorough the production of flowers and eventually seeds [107]. Certain degree of clonal propagation has been reported in natural populations that might serve as a bet-hedging reproductive strategy at higher elevations [108]. This is probably through adventitious root production on stem, since they haven't been reported to produce special organs to aid this process [106,108]. *A. alpina* is distributed across the majority of the European alpine habitats, eastern Africa, the Anatolian peninsula and the eastern North America, which have a varied climate [109,110].

Genetic variations in flowering time regulation is a trait affecting adaptation of *A. alpina* [111]. The *A. alpina* ecotype Paj obligatorily requires vernalization to flower and produces fewer seeds [107]. In contrast, the perpetually flowering ecotypes, Dor, Tot and Wca, flower continuously throughout the plant life cycle without the requirement of vernalization to flower. These natural variations are conferred by allelic differences of the *PERPETUALLY FLOWERING 1* (*PEP1*) gene [111]. Mutation of this gene in seasonally flowering *A. alpina* accession Paj leads to perpetual flowering phenotype [107]. It is assumed that genetic variations in adventitious rooting would help these plants to adapt through clonal propagation in the different environmental conditions, leading to the observed divergence in life-history strategies.

1.6. Auxin induces adventitious root development in diverse species

The earliest protocol to induce adventitious roots on *A. thaliana* required seedlings grown on vertical petri-plates followed by removal of root tips [112]. The recent protocols require etiolated seedlings treated to light leading to adventitious root formation on the elongated hypocotyls [50,51]. Many studies have used the classical system of de-rooted plants or stem cuttings of different species to study adventitious root formation [19,63,113,114]. Most of these studies have benefitted from exogenous auxin application to promote adventitious root formation.

Auxin application during adventitious rooting has helped dissect genes and pathways regulated during auxin mediated adventitious rooting. It was the very first hormone characterised for regulating adventitious root production [44,115]. Auxin analogues are applied in the form of solution or powder in horticulture to induce adventitious roots on plant cuttings [116]. Indole-3-butyric acid (IBA) is the most commonly used auxin analogue due to its greater stability and higher effect on adventitious root induction in comparison to the natural analogue indole-3-acetic acid (IAA). Exogenous auxin application promotes root formation on stem cuttings in several species [27,117–124]. Auxin also affects the de novo root generation in leaves in *A. thaliana* and *Morinda citrifolia* [122,125].

Though very uncommon, auxin spray was used to induce adventitious roots on cuttings of herbaceous perennials in two previous studies [116,126]. Recent studies comparing the different methods of hormone delivery including application of auxin at the base of the cutting, shoot-tip drench, foliar spray and stem injection concluded that foliar spray was the most efficient way [127,128]. Auxin spray has been used to investigate auxin sensitivity and response in *A. thaliana* and Scots pine [128–131]. Incidentally, one study found a positive correlation between auxin applied via spray on the leaves of intact plants and adventitious rooting in *Rumex* species [132]. Since auxin foliar spray is practiced in agricultural industries to induce adventitious root, auxin spray is a promising tool to study adventitious rooting on intact plants as well as cuttings.

1.7. Genetic approaches to study adventitious root formation

Induced mutations give us the opportunity to identify new genes participating in the phenotype of interest. Mutagenesis can be induced by various approaches involving chemical, radiations and insertional methods [133]. Both forward and reverse genetics approaches are valuable to search for

genes affecting a desired phenotype. Adventitious rooting mutants have helped discover genetic and proteomic regulators of adventitious rooting [24,26,47,50–54,62,67,68,72–74,134–146]. Mutants defective in auxin signalling and transport have time and again proved the importance of auxin in the adventitious root development in different species [51,65,147]. Genes affecting the developmental phases of adventitious rooting have also been identified using mutants [148]. One of the recent studies explored the role of myosin in adventitious root development by creating a knock-out line affecting three myosin encoding genes and a rescue transgenic line [149]. Therefore, genetic approaches provide a valuable tool to characterise molecular regulators participating in adventitious root development in *A. alpina*.

1.8. Research aims

Adventitious root development has not been characterised in alpine perennials. *A. alpina*, with its short juvenile phase, provides a model to investigate the evolutionary ecology of alpine plants including the trade-offs between sexual and asexual reproduction, particularly in the light of recent research showing the possibility of transcriptomic studies in this species [150]. The basic understanding of flowering time regulation in *A. alpina* also aids us examine the dependence of adventitious root formation on the developmental stages of plants. Naturally occurring genetic variation reported earlier in *A. alpina* help uncover the effect of genotype, age and environmental conditions on adventitious root development [111]. The knowledge gathered from the annual *Arabidopsis thaliana* would help further our understanding of natural occurrence of clonal growth thought adventitious rooting in the closely related perennial *A. alpina*.

Firstly, the development and calibration of a protocol to induce adventitious root formation robustly was required. Secondly, this would be followed by exploring adventitious rooting in natural variations of *A. alpina*. Thirdly, the thesis aims at understanding the molecular mechanisms regulating this process. Finally, the aim of this study was to discover the effect of natural stimuli on adventitious rooting in *A. alpina*.

2. RESULTS

2.1. *A. alpina* shows natural variation in adventitious rooting

A. alpina can produce adventitious roots in nature, whereas protocols to induce adventitious root formation in plants grown in controlled greenhouse conditions was not established. This chapter focuses on the study of adventitious root formation in ecotypes and the development and optimization of protocol that would ensure the robust production of adventitious roots.

2.1.1. *A. alpina* produces adventitious roots in the greenhouse

The seasonally flowering ecotype Paj along with the perpetually flowering ones, namely, Dor, Tot and Wca, were grown in a long day greenhouse to investigate natural variation for adventitious rooting in *A. alpina* (Figure 2-1A, C-E). The perpetually flowering ecotypes in this study carry mutant alleles of the *PEP1* gene [111]. Therefore, the perpetually flowering *pep1-1* mutant in the well-characterised Paj background was included in this study to gain further insight into the correlation between flowering behaviour and clonal propagation through adventitious root production (Figure 2-1B). Contrary to its background, *pep1-1* flowers in long days similar to the ecotypes Dor, Tot and Wca without the requirement of vernalization [111] (Supplementary Figure 7-1). The number of plants with adventitious roots on the hypocotyl, the main stem and the axillary branches were scored on six-week old plants. At this developmental stage, adventitious roots were observed on the hypocotyls and the main stem of the plants, however none of the ecotypes showed adventitious roots on the axillary branches (Figure 2-1F-I). Interestingly, *pep1-1* compared to Paj showed increased potential to develop adventitious roots on the hypocotyl. On the contrary, excluding Tot, all ecotypes developed adventitious roots on the hypocotyl at genotype-specific frequencies, with *pep1-1* showing the highest potential to develop adventitious roots on the hypocotyl (Figure 2-1F, I). Additionally, unlike the other ecotypes, all Wca plants had adventitious roots on the second internode from the cotyledons (Figure 2-1H). The perpetually flowering ecotypes, Dor, Tot and Wca, and the *pep1-1* mutant developed adventitious roots on the main stem and the hypocotyl at different frequencies suggesting that the quantitative and qualitative

difference between ecotypes to develop adventitious roots is not influenced by their flowering behaviour.

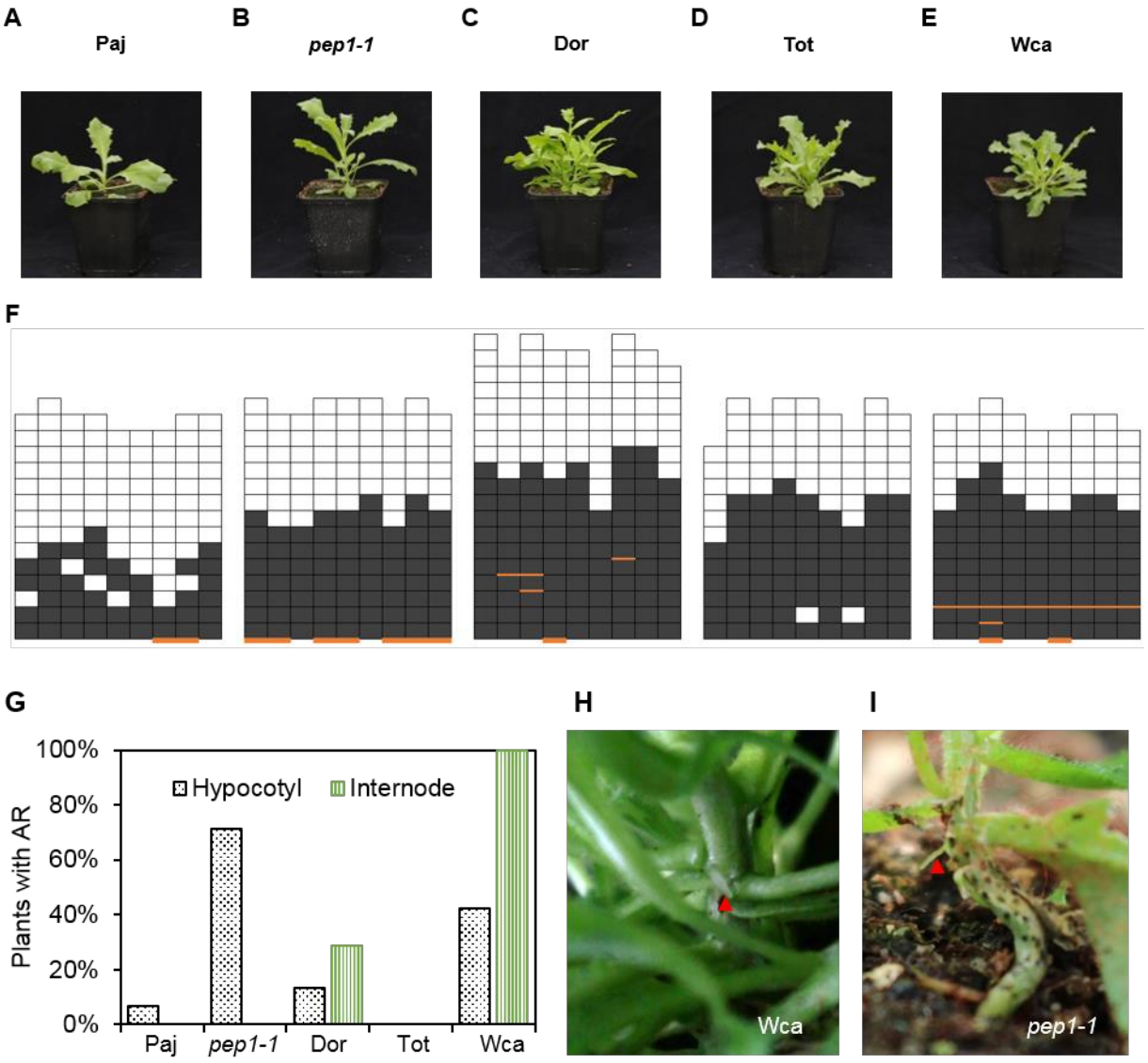

Figure 2-1. **Natural variation for adventitious rooting in *A. alpina*.**
(A) Paj, **(B)** *pep1-1*, **(C)** Dor, **(D)** Tot and **(E)** Wca grown in long days for 6 weeks. **(F)** Graphical representation showing presence of adventitious roots in the accessions and the *pep1-1* mutant. Each column represents a plant of **(A)** Paj, **(B)** *pep1-1*, **(C)** Dor, **(D)** Tot and **(E)** Wca, with each box representing a leaf axil and the lines between boxes in a column representing an internode. The presence of branches (gray boxes) and adventitious roots on the main stem (thick orange lines in a column) were scored in six-week old long day-grown plants. The thick orange lines at the bottom represent adventitious roots on the hypocotyl. **(G)** Percentage of plants with adventitious roots on the hypocotyl and on the main stem in *A. alpina* accessions and the *pep1-1* mutant. Plants did not produce adventitious roots in the axillary branches. Results are shown as an average of 45 plants. In long days, adventitious roots (red arrowhead) were found on the **(H)** main stem of Wca plant and **(I)** hypocotyl of *pep1-1* plants.

2.1.2. *GH3.3* and *GH3.6* homologs during adventitious rooting in natural accessions of *A. alpina*

Auxin conjugating *GH3* genes, *GH3.3*, *GH3.5* and *GH3.6*, regulate adventitious rooting in *A. thaliana* [52]. To explore whether the increased potential of Wca to develop adventitious roots on the main stem correlated with the expression of the auxin inducible *GH3* genes, the homologues of *GH3.3*, *GH3.5* and *GH3.6* were searched for in the *A. alpina* genome. While the homologue of *GH3.5* was not found annotated, homologues of *GH3.3* (*AaGH3.3*) and *GH3.6* (*AaGH3.6*) were identified and sequenced in the *A. alpina* genome. Differences were present in the coding sequences of *AaGH3.3* among Dor, Paj, Tot and Wca. Whereas the coding sequence of *AaGH3.6* was fully conserved among Paj and Wca, the corresponding Dor and Tot *AaGH3.6* sequences are similar to each other however showed multiple base pair differences in comparison to the Paj *AaGH3.6* (Supplementary Figure 7-2A-B).

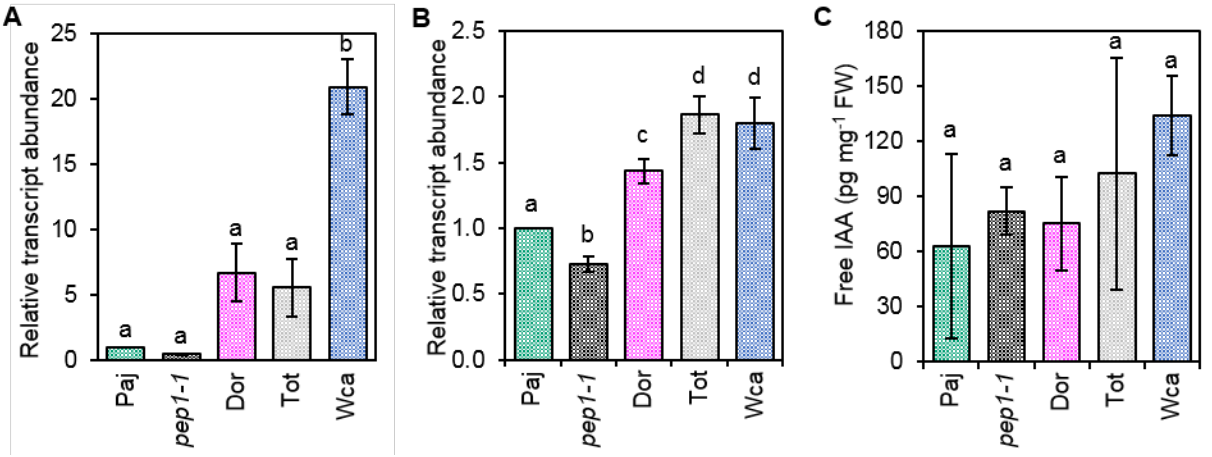

Figure 2-2. **Transcript levels of homologs of *GH3.3* and *GH3.6*, and IAA content in *A. alpina* accessions.**
The transcript abundance of the homologues of **(A)** *GH3.3* and **(B)** *GH3.6* quantified by quantitative RT-PCR in the main stem of 6-week-old Paj, *pep1-1*, Dor, Tot and Wca. *AaPP2A* was used as the house-keeping gene. Three biological replicates were used for this study. A one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post *hoc*-test with Benjamini correction was used to get the values significantly different ($P < 0.05$) as presented in Supplementary Table 1 & 2. **(C)** The amount of endogenous free IAA in the main stem of 6-week-old Paj, *pep1-1*, Dor, Tot and Wca. Error bars indicate SD of three biological replicates. An ANOVA on the data indicated no significant difference across the data as shown in Supplementary Table 3. FW denotes fresh weight.

The examination of the expression levels in the main stems of the six-week old ecotypes and the *pep1-1* mutant was investigated (Figure 2-2A-B; Supplementary Table 1; Supplementary Table

2). *AaGH3.3* showed significantly higher expression levels in Wca, while *AaGH3.6* expression significantly differed in Dor, Tot and Wca. To examine if higher *GH3.3* and *GH3.6* expression affected the endogenous free IAA content during adventitious rooting and otherwise, the level of free endogenous IAA (pg mg^{-1} of Fresh Weight, FW) was measured in the main stem of each genotype (Figure 2-2C; Supplementary Table 3). There was no significant difference in the free endogenous IAA levels across the ecotypes.

2.1.3. Auxin spray induces adventitious root formation in an age-, day length, -dosage- and genotype-dependent manner

For a robust induction of adventitious root production, intact plants were sprayed with 1-Naphthaleneacetic acid, 1-NAA. Initially the protocol was primarily optimised by treating Paj plants with 1-NAA and the inactive analogue 2-NAA to ensure that the induction of adventitious roots by auxin as a biologically relevant process (Figure 2-3). Plants sprayed with 1-NAA every week until 2 weeks produced adventitious roots on the main stem and the branches. However, 2-NAA sprayed plants did not produce adventitious roots indicating that auxin (1-NAA) spray induces adventitious root formation in *A. alpina*.

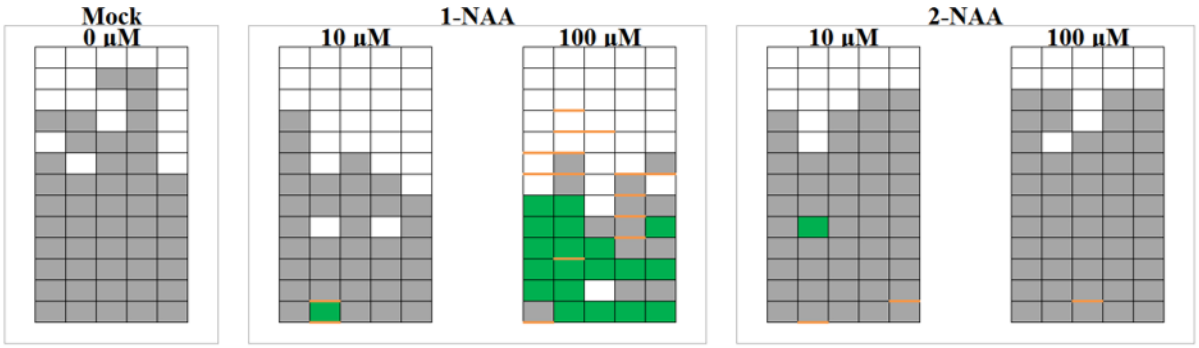

Figure 2-3. **Auxin spray promotes adventitious rooting in Paj.**
Plants were sprayed with 10 and 100 µM of the auxin analogues, 1-NAA and 2-NAA, every week until 2 weeks. The presence of adventitious roots on the main stem (until 12 expanded internodes) and branches was scored in 8-week old Paj plants 3 weeks after spray. Each column represents a plant, with each box representing a leaf axil and the lines between boxes in a column representing an internode. Branches are denoted as gray boxes. Adventitious roots on the internodes are represented as thick orange lines in a column, whereas green boxes represent branches with adventitious roots.

Further experiments were designed to induce adventitious roots following a one-time auxin spray application on both eight-week old Paj and *pep1-1* plants. To examine the effect of day-length on

adventitious root formation, plants grown in long-day (16 h light/ 8 h dark) and short-day (8 h light/ 16 h dark) conditions were sprayed with a control solution without auxin and a 100 µM auxin solution. Relative to plants grown in long-day condition, short-day grown plants produce adventitious roots on more internodes, suggesting that one-time auxin spray is a robust technique (Figure 2-4). However, since the short-day grown eight-week old plants did not produce branches at the time spray, adventitious roots were not observed on branches. Further studies to understand adventitious root development were carried out under long day length conditions that promotes adventitious roots on the main stem as well as the branches.

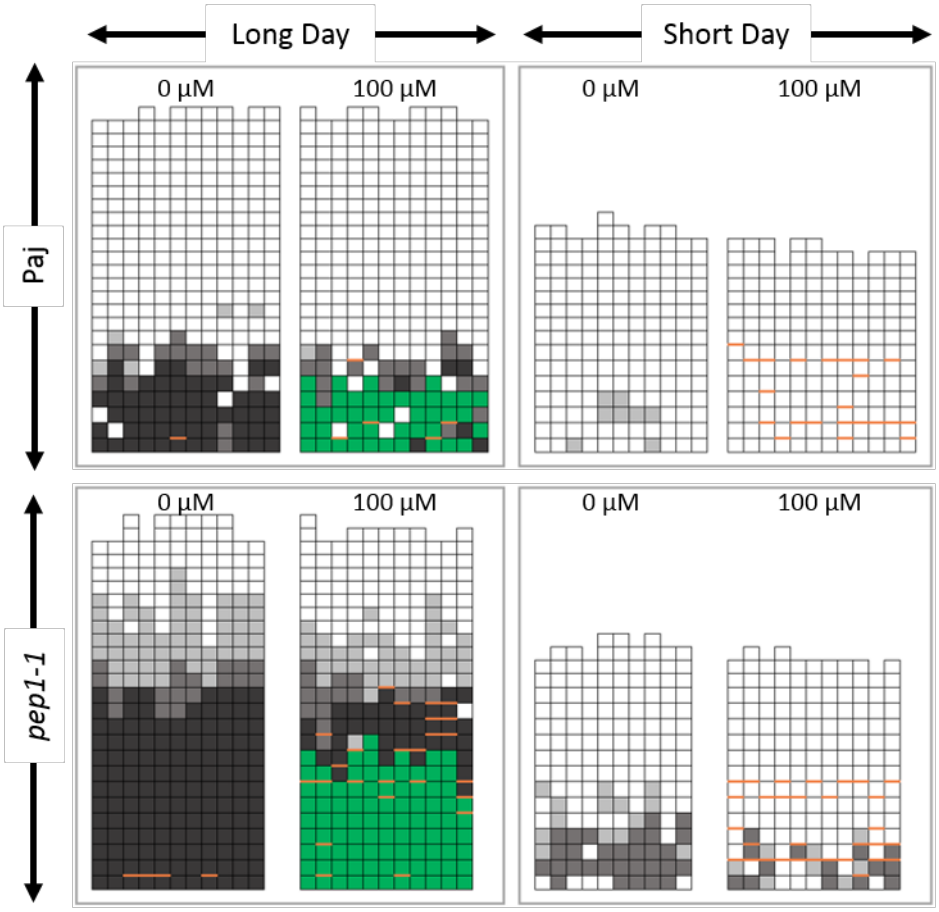

Figure 2-4. **Day-length effect on adventitious root formation.**
Eight-week old Paj and *pep1-1* plants grown in long-day (16 h light/ 8 h dark) and short day (8 h light/ 16 h dark) conditions were sprayed with 0 and 100 µM 1-NAA. Graphical representation of plants 2 weeks after spray. Each column represents a plant, with each box representing a leaf axil and the lines between boxes in a column representing an internode. Branches are denoted as gray boxes and adventitious roots on the internodes as thick orange lines in a column. Green boxes represent branches with adventitious roots.

An attempt to induce adventitious roots by applying auxin on three- and five-week old Paj plants grown in long day greenhouse was unsuccessful due to the absence of a definitive main stem or branches (Figure 2-5). On the contrary, adventitious roots were produced on the main stem of five-week old *pep1-1* plants. Therefore, the effect of spraying auxin on older seedlings, i.e. six-week and eight-week old, was investigated. This provided the opportunity to compare the propensity to produce adventitious roots between six-week old vegetative *pep1-1* plants and eight-week old *pep1-1* plants having undergone the vegetative-to-flowering transition, whereas Paj plants were vegetative. The timing of floral transition in *pep1-1* was evaluated by quantifying *AaLFY* in the apices of plants aged two to eight weeks (Figure 2-6). *AaLFY* was upregulated in eight-week old *pep1-1* plants, whereas Paj did not show any upregulation.

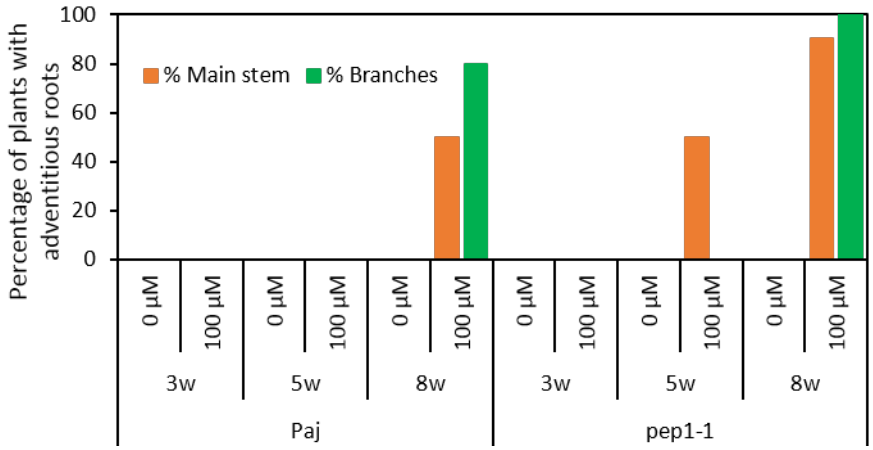

Figure 2-5. **Auxin spray promotes adventitious rooting in Paj and *pep1-1* in an age-dependent manner.**
Paj and *pep1-1* plants of 3, 5 and 8 weeks were sprayed with mock (0 µM) / 100 µM 1-NAA. The presence of adventitious roots on the main stem (orange) and branches (green) was scored 3 weeks after auxin spray.

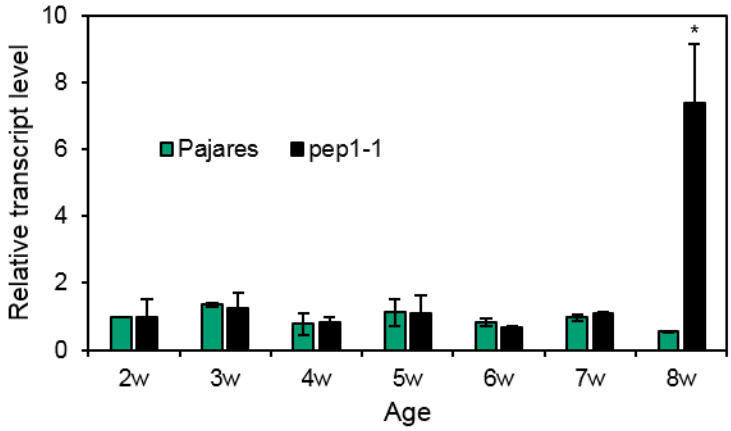

Figure 2-6. **Flowering transition of *pep1-1*.**
The abundance of *AaLFY* with respect to *AaPP2A* in 2, 3, 4, 5, 6, 7 and 8 week-old Paj and *pep1-1* plants. Three biological replicates were used for this study. Differences were tested using a Student's t-test with a significance of p-value < 0.05.

1-NAA applied at 10, 20, 50 and 100 µM concentrations was applied using spray and the occupancy of axillary branches and internodes on the main stem with adventitious roots was recorded one, two, three and five weeks after spraying auxin in six- and eight-week old Paj and *pep1-1* plants. Both *pep1-1* and Paj plants developed adventitious roots in the internodes on the main stem after the application of auxin in a dosage dependent manner, such that more internodes developed adventitious roots following the application of higher concentration of 1-NAA (Figure 2-7; Supplementary Table 4, 5). To consider the effect of age and the resulting architecture of the genotypes, the frequency of internodes or branches occupied by adventitious root are shown. Additionally, both genotypes had more axillary branches with adventitious roots when sprayed at the age of eight-weeks than at the age of six-weeks. While no substantial differences were observed in the branches with adventitious roots in six-week old Paj and *pep1-1*, eight-week old plants responded earlier to produce adventitious roots on branches. Similar to internodes, the adventitious roots on branches were always seen two weeks after spray irrespective of the concentration of auxin spray.

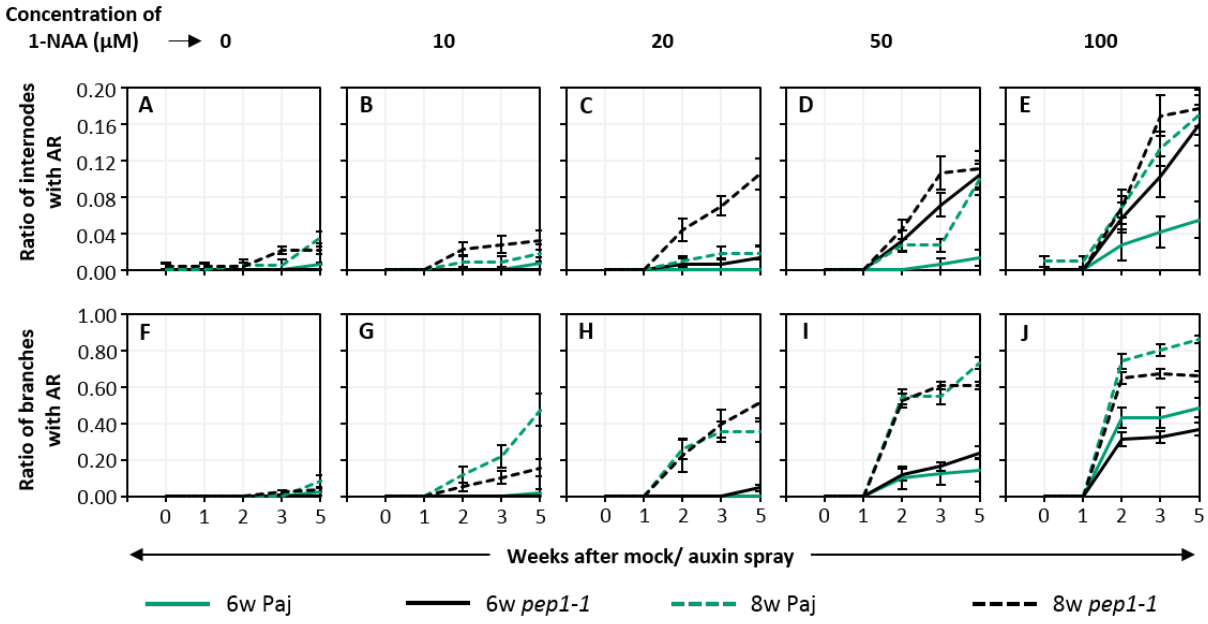

Figure 2-7. **Auxin spray induces adventitious roots in a dosage and age dependent manner in Paj and *pep1-1*.**
Proportion of **(A-E)** internodes and **(F-J)** branches with adventitious roots after the application of 0, 10, 20, 50 and 100 µM 1-NAA relative to before spray in six-week (6w) and eight-week (8w) old Paj and *pep1-1* plants. Plants were scored before spray and 1, 2, 3 and 5 weeks after spray. Ten plants were characterized for each accession/mutant for each treatment. Statistical analyses are presented in Supplementary Table 4.

Another mutant allele of *PEP1* in *A. alpina*, *pep1-2,* was investigated for adventitious root formation. Six-week old *pep1-2* plants produced adventitious roots on the hypocotyl with a higher frequency than *pep1-1* (Figure 2-8A). Application of auxin at concentrations of 10, 50 and 100 µM did not induce adventitious root production in these mutants (Figure 2-8B-D). These results suggest that the adventitious root development in *A. alpina* shows a dosage dependent response to auxin which differs among the genotypes and is dependent on the age of the plants and the day-length the plants were exposed to during the auxin spray application. Furthermore, it also shows that the main stem and the branches show different competence for responding to auxin spray. Finally, *PEP1* seems to participate in adventitious rooting as shown by the two mutants, *pep1-1* and *pep1-2*, although the effect seem to be allele specific.

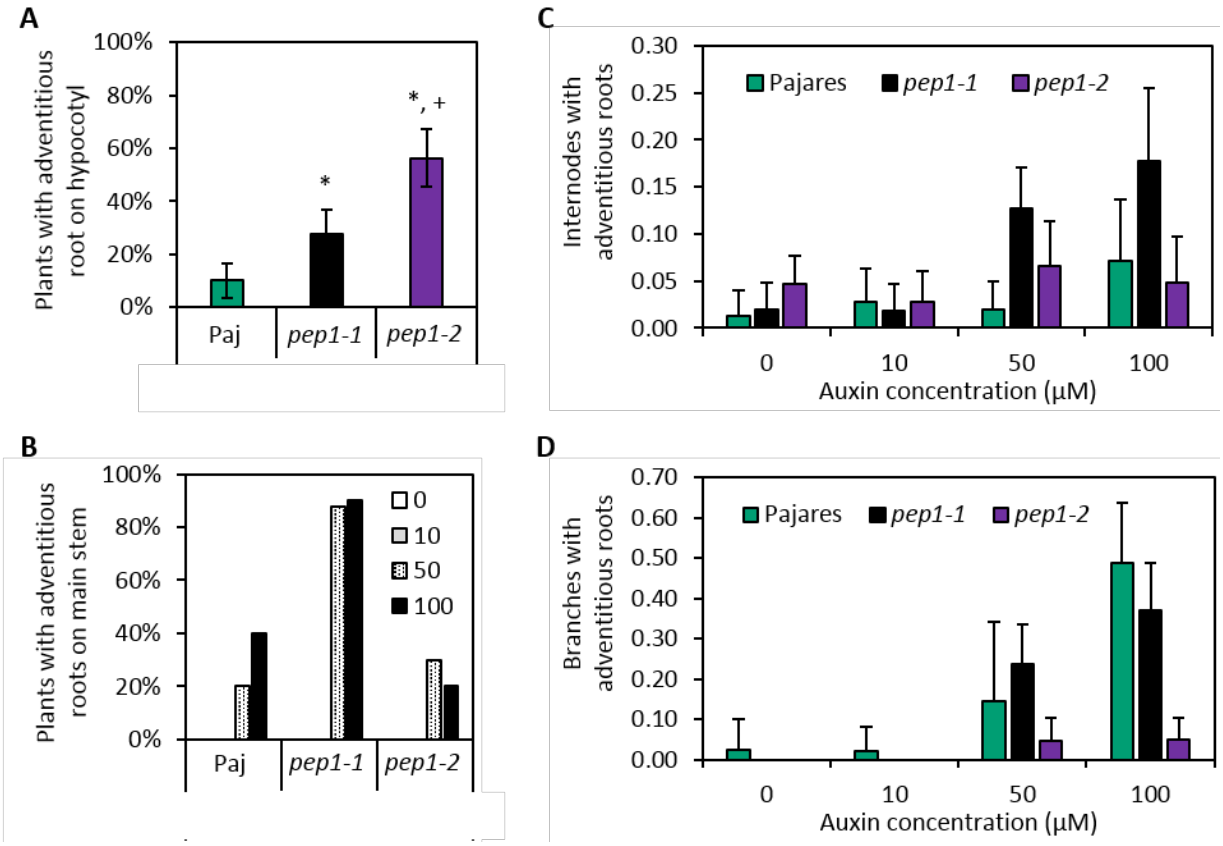

Figure 2-8. ***pep1-2* does not respond to auxin spray.**
(A) Percentage of six-week old Paj, *pep1-1* and *pep1-2* plants with adventitious roots on the hypocotyl. Statistical differences were obtained using a Student's t-test. Here, * and + represent significance relative to Paj and *pep1-1*, respectively. **(B)** Percentage of six-week old Paj, *pep1-1* and *pep1-2* plants with adventitious roots on the main stem 5 weeks after application of 10, 50 and 100 µM 1-NAA. Five weeks after auxin spray application, the ratio of **(C)** internodes and **(D)** branches producing adventitious roots relative to internodes and branches at the time of spray in Paj, *pep1-1* and *pep1-2*. The standard deviation corresponds to deviation within 10 plants.

2.1.4. Auxin spray affects *A. alpina* ecotypes in diverse ways

To test the sensitivity to auxin, the remaining ecotypes, Dor, Tot and Wca, were sprayed with different concentrations of 1-NAA, as earlier. Six-week old plants were used, since the effect of auxin at this developmental stage revealed a difference in response between Paj and *pep1-1*. Since the ecotypes show differences in the number of branches and internodes, the frequency of internodes or branches occupied by the adventitious roots is shown to nullify the difference in the developmental stage of the genotypes. Irrespective of mock or auxin application, similar number of internodes in Wca plants were occupied by adventitious roots (Figure 2-9; Supplementary Figure 7-3; Supplementary Table 6, 7). Application of 1-NAA on the ecotype Tot promoted the

formation of adventitious roots in the axillary branches, whereas it did not influence adventitious root production on the main stem. Auxin spray application did not affect adventitious root formation in the ecotype Dor, since similar number of internodes on the main stem or axillary branches occupied by adventitious roots after mock or 1-NAA treatment. To check whether this phenotype was due to a technical problem during auxin spraying, branching, a trait regulated by auxin was scored [151]. In all genotypes, including Dor, application of 1-NAA reduced the number of branches suggesting that the lack of auxin response observed in Dor is specific to adventitious rooting.

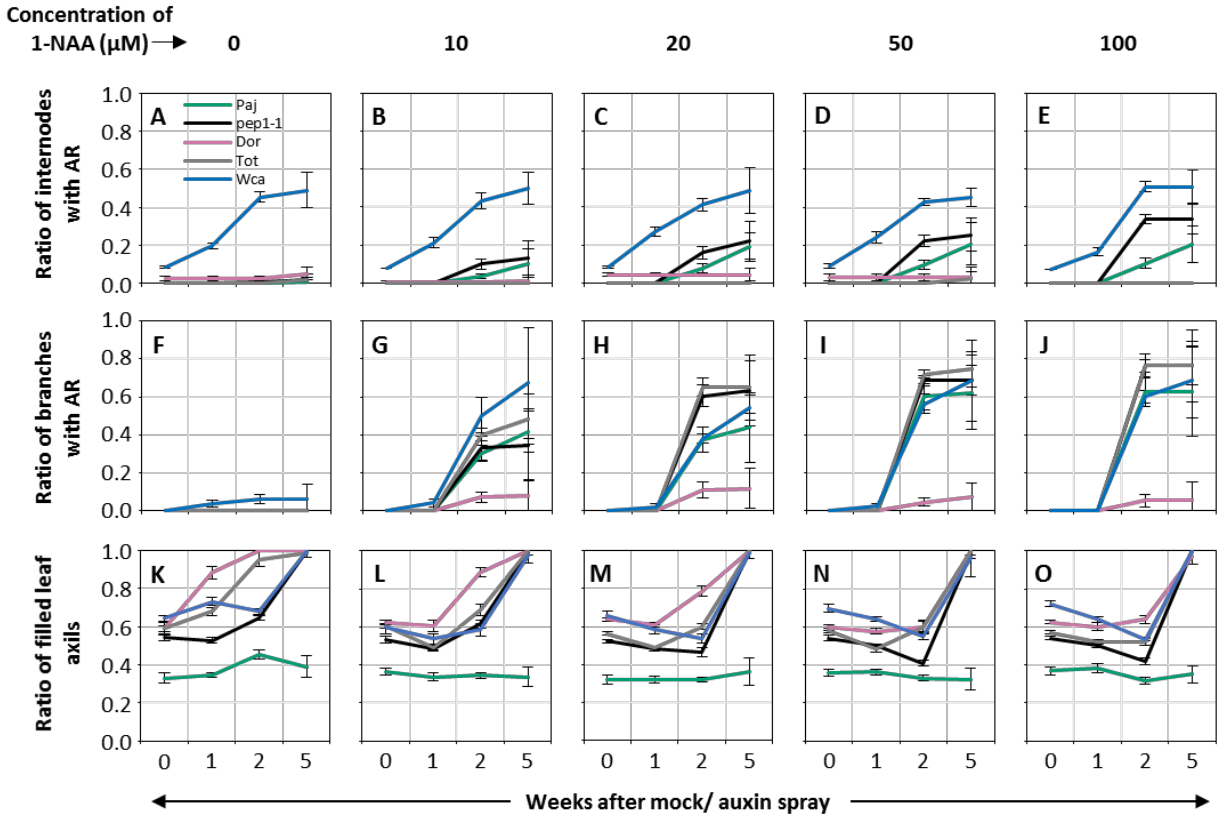

Figure 2-9. **Auxin spray induces adventitious roots in a dosage dependent manner in *A. alpina* accessions and *pep1-1*.**
Proportion of **(A-E)** internodes with adventitious roots, **(F-J)** branches with adventitious roots, and **(K-O)** leaf axils filled with branches after the application of 0, 10, 20, 50 and 100 µM 1-NAA relative to before spray in six-week old Paj, *pep1-1*, Dor, Tot and Wca plants. Plants were scored before spray and 1, 2 and 5 weeks after spray. Nine plants were characterized for each accession/mutant for each treatment. Statistical analyses are presented in Supplementary Table 5.

2.1.5. Identification of *A. alpina* mutants affected in adventitious rooting

The aim was to identify genes that participate in adventitious root formation supporting the clonal propagation of *A. alpina*. Auxin is a central player during adventitious root formation. Mutations in genes participating in auxin homeostasis can be embryo lethal as can be concluded from various studies [135,152–156]. As such, an EMS mutagenesis population might seem as an ineffective prospect to look for auxin-dependent adventitious root regulators in *A. alpina*. However, auxin spray gives us the opportunity to hunt for negative regulators of adventitious root formation in *A. alpina*, which do not spontaneously form adventitious roots in greenhouse conditions. In the case of a non-functional negative regulator, the density of adventitious rooting would be enhanced; besides adventitious roots might form on sections of the stem that have not been known to like the lower internodes of the main stem.

In nature, *A. alpina* grows more rosette-like but produces creeping branches which would eventually produce adventitious roots possibly foraging for nutrients in the harsh conditions. The induction of adventitious roots on *A. alpina* in the greenhouse conditions was quite ineffective since the plants were staked and stood upright. Auxin spray is a robust protocol to induce adventitious roots on the internodes of the branches and the main stem. In this study, the *pep1-1* mutant upon auxin spray shows higher response to adventitious rooting on branches and the main stem.

To study the molecular mechanism regulating adventitious rooting in *A. alpina*, ethyl methane sulfonate (EMS)-induced mutants in the *pep1-1* background were screened for mutants showing adventitious root developmental phenotype. Since *pep1-1* plants showed higher response to auxin spray for adventitious rooting, the screen focused on mutants that would not produce adventitious roots post repeated auxin spray. The absence of adventitious roots might be a result of a loss-of-function mutations in the positive regulator or a gain-of-function mutations in the negative regulator of adventitious root formation.

The screening was done in 3 phases with *pep1-1* being the control. The plants were screened for phenotypic differences before auxin treatment. Several mutants showing phenotypic differences in comparison to the *pep1-1* mutant, six weeks after sowing, were discovered. Among the plants, plants affected in growth rate, height, leaf shape, branching, flowering and apical dominance were

discovered. Some plants displayed differences in more than one phenotype. Most of the population was comprised of dwarf plants and plant with reduced growth depicted by fewer leaves, thinner stem and delayed branching. None of plants produced adventitious roots spontaneously in the absence of auxin. It indicates that probably more pools need to be screened.

Leaf curling was observed in the ecotypes for *A. alpina* plants treated with auxin. Auxin dependent leaf curling upon higher auxin levels has been described in several studies [142,155,157–159]. Auxin spray caused curling of leaves on all plants suggesting the auxin spray was saturating and verifying that all the plants were sprayed. The mutants were distributed into three categories: No AR, No AR (+) and others (Table 2-1).

Table 2-1. Categories of EMS mutants

Category	**Screen I**	**Screen II**	**Screen III**	**Total**
Screened	**765**	**585**	**420**	**1770**
Others	81	11	0	90
No AR	19	15	2	36
No AR (+)	26	8	16	52
Total	126	34	18	178

Table 2-1. **Summary of categories of mutants identified in the EMS screens.**
The total number of M1 families screened is represented below each screen. Mutants denoted 'Others' have phenotypes other than adventitious root related. 'No AR' mutants do not have visible adventitious roots on the main stem and the branches. 'No AR (+)' mutants did not produce adventitious roots and showed other phenotypic differences.

There were plants that looked like *pep1-1* plants but did not produce adventitious roots which were assigned to the 'No AR' category. While some others that did not produce adventitious roots had phenotypic differences already prior to auxin spray were categorized as 'No adventitious root (+)'. The plants that showed phenotypic differences before auxin spray were categorized as 'others'. The whole list of mutants characterized in this study are tabulated in the Supplementary Table 8.

The mutants that did not develop adventitious roots after auxin spray, a total of 40, were grown for re-checking the absence of adventitious roots upon auxin spray. Surprisingly, several of these mutants had plants producing adventitious roots post auxin treatment. It is possible that the putative mutants identified during the first screen were false positives. It is also possible that selected

mutant were lethal in homozygous lines and therefore, were absent in this screen. Overall, it suggests that further screens are required to identify an adventitious rooting regulator.

Among the mutants showing adventitious rooting defects, apart from non-adventitious rooting mutants, the plants delaying adventitious root production seem interesting. Mutations in genes regulating the induction, the dedifferentiation of shoot based cells into stem cells and the priming of the root primordium might affect this phenotype. The influence of mutations in cell cycle related genes might be too drastic to be only affecting adventitious rooting. On the other hand, there are mutants that produce adventitious roots only on branches upon auxin spray. This behaviour is similar to *A. alpina* plants of Tot accession sprayed with auxin which behave similarly. Detailed mapping of Tot or these mutants would further be required to understand the bias regulating adventitious rooting in the main stem and branches.

2.2. Differential regulation of hormonal responses regulates adventitious root formation in vernalized *A. alpina* following auxin spraying

Auxin spray promotes adventitious root formation in seasonally flowering vegetative Paj plants and the perpetually flowering ecotypes. Paj plants undergo vegetative to flowering transition while exposed to 12 weeks of vernalization, and flower at the end of vernalization. This section centres around the effect of auxin spraying on flowering Paj plants at the end of vernalization followed by a transcriptomic study aimed at understanding the regulation of adventitious root formation in *A. alpina*.

2.2.1. Auxin spray induces adventitious root development on specific internodes of vernalized *A. alpina* plants

To determine the response of flowering Paj plants to the application of auxin, plants vernalized for 12 weeks were sprayed with different concentrations of 1-NAA (10, 20, 50 and 100 μM) and a control solution (0 μM). Plants were then scored for the presence of adventitious roots 1, 2 and 3 weeks after the spray. Adventitious roots appeared on the main stem and on axillary branches 1–3 weeks after auxin spray, with a clear dose-dependent response in terms of the number of plants, branches and internodes responding to auxin (Figure 2-10).

Even though the whole plant was sprayed with auxin, the adventitious roots formed in 1–3 specific internodes, regardless of the applied auxin concentration (elongated internodes between nodes 11 and 15; Figure 2-10). Adventitious roots preferentially developed on the uppermost elongated internodes characterized by the presence of dormant axillary buds in the leaf axils (Figure 2-10) [160,161]. Overall, these results suggest that auxin can induce adventitious root development in vernalized *A. alpina* in a dose-dependent manner and that the internodes along the main stem axis differ in their capacity to initiate adventitious roots in response to auxin.

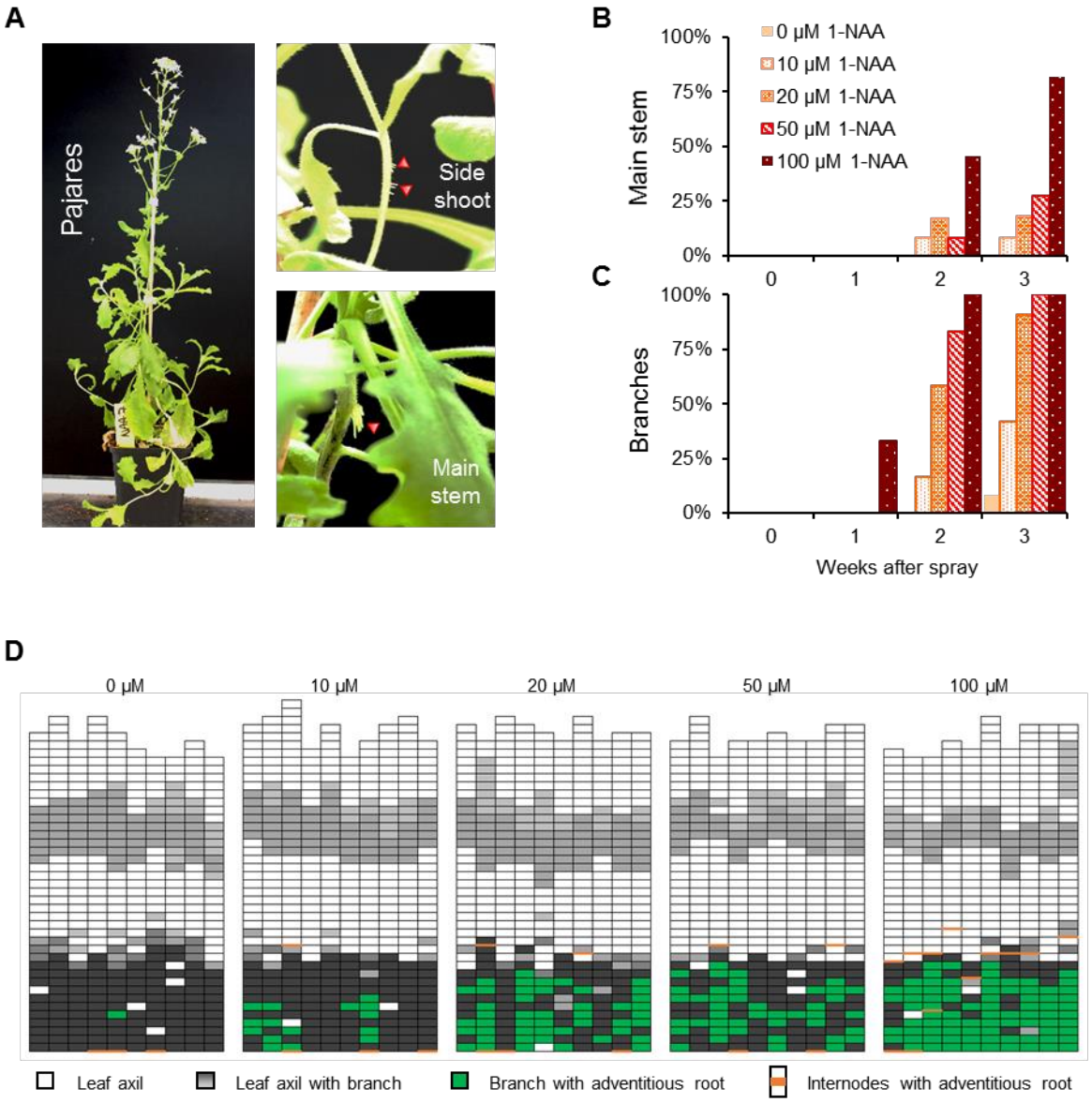

Figure 2-10. **Auxin spray induces adventitious rooting in vernalized *A. alpina*.**
(A) Adventitious roots on the main stem and branches of a 12-week vernalized *A. alpina* (accession *Paj*) shown with red arrow heads 2 weeks after auxin (100µM 1-NAA) spray. **(B, C)** Quantification of plants (n=10) with adventitious roots on the main stem (upper) and branches (lower) 3 weeks after auxin (1-NAA) spray on 12-week vernalized *A. alpina* plants with concentrations of 0µM, 10µM, 20µM, 50µM and 100µM. **(D)** Schematic representation of *A. alpina* plants (n=10), 3 weeks after spray, showing presence and absence of branches and adventitious roots in different zones of the plant. The plants were sprayed with 0, 10, 20, 50 and 100 µM 1-NAA after 12 weeks of vernalization. Each box represents a leaf node. Grey boxes represent leaf axils filled with branches such that the newer the branch, the lighter the shade of grey. Orange lines represent adventitious roots on the main stem.

2.2.2. Transcriptomic profiling after auxin spray application reveals hormonal signalling as a determinant of adventitious rooting in *A. alpina*

Histological analysis revealed the presence of adventitious root primordia in the vascular cambium tissue in the stems harvested from the rooting zone 120 h after auxin spray (Figure 2-11). The transcriptomic profiles of internodes with and without the capacity to initiate adventitious roots after auxin spray application to determine the factors regulating primordia formation. The plants were grown for eight weeks in a long-day greenhouse, vernalized for 12 weeks and then sprayed once with 10 μM 1-NAA or the corresponding control. Before and after spraying, two samples were harvested from the same plants at different times (6, 24, 72 and 120 h): the "rooting zone" (a pool of two extended internodes below the compact zone with the potential to produce adventitious roots after auxin application) and the "non-rooting zone" (a pool of two internodes below the rooting zone, lacking the potential to produce adventitious roots after treatment). Comparisons between the zones were expected to identify genes that regulate competence to adventitious rooting in response to auxin spray, whereas comparisons between the auxin spray and controls were expected to identify auxin-regulated genes. The experimental setup also controlled for the induction of genes by wounding during sample collection, given that the same genes would be induced in the control spray treatment lacking auxin.

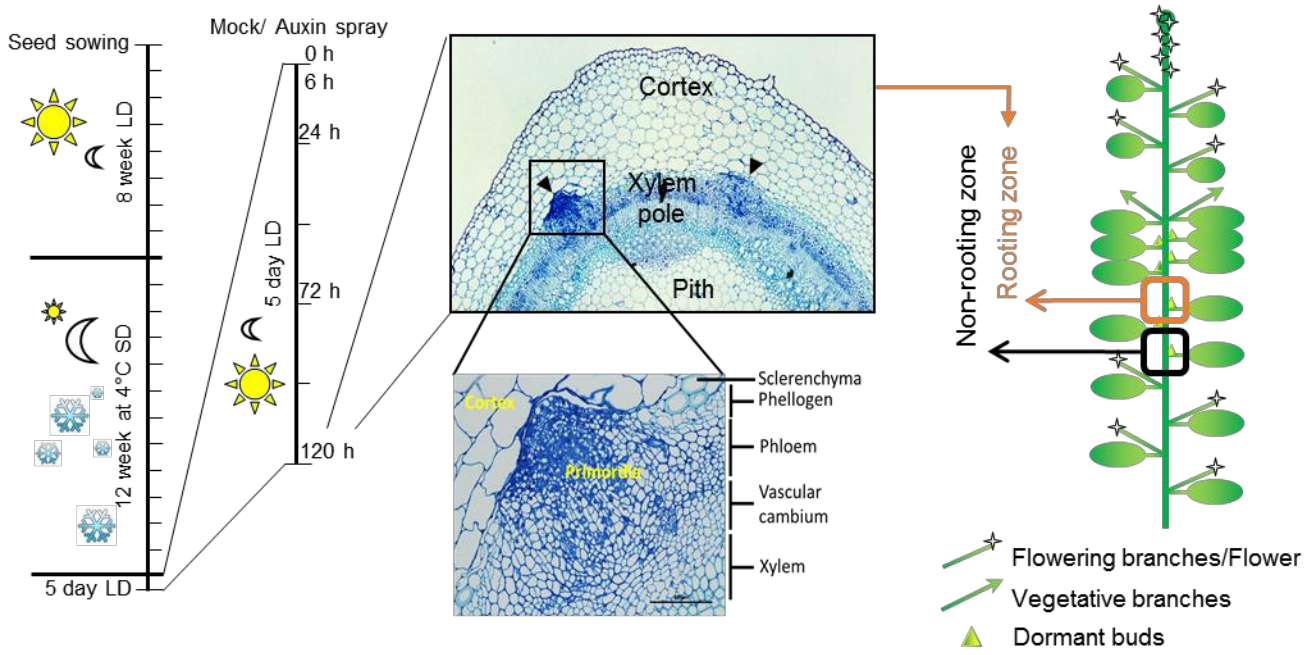

Figure 2-11. **Overview of the experimental set-up for sample collection.**
The plants were grown at 22°C in long days for 8 weeks before being transferred to 4°C for a period of 12 weeks in short days. The plants were transferred back to LD conditions and sprayed with mock/auxin solutions. The rooting zone and the non-rooting zones were collected before spray, 6 hours, 24 hours, 72 hours and 120 hours after spraying. At 120 hours, primordium (black arrow heads) formation could be seen on the stem cross-section on the main stem.

Nearly 12.5 million reads were sequenced from each library, among which ~89.6% could be mapped to the *A. alpina* Paj reference genome. The ratio of genes mapped and the genes aligning to multiple regions are tabulated in Supplementary Table 9. Neither the sample zone nor the auxin treatment affected the number of reads per sample, suggesting that these factors did not cause a change in the overall transcriptional activity.

Clustering of the expression profile data resulted in five groups defined by the timing relative to auxin treatment and the rooting response, i.e. before spraying (0 h), 6 h after spraying, 24 h after spraying, the zones 72 and 120 h after spraying that will not produce adventitious root and the zones 72 and 120 h after spraying that will produce adventitious roots. Comparison of the rooting and non-rooting zones in the mock treatment experiment indicated transcriptomic differences between samples that were non-auxin-spray dependent. At the initial time point (0 h), more than 700 genes were differentially expressed, which may explain the differences in competence to

adventitious root formation after auxin treatment (Table 2-2). The transcriptomic difference between the two zones was maintained at all subsequent time-points, with 1036, 876, 833 and 450 differentially expressed genes at 6, 24, 72 and 120 h after control treatment, respectively. Auxin treatment nearly doubled the number of differentially expressed genes detected 6 h after spray, suggesting an early transcriptome reprogramming by auxin (Table 2-2). Only 200 genes were differentially expressed when comparing the rooting and non-rooting zones at this stage after auxin treatment (Table 2-2). Around 72 and 120 h after treatment there was a clear separation between the rooting and non-rooting zones. Overall, these data indicate that the auxin response was saturated 6 h after spraying and that the expression of several genes promoting adventitious rooting might change dramatically 72 and 120 h after auxin spray. It therefore appears that the auxin response was dominant in the early phase, possibly affecting the expression of several genes associated with dedifferentiation and redifferentiation, but this had mostly worn off after 6 h, given the greater similarity between the auxin-sprayed rooting zone and corresponding mock treatment than between the auxin-sprayed rooting and non-rooting zones. The effect of rooting was observed 72 h after spraying (Figure 2-12). At these later time points (72 and 120 h), the auxin-sprayed rooting and non-rooting internodes showed significant differences in their transcriptomic profiles.

Table 2-2. Number of upregulated and downregulated genes

Condition 1	Condition 2	Upregulated	Downregulated	Total	% Up	% Down
EV-0-R	**EV-0-NR**	394	306	700	56.29	43.71
	6h-M-R	1094	1573	2667	41.02	58.98
	6h-A-R	1917	2506	4423	43.34	56.66
	24h-M-R	1633	1728	3361	48.59	51.41
	24h-A-R	1802	1972	3774	47.75	52.25
	72h-M-R	1457	1357	2814	51.78	48.22
	72h-A-R	2019	1697	3716	54.33	45.67
	120h-M-R	1631	1465	3096	52.68	47.32
	120h-A-R	1848	1743	3591	51.46	48.54
EV-0-NR	**6h-M-NR**	892	1372	2264	39.4	60.6
	6h-A-NR	1723	2409	4132	41.7	58.3
	24h-M-NR	1375	1662	3037	45.27	54.73
	24h-A-NR	1433	1672	3105	46.15	53.85
	72h-M-NR	2021	1424	3445	58.66	41.34
	72h-A-NR	1787	1734	3521	50.75	49.25
	120h-M-NR	1435	1411	2846	50.42	49.58
	120h-A-NR	1906	1742	3648	52.25	47.75

6h-M-R	**6h-M-NR**	563	473	1036	54.34	45.66
	6h-A-R	686	784	1470	46.67	53.33
	24h-M-R	1347	950	2297	58.64	41.36
	72h-M-R	2367	1819	4186	56.55	43.45
	120h-M-R	2464	1828	4292	57.41	42.59
6h-M-NR	**6h-A-NR**	965	1175	2140	45.09	54.91
	24h-M-NR	1503	1057	2560	58.71	41.29
	72h-M-NR	2682	1473	4155	64.55	35.45
	120h-M-NR	2193	1495	3688	59.46	40.54
6h-A-R	**6h-A-NR**	157	64	221	71.04	28.96
	24h-A-R	1719	1158	2877	59.75	40.25
	72h-A-R	3068	2542	5610	54.69	45.31
	120h-A-R	2753	2369	5122	53.75	46.25
6h-A-NR	**24h-A-NR**	1943	1592	3535	54.96	45.04
	72h-A-NR	2854	2485	5339	53.46	46.54
	120h-A-NR	2837	2278	5115	55.46	44.54
24h-M-R	**24h-M-NR**	452	415	867	52.13	47.87
	24h-A-R	625	578	1203	51.95	48.05
	72h-M-R	1417	1190	2607	54.35	45.65
	120h-M-R	1607	1370	2977	53.98	46.02
24h-M-NR	**24h-A-NR**	244	221	465	52.47	47.53
	72h-M-NR	1690	809	2499	67.63	32.37
	120h-M-NR	1374	1076	2450	56.08	43.92
24h-A-R	**24h-A-NR**	535	606	1141	46.89	53.11
	72h-A-R	1396	1045	2441	57.19	42.81
	120h-A-R	1317	1092	2409	54.67	45.33
24h-A-NR	**72h-A-NR**	911	777	1688	53.97	46.03
	120h-A-NR	1237	895	2132	58.02	41.98
72h-M-R	**72h-A-R**	820	596	1416	57.91	42.09
	72h-M-NR	658	175	833	78.99	21.01
	120h-M-R	357	287	644	55.43	44.57
72h-M-NR	**72h-A-NR**	297	843	1140	26.05	73.95
	120h-M-NR	363	812	1175	30.89	69.11
72h-A-R	**72h-A-NR**	377	572	949	39.73	60.27
	120h-A-R	291	503	794	36.65	63.35
72h-A-NR	**120h-A-NR**	538	331	869	61.91	38.09
120h-M-R	**120h-A-R**	528	749	1277	41.35	58.65
	120h-M-NR	225	225	450	50	50
120h-M-NR	**120h-A-NR**	534	346	880	60.68	39.32
120h-A-R	**120h-A-NR**	567	380	947	59.87	40.13

Table 2-2. **Table showing the number of upregulated and downregulated genes between different time points and treatments.**
The name of the samples are in the form 'Time-Treatment-Zone'. The time-points in this study include 'End of Vernalization' (EV), 6 hours (6h), 24 hours (24h), 72 hours (72h) and 120 hours (120h) after spray. Rooting and non-rooting zones are denoted as R and NR, respectively. The spray treatments are denoted as 0 (no spray), M (mock) and A (auxin, 1-NAA).

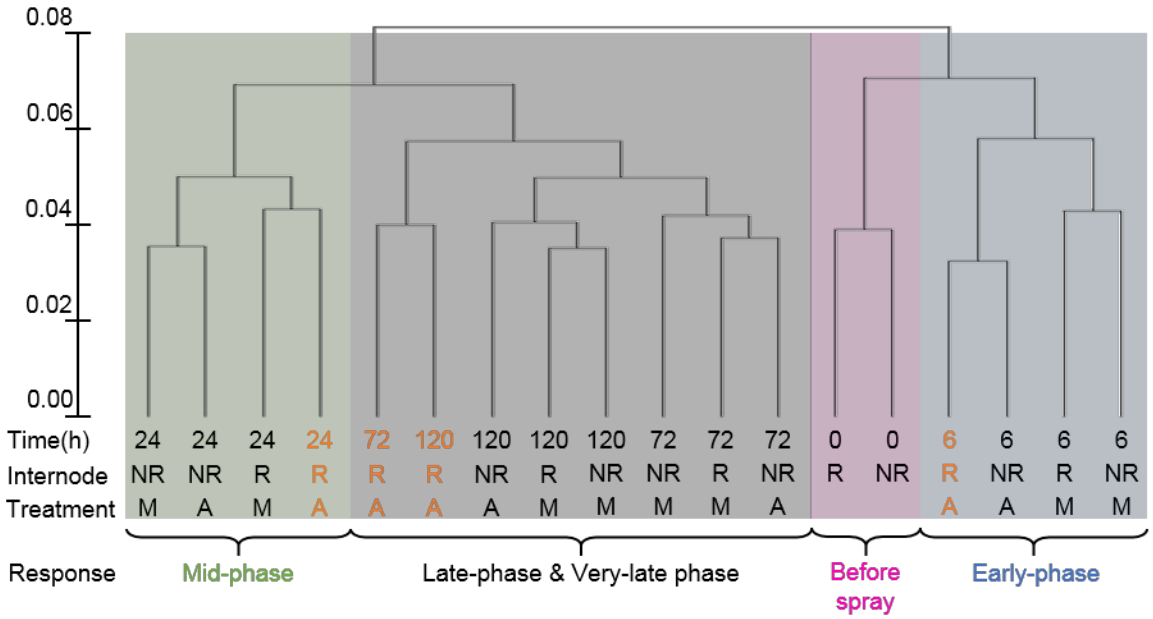

Figure 2-12. **Clustering of the expression profiles.**
The tree shows the distribution of the rooting (R) and the non-rooting (NR) zones at the end of vernalization, and 6, 24, 72 and 120 hours after mock (M)/auxin (A, 1-NAA) spray. The cluster is divided into sub-clusters representing the early-, mid-, late- and very late- phases of adventitious root formation. The cluster was generated using the R package "cummeRbund" to determine the relationship between conditions including time, treatment and zone.

Further analysis of the 9148 differentially expressed genes with orthologues in *A. thaliana* (72.53% of all differentially expressed genes in *A. alpina*) was done using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) Mapper. Nearly 4000 genes associated with KEGG pathways revealed an enrichment of genes participating in metabolic pathways (18.9%), mainly the metabolism of carbon (2.93%), purines (1.64%), starch and sucrose (1.62%), amino and nucleotide sugars (1.45%), cysteine and methionine (1.41%), glutathione (1.27%) and pyrimidine (1.08%) (Figure 2-13; Supplementary Table 10). Genes related to the biosynthesis of secondary metabolites (11.38%), amino acids (2.60%), phenylpropanoids (1.81%), and ribosomes (1.52%) were also enriched in these samples. The third most enriched category was plant hormone signal transduction (3.71%). Gene Ontology enrichment analysis was applied to gain insight into the various biological processes that might play a role during adventitious rooting in *A. alpina*. The GO categories plant organ development (GO:0099402), root development (GO:0048364), lateral root development (GO:0048527) and root system development (GO:0022622) were upregulated specifically in the rooting zone, only after auxin treatment (Figure 2-14). Several rooting-associated genes are auxin

responsive, and therefore were also upregulated in the non-rooting zone, however only 6 h after spraying. Interestingly, 24 h after spraying, root development genes were expressed specifically in the auxin-treated samples of the rooting zone, suggesting that the root primordium formation may have been induced already at this point. In addition, the expression of the homologue of *LATERAL ROOT PRIMORDIUM 1* (*LRP1*) in *A. alpina,* which is considered as a root primordia marker gene, was upregulated 6 h after auxin spray, with expression levels increasing up to 72 h (Figure 2-15A). Like *AaLRP1,* several other root development genes were also upregulated in the rooting zone soon after the auxin spray (Figure 2-15B). These results suggest that the development of adventitious root primordia in *A. alpina* might occur at an earlier stage than 72 h after auxin spray.

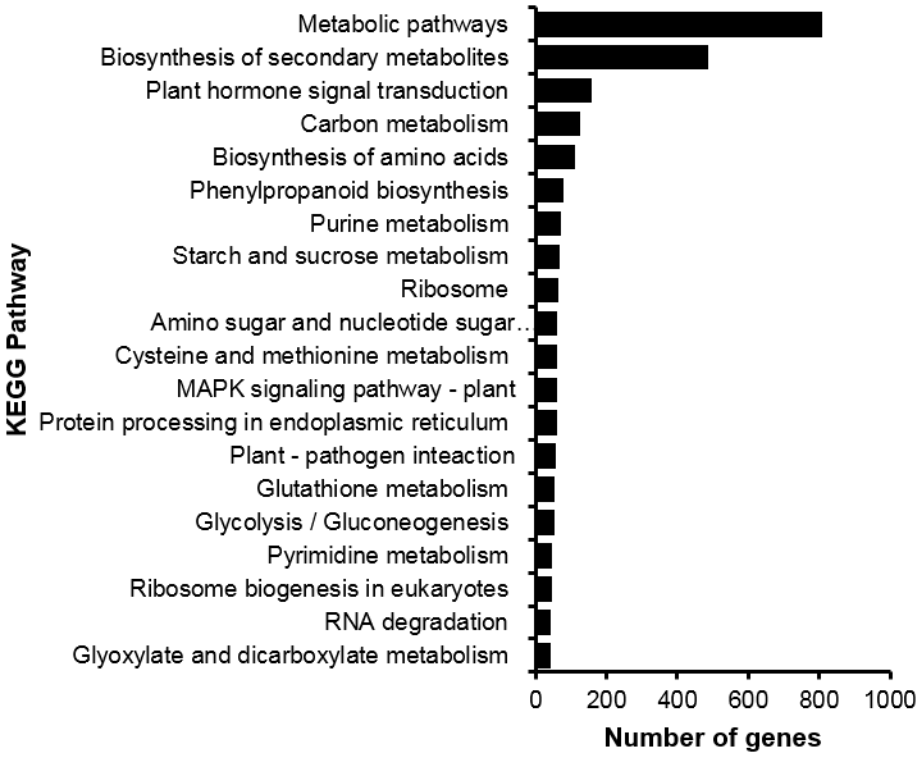

Figure 2-13. **Composition of the genes identified in the transcriptome study.**
Bar plot showing the constitution of differentially regulated genes in this transcriptome data in the form of KEGG categories and the number of genes in each category. Only the categories with more than 5% of the total number of genes in the KEGG pathway with the highest number of genes having *A. thaliana* orthologues are shown here.

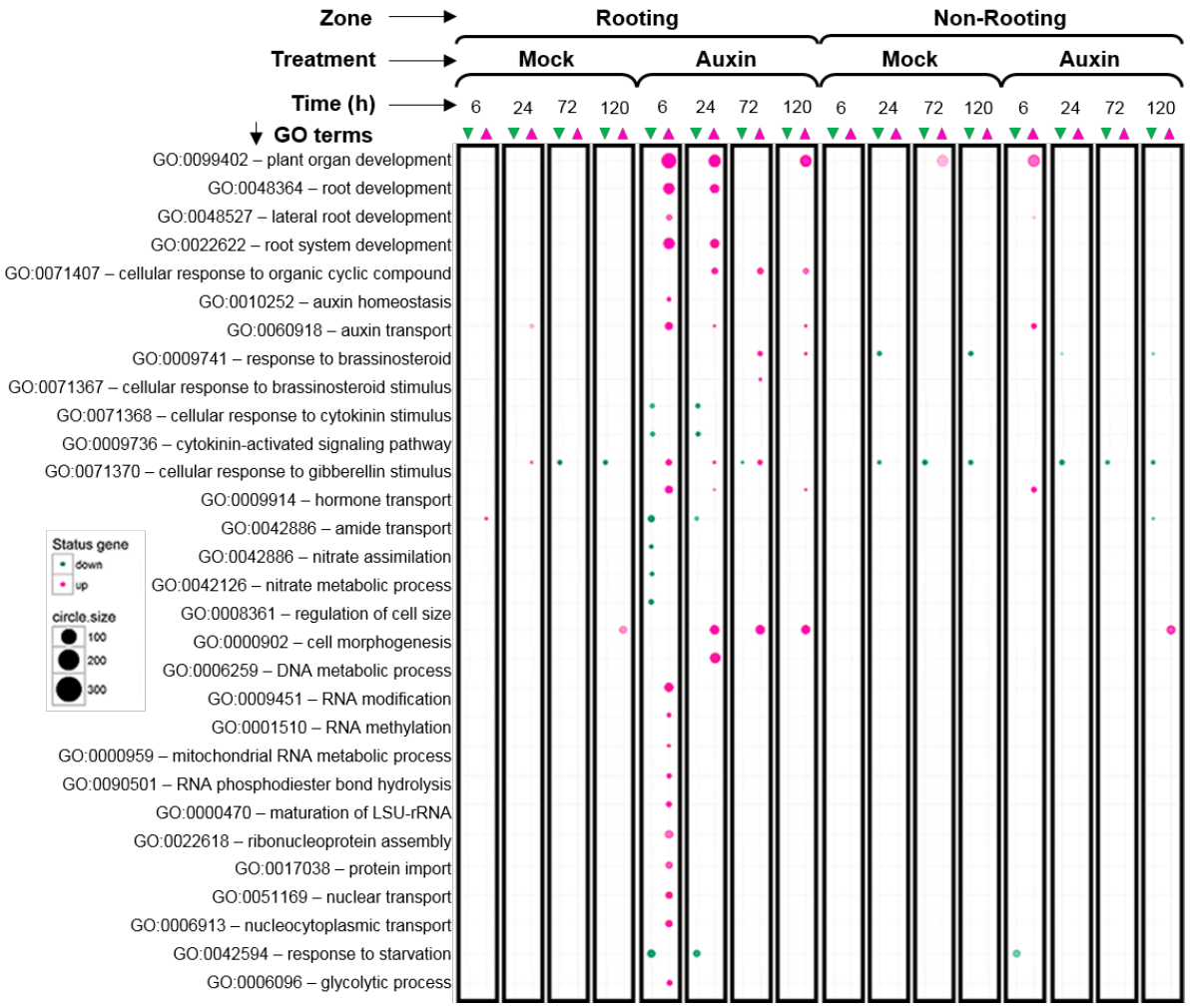

Figure 2-14. **Bubble Chart showing the enriched GO terms.**
The upregulated and downregulated genes in the rooting and the non-rooting zone at 6, 24, 72, 120 hours after auxin spray relative to the end of vernalization were analysed. Magenta represents upregulated and green represents downregulated GO terms and the size of the circle denotes the number of genes participating. The GO terms selected here are differentially enriched in the auxin sprayed rooting zone only. The transparency of the circle represents the confidence with respect to the set p-value of 0.01. The GO terms showing differential regulation between the rooting and the non-rooting zone post treatment are selectively shown here.

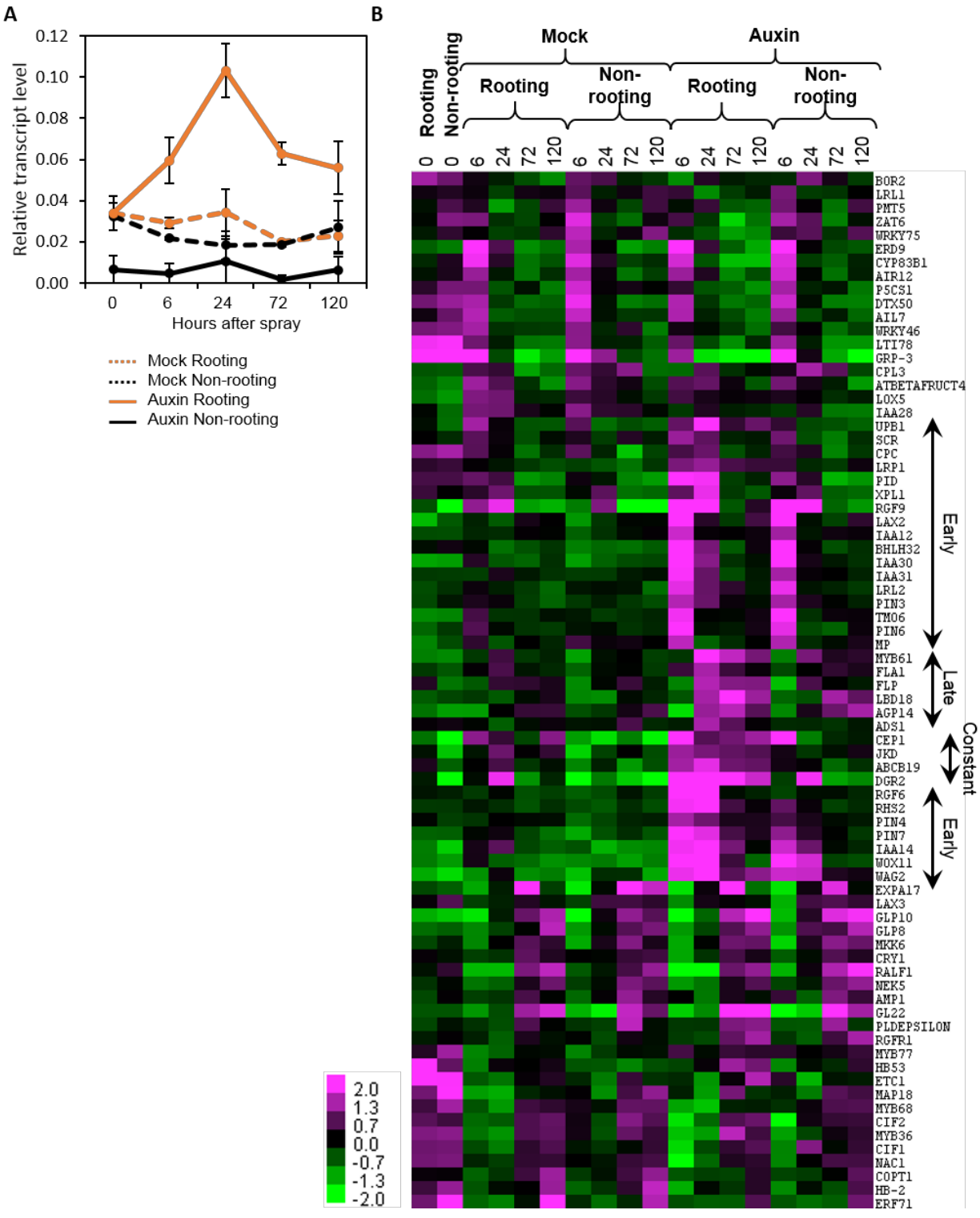

Figure 2-15. **Regulation of root associated genes in the *A. alpina* main stem during adventitious root development.**
(A) Relative abundance of *AaLRP1* was studied in response to mock/auxin spray in the rooting and the non-rooting zone of Paj plants using quantitative RT-PCR. Three biological replicates were used in this study. *AaPP2A* was used as the house-keeping gene. **(B)** Heat map of the expression pattern of 76 genes

out of the 525 genes in *A. thaliana* that are associated with the root system development (GO:0022622) with log_2Fold-change ≥ 2 and the difference between the maximum and the minimum value ≥ 2. The heat map was generated with CLUSTER3.0 and was analysed with TREEVIEW. Changes in the expression pattern are depicted as shown in the scale. Green represents downregulation and magenta represents upregulation of expression levels. The heat map shows the expression levels in rooting (R) and non-rooting (NR) zones at the end of vernalization (0), and at 6h, 24h, 72h and 120h after mock/auxin (1-NAA) spray.

The GO categories hormone signalling and transport (GO:0009914) were also enriched in the rooting zone after auxin treatment. The GO term auxin transport (GO:0060918) was enriched in the rooting zone at most time points, but predominantly 6 h after auxin treatment. Genes associated with auxin homeostasis (GO:0010252) were upregulated 6 h after auxin spraying specifically in the rooting zone. In addition, genes related to cytokinin signalling (GO:0009736) and response (GO:0071368) were downregulated 6 and 24 h after spraying, whereas the response to brassinosteroids (GO:0009741 & GO:0071367) was evident at the 72 and 120 h time points. Genes associated with the response to gibberellin (GO:0071370) were enriched 72 h after auxin application in the rooting zone but were downregulated in the non-rooting zone whether or not the auxin spray was applied. Overall, these results suggest that auxin, brassinosteroid and gibberellin act as stimulators, whereas cytokinin signalling might take the role of an inhibitor during adventitious rooting in *A. alpina*.

2.2.3. Adventitious root induction and initiation take place 6 and 24 h after auxin spraying

To gain insights into the basis of adventitious root induction, genes differentially expressed between the rooting and the non-rooting zones at early stages after auxin spray application were examined. Six hours after treatment, although several genes were differentially expressed in both zones compared to before treatment, only a few genes differentially expressed between the rooting and non-rooting zones were detected (Table 2-2). Specifically, only 64 genes were upregulated, and 157 genes were downregulated in the rooting zone. Among these genes, 33 (upregulated) and 112 (downregulated) had homologues in *A. thaliana*. The genes upregulated in the rooting zone included the homologs of the ethylene biosynthesis gene *AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE 8* (*ACS8*) and the ethylene response factor *ERF022*. In addition, *METHYL ESTERASE 1* (*MES1*) and the auxin binding *GERMIN 3* (*GER3*) were downregulated in

the rooting zone. Interestingly, the *A. alpina* homologues of *HECATE 1* (*HEC1*) and *FLAVIN MONOOXYGENASE* (*FMO*) were differentially expressed between the rooting and non-rooting internodes, suggesting that auxin transport might differ between samples at this time point.

Comparison of the 0 h (before application) and 6 h (after application) samples identified 414 upregulated and 399 downregulated genes in the rooting zone (Figure 2-16A, B; Supplementary Figure 7-4). GO enrichment to dissect the pathways that promote adventitious rooting revealed the enrichment of the GO terms for nuclear transport (GO:0051169), protein import (GO:0017038), protein transport (GO:0015031), nucleocytoplasmic transport (GO:0006913), protein targeting (GO:0006605) and intracellular protein transport (GO:0006886). In addition, GO terms associated with transcription and translation were also enriched among the upregulated genes (GO:0090304, GO:0034660, GO:0022618, GO:0016070, GO:0010608, GO:0010468, GO:0009451, GO:0006417, GO:0006396 and GO:0006364). In contrast, GO terms enriched among the downregulated genes included response to cytokinin (GO:0009735) and protein modification processes (GO:0036211 & GO:0006464). A search for interesting differentially expressed genes among these groups showed upregulation of homologs of the auxin-response genes *AUXIN RESPONSE FACTOR 10* (*ARF10*), *ARF19*, *GRETCHEN HAGEN 3.17* (*GH3.17*) and *NAKED PINS IN YUC MUTANTS 4* (*NPY4),* and SMALL AUXIN UPREGULATED RNA (SAURs) (*SAUR8*, *SAUR27* and *SAUR76*) and the downregulation of cytokinin signalling genes *ARABIDOPSIS THALIANA RESPONSE REGULATOR* 5 (*ARR5), ARR7*, *ARR15*, *SOB FIVE-LIKE 2* (*SOFL2*) and *WOODEN LEG* (*WOL*).

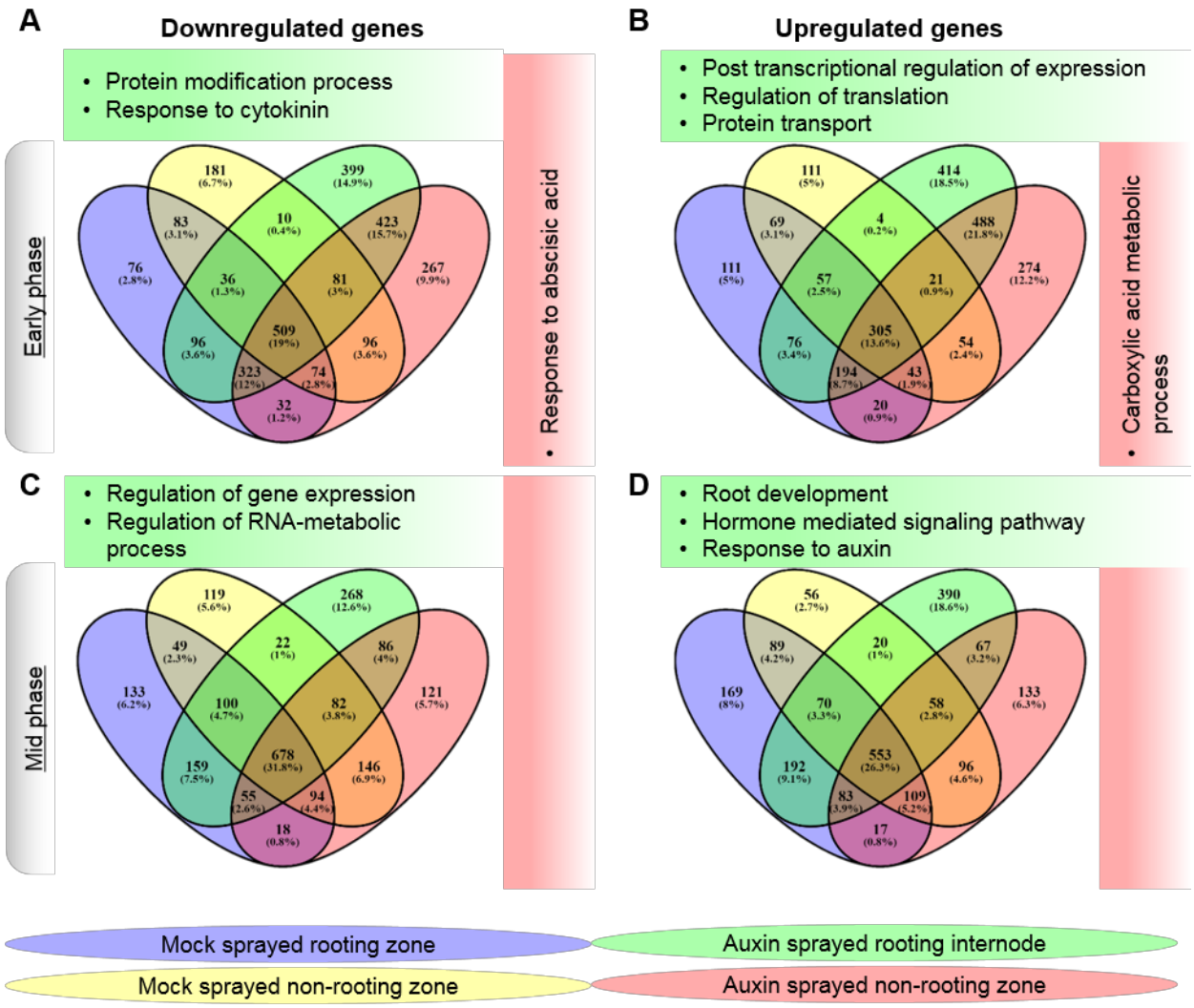

Figure 2-16. **Comparison of downregulated and upregulated during adventitious rooting.** The Venn diagram shows number of genes regulated at **(A, B)** 6 and **(C, D)** 24 hours after mock/auxin (1-NAA) spray relative to end of vernalization in the rooting and non-rooting zone. Selected GO terms (p-value < 0.05) enriched in the list of gene specific to auxin sprayed rooting and non-rooting zone are shown in the green and red boxes adjacent to the Venn diagram. The percentage of number of genes in each group is shown below the number of genes.

Comparison of the 0 h (before application) and 24 h (after application) samples produced 606 upregulated and 535 downregulated genes in the rooting zone (Figure 2-16C, D; Supplementary Figure 7-5), among which 472 of the upregulated genes and 386 of the downregulated genes had homologues in *A. thaliana*. At this stage, the transcriptome of the rooting and non-rooting internodes treated with auxin was remarkably different (Table 2-2). Homologues of genes

associated with the root meristem such as *CELLULOSE SYNTHASE-LIKE A15* (*CSLA15*), *C-TERMINALLY ENCODED PEPTIDE 1* (*CEP1*), *JACKDAW* (*JKD*), *LATERAL ORGAN BOUNDARIES 16* (*LBD16*), *LBD18*, *LBD19*, *LRP1*, *MAGPIE* (*MGP*), ROOT MERISTEM FROWTH FACTOR 6 (*RGF6*), *SCARECROW* (*SCR*) and *WUSCHEL RELATED HOMEOBOX 11* (*WOX11*) were upregulated in the rooting zone. These results suggest that root meristem genes are already upregulated in the rooting zone around 24 h after auxin spray.

The differentially regulated genes between the rooting and non-rooting internodes also included those involved in auxin signalling and transport, such as homologues of the auxin repressors *Aux/IAAs* (*IAA1*, *IAA5*, *IAA6*, *IAA7*, *IAA14*, *IAA19*, *IAA29* and *IAA32*) which were upregulated in the rooting zone, except *IAA18*, which was downregulated in the rooting sample. The expression of auxin conjugating *GH3* genes, *GH3.1* and *GH3.6*, were upregulated in the rooting zone. Homologues of genes encoding auxin efflux carriers such as *ATP-BINDING CASSETTE B19* (*ABCB19*), *PIN-FORMED 7* (*PIN7*), *PIN-LIKES 5* (*PILS5*), *PINOID* (*PID*) and *TRANSPARENT TESTA 4* (*TT4*) were also highly expressed in the rooting zone. Many members of the SAUR family such as *SAUR1*, *SAUR10*, *SAUR11*, *SAUR15*, *SAUR27*, *SAUR28*, *SAUR29*, *SAUR35*, *SAUR50*, *SAUR52*, *SAUR66*, *SAUR67* and *SAUR76* were upregulated in the rooting zone, whereas *SAUR33*, *SAUR36* and *SAUR59* were downregulated. The homologues of the auxin-binding GERMIN-LIKE PROTEIN (GLP) encoding genes, *GLP8* and *GLP10,* were also downregulated in the rooting zone. These results suggest that auxin signalling and transport is enhanced in the rooting zone at 24 h after spray.

Apart from auxin, homologues of genes associated with abscisic acid, ethylene, cytokinin, brassinosteroid and gibberellic acid were also differentially expressed between the rooting and the non-rooting samples. Cytokinin degrading *CYTOKININ OXIDASE 3* (*CKX3*), the abscisic acid synthesis genes *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 3* (*NCED3*) and *NCED5*, the gibberellic acid synthesis gene *GIBBERELLIN-20-OXIDASE 2* (*GA20OX2*) and ethylene production induced by auxin through *ACS4* were upregulated in the rooting zone compared to non-rooting zone. The brassinosteroid signalling genes *BRASSINOSTEROID ENCHANCED EXPRESSION 1* (*BEE1*), *BEE2*, *BRASSINOSTEROID-SIGNALING KINASE 5* (*BSK5*), the ethylene signalling genes *ERF014* and *ERF022*, the gibberellic acid signalling genes *GA-STIMULATED ARABIDOPSIS 4* (*GASA4*), *GASA6*, *GASA14* and *RGA-LIKE PROTEIN 3* (*RGL3*),

and the cytokinin signalling genes *LONELY GUY 4 (LOG4), LOG7* and *RR5* were enhanced in the rooting zone.

Genes specific to the rooting zone that were upregulated (390) and downregulated (268) at 24 h after auxin spray compared to before spray were investigated next (Figure 2-16C, D; Supplementary Figure 7-5). Among the upregulated genes, GOs associated with plant organ development, root development, root morphogenesis (GO:0010015), DNA replication and transcription were enriched. Additionally, genes participating in hormone signalling such as auxin signalling and response (GO:0009733, GO:0009734, GO:0009755, GO:0032870 and GO:0071365) were also enriched among the upregulated genes. These results suggest that induction of adventitious rooting may take place 6 h after auxin treatment and the initiation of adventitious roots 24 h after auxin spray application.

2.2.4. Adventitious root elongation takes place 72 and 120 h after auxin spray

To get insights on the molecular mechanisms involved at later stages of adventitious rooting, genes differentially expressed between the rooting and non-rooting zones 72 and 120 h after auxin spraying were examined. The root meristem markers (*CEP1*, *FAF2*, *JKD*, *LBD18*, *LBD33*, *LOB*, *LRP1*, *MGP* and *WOX11*) continued to be highly expressed in the rooting zone signifying the continuation of the root primordium development.

Homologs of auxin-response genes such as *IAA5*, *IAA6*, *IAA7* and *IAA14* were also enriched in the rooting zone. The expression of genes associated with auxin homeostasis such as *GH3.1*, *GH3.6, GH3.9* and *MES18*, and auxin transport such as *ABCB19* and *PINOID* was enhanced in the rooting zone. The expression of members of homologs of the SAUR family (*SAUR1*, *SAUR6*, *SAUR9*, *SAUR10*, *SAUR11*, *SAUR15*, *SAUR16*, *SAUR27*, *SAUR28*, *SAUR29*, *SAUR50*, *SAUR51*, *SAUR52*, *SAUR54*, *SAUR66*, *SAUR67* and *SAUR76*) remained enhanced in the rooting zone, but not in the non-rooting zone. The homologs of ethylene biosynthesis genes *ACS4* and *ACC OXIDASE 5* (*ACO5*), and ethylene signalling genes *ERF022*, *ERF38* and *ERF53* were strongly expressed in the rooting zone. A similar enhancement was observed for brassinosteroid synthesis gene *BRASSINOSTEROID-6-OXIDASE 2* (*BR6OX2*), and brassinosteroid signalling genes *BEE2*, *BRI1 SUPPRESSOR 1* (*BRS1*) and *BRI1-5 ENHANCED 1* (*BEN1*). On the contrary, the methyl IAA esterase *MES9*, the strigolactone synthesis gene *CCD7*, the cytokinin signalling gene *ARR7*, the

ethylene signalling gene *ERF6*, the gibberellic acid synthesis gene *GA3OX* and the jasmonic acid synthesis gene *LOX4* were downregulated in the rooting zone.

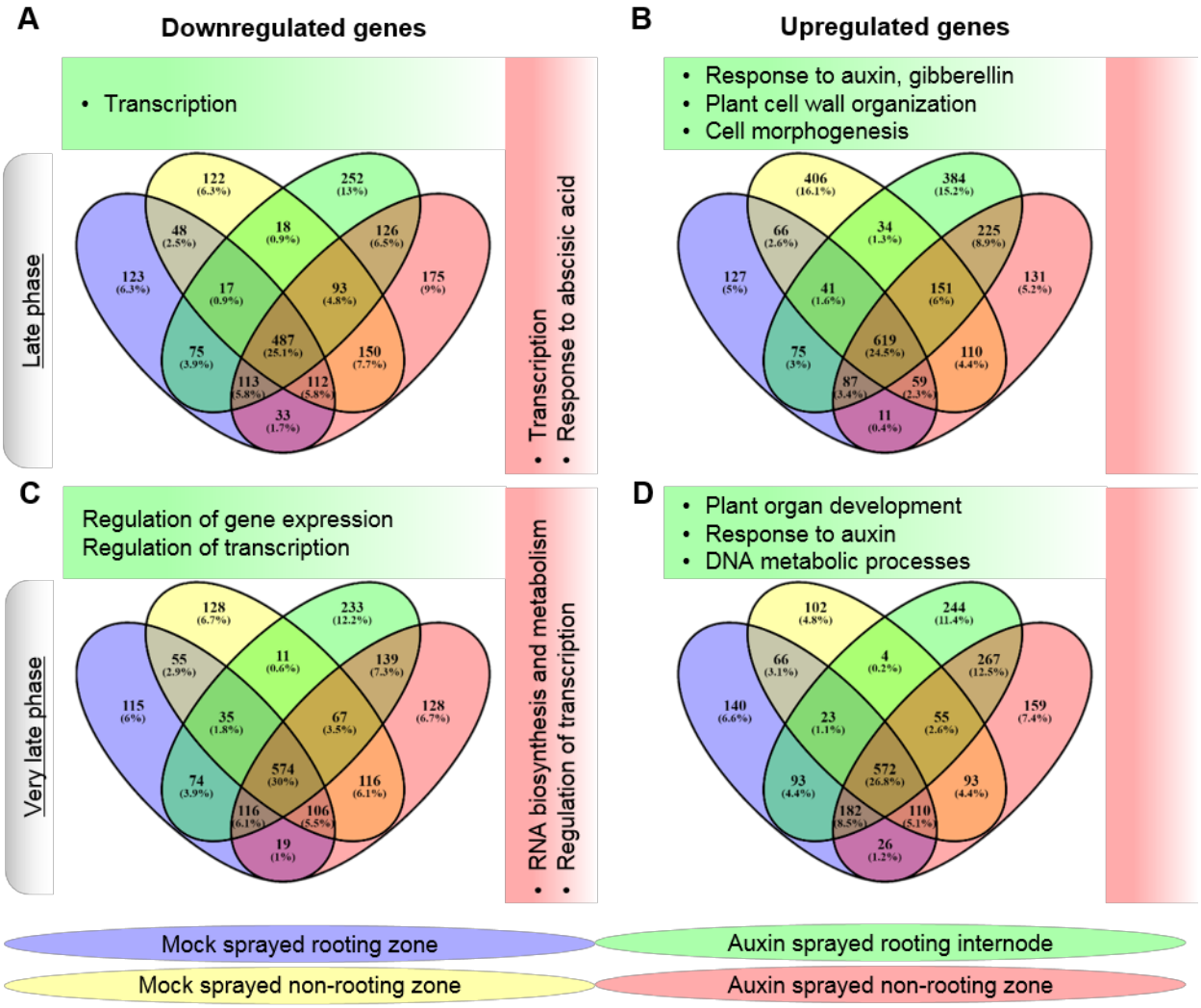

Figure 2-17. **Comparison of downregulated and upregulated during adventitious rooting.** The Venn diagram shows number of genes regulated at **(A, B)** 72 and **(C, D)** 120 hours after mock/auxin (1-NAA) spray relative to end of vernalization in the rooting and non-rooting zone. Selected GO terms (p-value < 0.05) enriched in the list of gene specific to auxin sprayed rooting and non-rooting zone are shown in the green and red boxes adjacent to the Venn diagram. The percentage of number of genes in each group is shown below the number of genes.

Genes upregulated (384) and downregulated (252) in the rooting zone 72 h after auxin spray application relative to before spraying were investigated (Figure 2-17A, B; Supplementary Figure 7-6). Among the 384 upregulated genes, the enriched GOs obtained were related to cellular

response to lipid (GO:0071396), cellular polysaccharide metabolic process (GO:0044264), cell wall modification (GO:0042545), unidimensional cell growth (GO:0009826), plant-type cell wall organization (GO:0009664) and cell morphogenesis (GO:0000902) (Figure 2-17A). In addition, GO terms associated with cellular response to hormonal stimulus (GO:0032870), hormone mediated signalling pathway (GO:0009755), response to gibberellin (GO:0009739) and response to auxin (GO:0009733) were also detected among the upregulated genes. Among the 252 downregulated genes GOs associated mainly to RNA processes (GO:2001141, GO:0051252, GO:0032774, GO:0006355 and GO:0006351) were enriched (Figure 2-17B). Interesting candidates among the differentially expressed genes included the auxin transport gene *PIN7*; the auxin signalling gene *IAA1* and SAURs (*SAUR1*, *SAUR9, SAUR15*, *SAUR27*, *SAUR30*, *SAUR66* and *SAUR76*), and ERFs (*ERF014*, *ERF022* and *ERF115*). The expression of the homologues of the cytokinin signalling genes, *ARR6* and *ARR12*, was reduced in the rooting zone.

The transcriptional make-up of the rooting zone was very similar 72 and 120 h after spraying. Genes related to auxin signalling such as the auxin amido-synthases *GH3.2* and *GH3.6*, auxin mediated transcription regulators, *SHORT HYPOCOTYL 2* (*SHY2*) and *IAA7*, and the auxin transport regulators *ABCB19* and *PINOID* were upregulated in the specifically in the rooting zone. Genes that were specifically upregulated (244) and downregulated (233) in the rooting zone compared to before auxin spray were also investigated (Figure 2-17C, D; Supplementary Figure 7-7). Among the upregulated genes GO categories related to auxin response (GO:0009733), DNA metabolic processes (GO:0006259) and plant organ development (GO:0099402) were enriched. Among the downregulated genes, GO categories related to RNA processes (GO:2001141, GO:0051252, GO:0032774, GO:0016070, GO:0010468, GO:0006355 and GO:0006351) were enriched (Figure 2-17C, D). Homolog of the ethylene signalling gene *ERF53* was upregulated in the rooting zone. The transcript level of the homologs of genes such as the Abscisic acid transporter *ABCG40*, the ethylene transcription factors *ERF1*, *ERF2*, *ERF6* and *ERF104*, was reduced in the rooting zone.

2.2.5. Members of the SAUR and AUX/IAA families are differentially regulated throughout adventitious rooting

To identify candidate genes that might play a role in adventitious rooting in *A. alpina*, a heat-map was generated using Cluster3.0. For obtaining the heat map, differentially expressed genes with log FPKM value greater than 2 and change in expression between two conditions greater than 1.5 were used as the input. A total of 6965 genes following these criteria were divided in four major clusters (Cluster I-IV) based on their expression patterns (Figure 2-18; Supplementary Figure 7-8). Each cluster was divided into several sub-clusters based on similarity in expression pattern of genes, out of which four sub-clusters were selected for further analyses (Figure 2-18B; Supplementary Figure 7-8). A total of 158 genes showed high expression 6 h after auxin spray but their expression was similar between the rooting and non-rooting internode (genes in yellow box in Figure 2-18B; Supplementary Table 11). GO categories enriched in this sub-cluster were related to hormone levels (GO:0010817), root development (GO:0048364), root morphogenesis (GO:0010015) and response to hormone (GO:0009725). Among the genes following this expression pattern were the auxin response genes *AUX/IAA1*, *AUX/IAA2*, *AUX/IAA12*, *AUX/IAA14*, *AUX/IAA19*, *AUX/IAA29*, *AUX/IAA30*, *AUX/IAA31*, *AUX/IAA32*, *GH3.1*, *GH3.2*, *GH3.3*, *SHORT ROOT* (*SHR*) and *WOX11*, and the auxin transport genes *AUX1*, *LAX2*, *PIN3*, *PIN4*, *PIN6* and *PIN7*. A total of 253 genes showed high expression 6 and 24 h after auxin spray and their expression differed between the rooting and non-rooting internode (genes in orange box in Figure 2-18B; Supplementary Table 9). GO categories enriched in this category included the regulation of organ growth (GO:0046620), the regulation of developmental growth (GO:0048638), the regulation of hormone levels (GO:0010817) and response to hormone (GO:0009725). Genes playing role in auxin signalling (*GH3.6*, *SAUR15*, *SAUR27*, *SAUR28*, *SAUR29*, *SAUR67*, *AUX*/*IAA5*, *AUX*/*IAA6*, *AUX*/*IAA9* and *AUX*/*IAA13*), ethylene biosynthesis genes (*ACS4*, *ACS8* and *ACS11*), brassinosteroid signalling (*BEE1*, *BEE2* and *BEE3*) and differentiation (*LRP1*, *JKD*, *RGF6*, *EARLY NODULIN-LIKE PROTEIN 8* and *ENODL17*) were members of this sub-cluster. The expression patterns of *SAURs* detected in this sub-set clustered together compared to a total of 47 *SAUR*s identified in *A. alpina* (Figure 2-19). The expression of 126 genes was consistently higher in the rooting relative to the non-rooting zone, even before auxin spray application (genes in green box in Figure 2-18B; Supplementary Table 11). GO categories enriched for this sub-set

of genes were GOs related to cuticle development (GO:0042335) and response to karrikin (GO:0080167). Candidates in this sub-cluster included *ABCB19*, *MGP*, *RGL2* and *SWEET13*. A total of 41 genes showed high expression 72 and 120 h after auxin spraying and their expression differed between the rooting and non-rooting zones (genes in blue box in Figure 2-18B; Supplementary Table 11). The GO terms enriched in the sub-cluster were phenylpropanoid metabolic process (GO:0009698), secondary metabolic process (GO:0019748), cell wall organization (GO:0071555) and cellular catabolic process (GO:0044248). The interesting candidates in this category included *LBD18*, *GLUTAMATE DEHYDROGENASE 3* (*GDH3*) and *SUGAR TRANSPORTER 14* (*STP14*).

In summary, our data suggests that even though the whole plant was auxin sprayed with auxin, several genes associated with auxin homeostasis, transport and signalling were differentially regulated between the rooting and non-rooting zones. These differences in hormonal signalling might contribute to the spatial patterns of adventitious rooting in *A. alpina* and differences in auxin response between internodes.

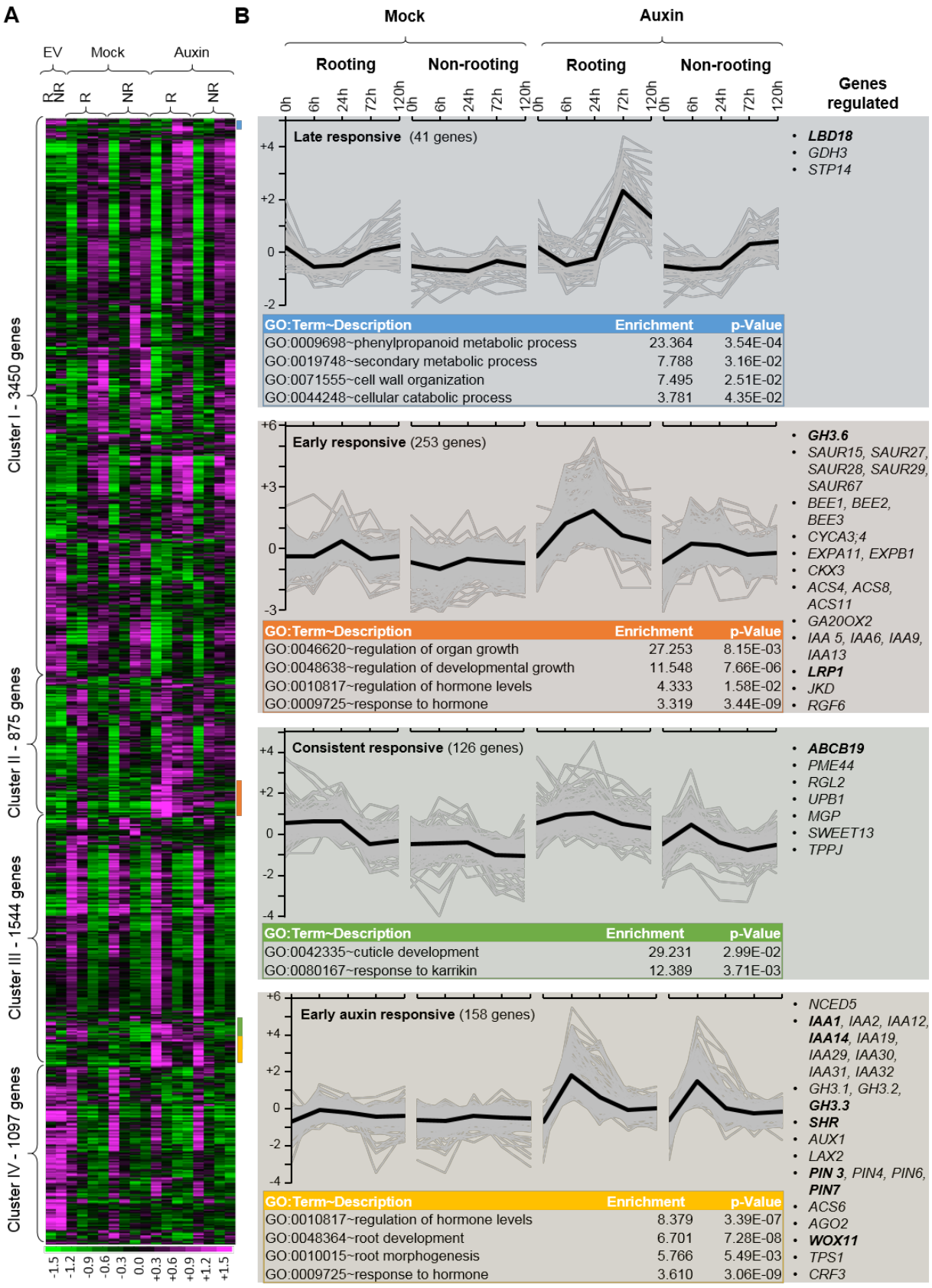
A
EV
Mock
Auxin
R
NR
R
NR
R
NR
Cluster I - 3450 genes
Cluster II - 875 genes
Cluster III - 1544 genes
Cluster IV - 1097 genes
-1.5
-1.2
-0.9
-0.6
-0.3
0.0
+0.3
+0.6
+0.9
+1.2
+1.5
B
Mock
Auxin
Rooting
Non-rooting
Rooting
Non-rooting
0h
6h
24h
72h
120h
Genes regulated
Late responsive (41 genes)
+4
+2
0
-2
GO:Term~Description
Enrichment
p-Value
GO:0009698~phenylpropanoid metabolic process
23.364
3.54E-04
GO:0019748~secondary metabolic process
7.788
3.16E-02
GO:0071555~cell wall organization
7.495
2.51E-02
GO:0044248~cellular catabolic process
3.781
4.35E-02
LBD18
GDH3
STP14
Early responsive (253 genes)
+6
+3
0
-3
GO:0046620~regulation of organ growth
27.253
8.15E-03
GO:0048638~regulation of developmental growth
11.548
7.66E-06
GO:0010817~regulation of hormone levels
4.333
1.58E-02
GO:0009725~response to hormone
3.319
3.44E-09
GH3.6
SAUR15, SAUR27, SAUR28, SAUR29, SAUR67
BEE1, BEE2, BEE3
CYCA3;4
EXPA11, EXPB1
CKX3
ACS4, ACS8, ACS11
GA20OX2
IAA 5, IAA6, IAA9, IAA13
LRP1
JKD
RGF6
Consistent responsive (126 genes)
+4
+2
0
-2
-4
GO:0042335~cuticle development
29.231
2.99E-02
GO:0080167~response to karrikin
12.389
3.71E-03
ABCB19
PME44
RGL2
UPB1
MGP
SWEET13
TPPJ
Early auxin responsive (158 genes)
+6
+4
+2
0
-2
-4
GO:0010817~regulation of hormone levels
8.379
3.39E-07
GO:0048364~root development
6.701
7.28E-08
GO:0010015~root morphogenesis
5.766
5.49E-03
GO:0009725~response to hormone
3.610
3.06E-09
NCED5
IAA1, IAA2, IAA12, IAA14, IAA19, IAA29, IAA30, IAA31, IAA32
GH3.1, GH3.2, GH3.3
SHR
AUX1
LAX2
PIN 3, PIN4, PIN6, PIN7
ACS6
AGO2
WOX11
TPS1
CRF3

Figure 2-18. **Co-expression clustering of the differentially expressed genes during adventitious root development after auxin spray.**
(A) Heat map of the expression pattern of 6965 genes out of the 30690 identified in *A. alpina* with log_2Fold-change ≥ 2 and the difference between the maximum and the minimum value ≥ 1.5. The heat map was generated with CLUSTER3.0 and was analyzed with TreeView. Changes in the expression pattern are depicted as shown in the scale below the heat map. Green represents downregulation and magenta represents upregulation of expression levels. The heat map is divided into four major clusters (I, II, III & IV) based on the overall expression pattern as shown to the left of the heat map along with number of genes in each cluster. The heat map shows the expression levels in rooting (R) and non-rooting (NR) zones at the end of vernalization (EV), and at 6h, 24h, 72h and 120h after mock/auxin (1-NAA) spray. **(B)** The average normalised expression pattern of genes in sub-clusters selected from the four heat map clusters shown with colored highlights to the right of the heat map. The expression levels in rooting and non-rooting zones after treatment are normalized with Cluster3.0. The total number of genes, interesting genes regulated and GO terms (Benjamini p-value < 0.05) with *A. thaliana* orthologues are shown for each sub-cluster.

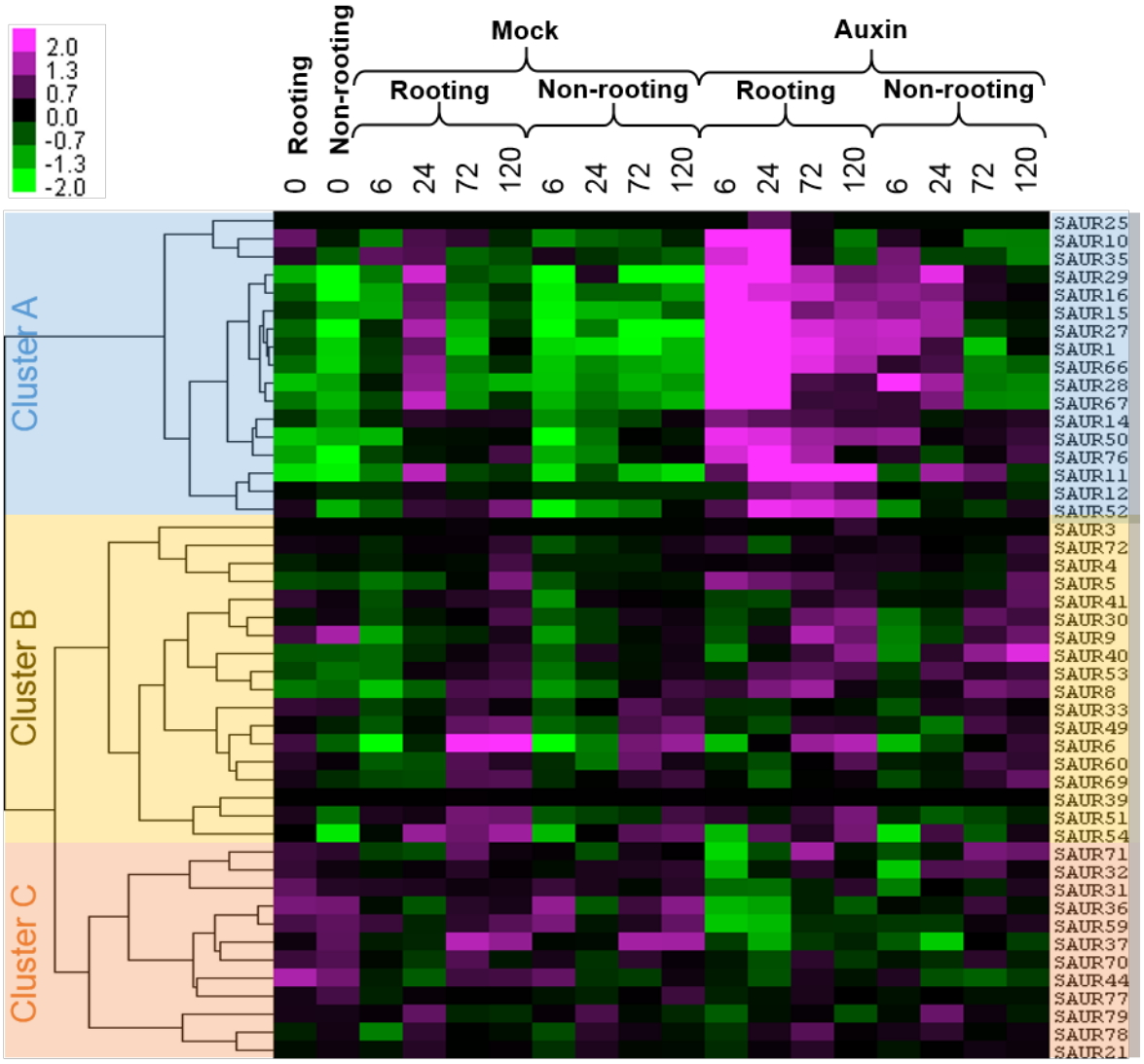

Figure 2-19. ***AaSAURs* are differentially regulated during adventitious rooting in *A. alpina*.**
Heat map of the expression pattern of the homologs of *AtSAURs* identified in *A. alpina* with $\log_2$Fold-change ≥ 2 and the difference between the maximum and the minimum value ≥ 2. The heat map was generated with Cluster3.0 and was analyzed with TreeView. Changes in the expression pattern are depicted as shown in the scale below the heat map. Green represents downregulation and magenta represents upregulation of expression levels. The heat map is divided into three clusters (A, B & C) based on the overall expression pattern as shown to the left of the heat map. The heat map shows the expression levels in rooting (R) and non-rooting (NR) zones at the end of vernalization (EV), and at 6h, 24h, 72h and 120h after mock/auxin (1-NAA) spray.

2.2.6. Differential auxin responsiveness between internodes before auxin spray defines spatial pattern of adventitious rooting

Since the adventitious rooting regulator *ABCB19* was detected among the genes showing consistent response to auxin spray, and was upregulated in the adventitious rooting internode already before auxin spray, the differentially expressed genes between the rooting and non-rooting zones at the end of vernalization was examined. Before auxin treatment, 394 genes were upregulated and 306 genes were downregulated in the rooting relative to the non-rooting zone (Table 2-2). Among these genes 276 (upregulated) and 243 (downregulated) had orthologues in *A. thaliana.* Interestingly, several GO categories associated with hormone signalling (GO:0032870 & GO:0009755; Figure 2-20A) were enriched in the rooting zone already before auxin spray. The GO 'response to auxin' (GO:0009733) was upregulated in the rooting zone already at the end of vernalization suggesting an enrichment of genes participating in auxin response. Besides *ABCB19*, the auxin-mediated transcriptional regulator *IAA7* was upregulated in the rooting zone whereas the expression of *IAA1* was reduced. The *FLAVONOL SYNTHASE 1* (*FLS1*), a flavonol biosynthesis gene, was also upregulated in the rooting zone. In addition, several *SAURs* (*SAUR1*, *SAUR6*, *SAUR10*, *SAUR16*, *SAUR29*, *SAUR51*, *SAUR52* and *SAUR54*) were upregulated in the rooting zone. The transcript levels of *GLP9*, encoding an auxin-binding protein, and *MES1* were downregulated in the rooting zone. To investigate differences in free endogenous IAA levels between the zones, its abundance in the rooting and the non-rooting zones at the end of vernalization was measured. IAA levels were similar between the rooting and the non-rooting zone suggesting that at the end of vernalization, although endogenous IAA levels do not differ, auxin response is enhanced between internodes (Figure 2-20B).

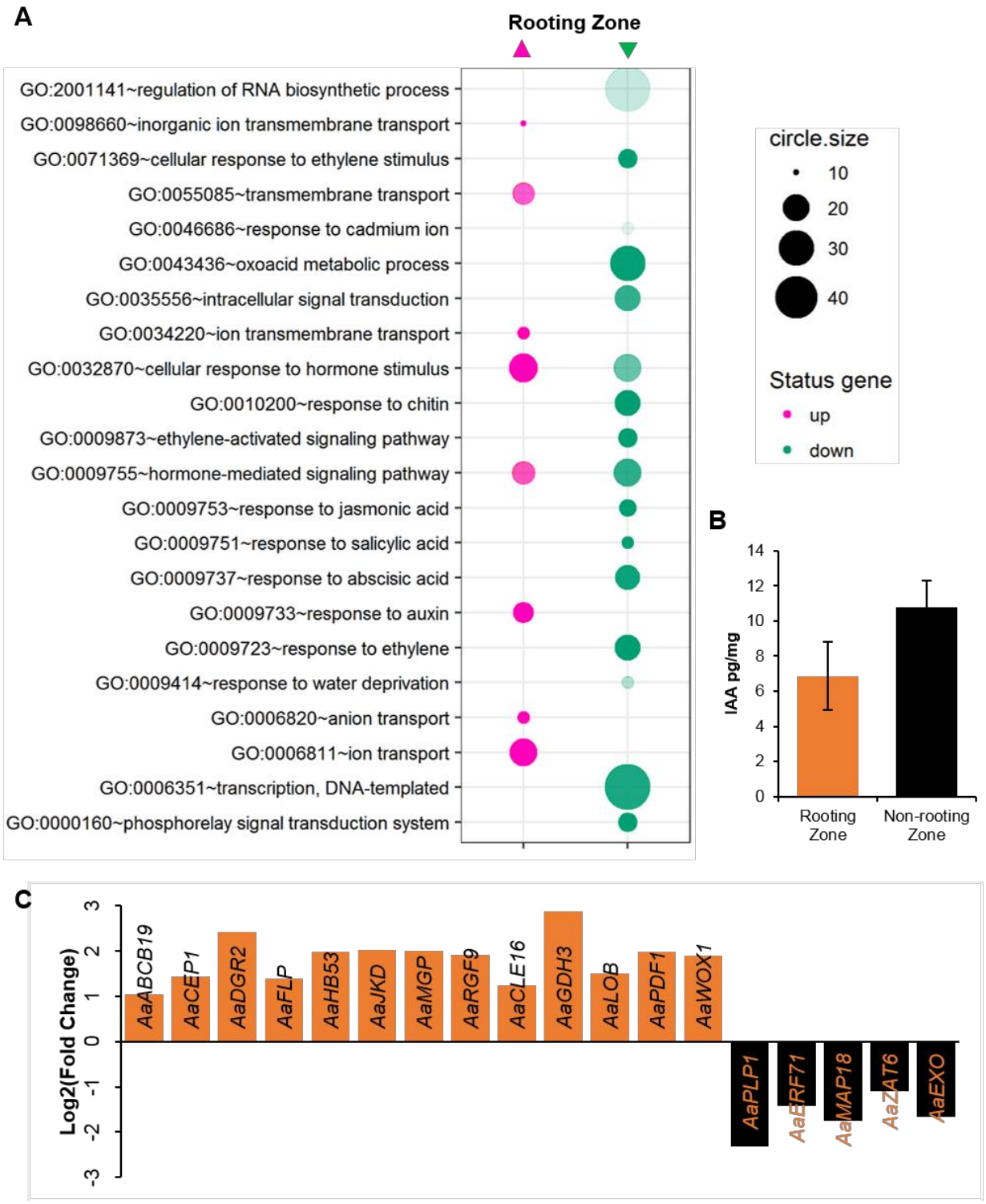

Figure 2-20. **Characterization of rooting and non-rooting zone at the end of vernalization.** **(A)** Bubble plot showing the enrichment of GO terms enriched among the upregulated and downregulated genes in the rooting zone at the end of vernalization relative to the non-rooting zone. Magenta represents upregulated and green represents downregulated GO terms and the size of the circle denotes the number of genes participating. The transparency of the circle represents the confidence with respect to the set p-

value of 0.01. **(B)** Quantification of auxin (IAA pg/mg) in the rooting and non-rooting zone at the end of vernalization. A student's t-test produced a p-value of 0.1517. **(C)** Log2(Fold change) of root development associated genes in the rooting zone at the end of vernalization. Genes upregulated and downregulated in the rooting zone are shown in orange and black bars, respectively.

To validate the significant enrichment of auxin responsive genes at the end of vernalization, all *A. alpina* differentially regulated genes soon after the auxin spray application were taken into consideration. A list of genes upregulated (1199) and downregulated (1542) 6 h after auxin spraying in both the rooting and non-rooting zones of *A. alpina* was generated. 196 and 171 of the auxin responsive genes were found in the upregulated and downregulated set of genes at the end of vernalization. These sets were found to be significantly enriched with a Fisher's Exact Test and also by randomization test (Krouk et al., 2010). This suggests 'response to auxin' genes are enriched in the upregulated genes.

The GO terms, response to abscisic acid (GO:0009737), ethylene (GO:0009723), jasmonic acid (GO:0009753) and salicylic acid (GO:0009751) were enriched in the non-rooting zone. The homologues of an abscisic acid transporter *ABCG40* and signalling gene *ABA REPRESSOR1* (*ABR1*), several ethylene response factors (*ERF1*, *ERF2*, *ERF6*, *ERF22*, *ERF71*, *ERF104* & *ERF105*), lipoxygenase genes, *LOX2* and *LOX4*, responsible for biosynthesis of jasmonic acid, salicylic acid responsive genes *PATHOGENESIS RELATED GENE 1* (*PR1*), *WRKY DNA BINDING PROTEIN 28* (*WRKY28*) and *SULPHOTRANSFERASE 12* (*SOT12*) were downregulated in the rooting zone. Similarly, the strigolactone synthesis genes *MAX3/CCD7* and *MAX4/CCD8* were downregulated in the rooting zone, whereas the expression of the brassinosteroid signalling genes *BEE2* and *BEE3* were enhanced in the rooting zone.

Among the differentially expressed genes, several homologs of root meristem associated genes were highly expressed in the rooting zone (Figure 2-20C). These included the homologs of *CLAVATA3/ESR RELATED 16* (*CLE16*), *JKD*, *RGF9*, *MGP* and *CEP1*. Apart from root meristem genes, genes associated with meristems such as *LATERAL ORGAN BOUNDARIES* (*LOB*), *PROTODERMAL FACTOR 1* (*PDF1*) and *WOX1* were also upregulated in the rooting zone. All in all, these results indicate that cells that have the identity of a root are present in the rooting zone at the end of vernalization.

2.3. *AaGH3.3* and *AaGH3.6* upregulated in the rooting zone during adventitious root development

Auxin signalling, metabolism and transport seem to be the important factors regulating adventitious root formation and have been considered as the central player in several plant species. It has been reported earlier in *A. thaliana* etiolated hypocotyls that the molecular network regulating adventitious rooting is composed of genes encoding transcription factors of the ARF family, namely, *ARF6*, *ARF7*, *ARF8*, *ARF9* and *ARF17*. *ARF6*, *ARF8* and *ARF17* further regulate the downstream genes of the Gretchen Hagen 3 family, *GH3.3*, *GH3.5* and *GH3.6*. This section focuses on testing whether these genes are differentially expressed between the rooting and the non-rooting zones during adventitious root formation, and if they respond to auxin spray in *A. alpina*.

2.3.1. Homologs of *ARF*s in *A. alpina*

BLAST search for homologs of *ARF*s in *A. alpina* genome yielded 20 *AtARF*-like genes (Figure 2-21). The homologs of *AtARF12-15, AtARF20-22 and AtARF23* were not found in *A. alpina.* Four novel *ARF*s were discovered containing auxin response and Aux/IAA binding domain, including a homolog (*Aa_G456320*) of the ARF10-ARF16-ARF17 family. Shared synteny advocates the evolutionary conservation and therefore the functional conservation. The synteny of genes neighbouring *ARF6, ARF8* and *ARF17* was examined in *Arabis alpina*. Synteny of *ARF17* neighbouring genes is conserved suggesting *AaARF17* to be orthologous to *AtARF17*, whereas synteny in *AaARF6* and *AaARF8* are not conserved. Their abundance is regulated by *miRNAs*, i.e. *miR160* regulates the abundance of *ARF17*, and *miR167* controls *ARF6* and *ARF8* levels. In *A. alpina*, the *miRNA* binding site in these three ARFs was conserved (Figure 2-21). Further characterisation including the transcript abundance was carried out to understand their role during adventitious root development.

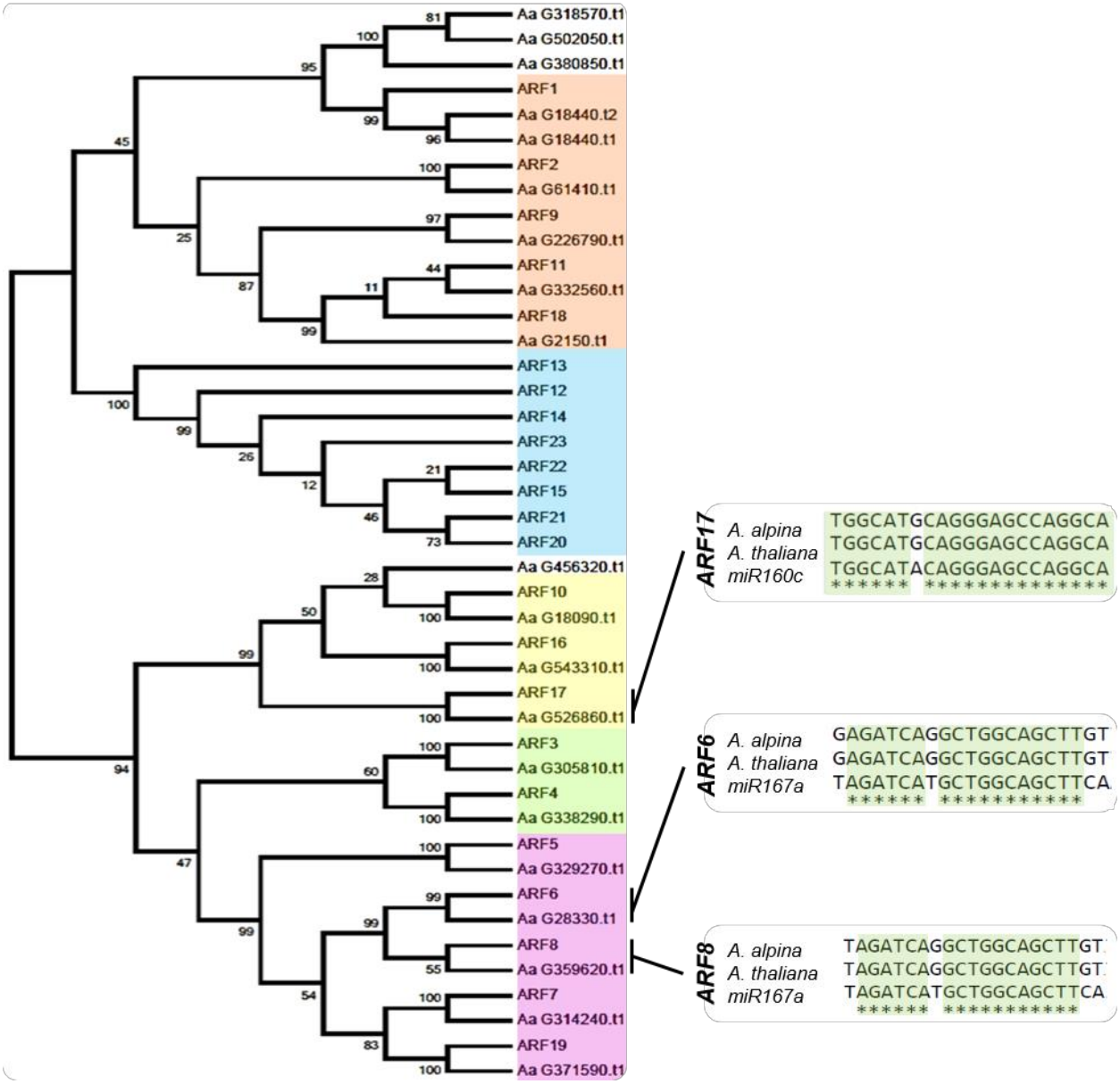

Figure 2-21. **ARFs in *A. thaliana* and *A. alpina.***
The ARFs are grouped by their similarity and family as in *A. thaliana*. The putative *A. alpina* ARFs are represented in red curly brackets. The evolutionary history was inferred using the Neighbour-Joining method in MEGA5. The optimal tree with the sum of branch length = 4.81012310 is shown. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The analysis involved 43 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 89 positions in the final dataset. Evolutionary analyses were conducted in MEGA5. Bootstrap value = 500. The *miRNA* binding sites of *ARF6*, *ARF8* and *ARF17* are highlighted in green.

2.3.2. Role of ARF and GH3 encoding genes during adventitious root development

In this study, exogenous auxin application did not affect the expression of *AaARF6*, *AaARF8* and *AaARF17*, irrespective of the zone sampled (Figure 2-22). Whereas auxin treatment led to the upregulation of the transcript abundance of the downstream genes *AaGH3.3* and *AaGH3.6*, along with auxin signalling genes such as *AaIAA3* and *AaIAA29* (Figure 2-23). Interestingly, the expression of *AaGH3.3* and *AaGH3.6* is higher in the auxin sprayed rooting zone relative to the non-rooting zone. Auxin might regulate the transcription of *AaGH3.3* and *AaGH3.6*, and other auxin response genes in a zone-dependent manner along with AaARF6, AaARF8 and AaARF17. Overall, the zone-dependent auxin response points to the presence of other competence factors during adventitious root development in *A. alpina*.

To investigate the role of *ARF6*, *ARF8* and *ARF17* in adventitious rooting, transgenic plants were generated with the over-expression or mimicry constructs of the *miRNA*s regulating the abundance of these *ARFs* in Paj and *pep1-1*. The transgenic plants will be selected post BASTA treatment.

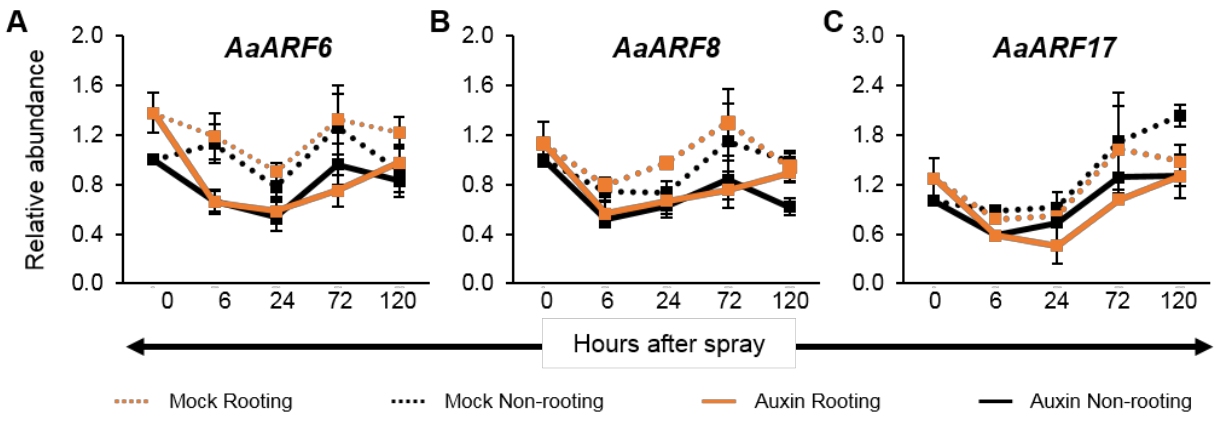

Figure 2-22. ***ARF* levels are unaffected during adventitious root formation.**
Expression pattern of **(A)** *AaARF6*, **(B)** *AaARF8* and **(C)** *AaARF17* in the main stem (rooting and non-rooting zone) determined using RT-qPCR. The values represent the mean and the standard deviation of three biological replicates. The zones were collected before spray, and 6, 24, 72 and 120 hours after spray.

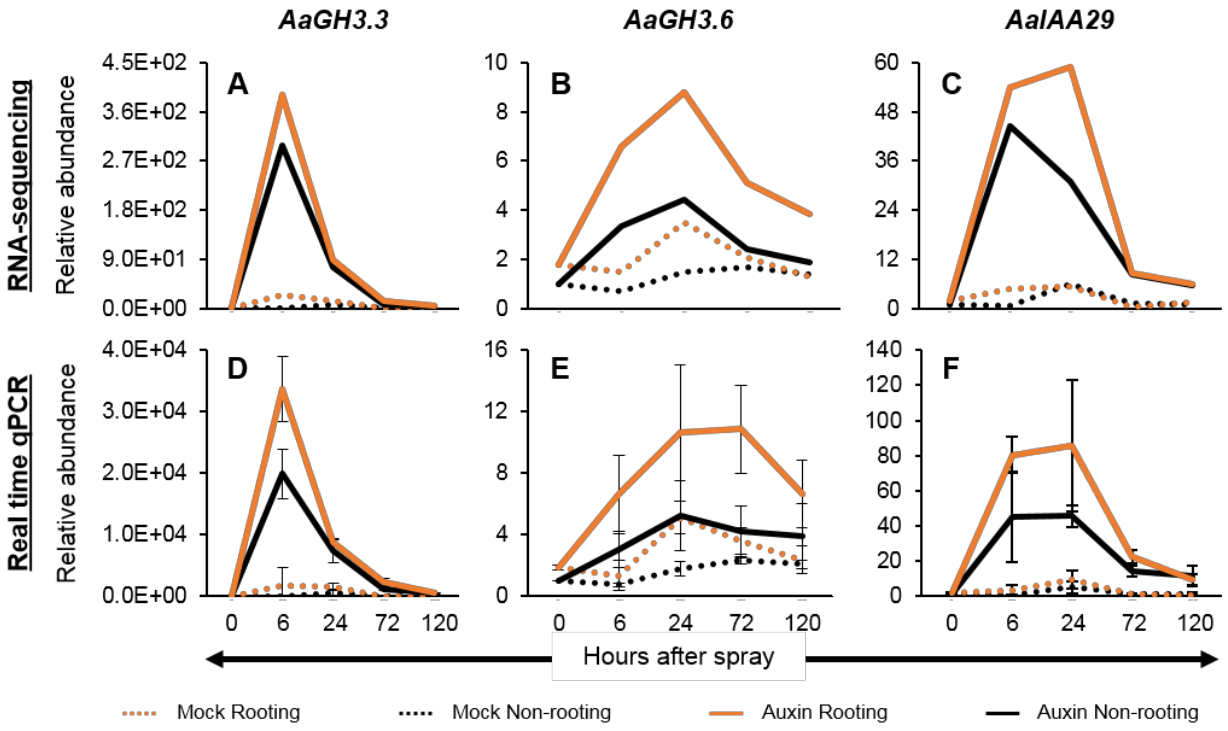

Figure 2-23. **Downstream regulators of root development are specifically upregulated in rooting zone.**
Expression pattern of **(A, D)** *GH3.3*, **(B, E)** *GH3.6* and **(C, F)** *IAA29* in the main stem (rooting and non-rooting zone) determined by **(A-C)** RNA-Sequencing and verification using **(D-F)** RT-qPCR. The values represent the mean and the standard deviation of three biological replicates. In the case of RNA-Sequencing, the FPKM value, and for qRT-PCR, the Ct value, were normalized to the respective values of *AaPP2A* (house-keeping gene) in the non-rooting zone at the end of vernalization.

2.4. Extended vernalization promotes adventitious rooting in *A. alpina*

2.4.1. Longer periods of cold induce adventitious roots in *A. alpina*

The higher transcript accumulation of meristem associated genes at the end of 12 week vernalization in the rooting zone suggested that prolonged exposure to cold might affect adventitious rooting in *A. alpina*. However, plants exposed to more than 12 weeks of vernalization in SD conditions did not develop adventitious roots (personal communication [161]). We tested the effect of prolonged exposure to cold under LD conditions, since it resembles ecological conditions in alpine summers during circumstances unfavourable for flowering. The presence of adventitious roots was scored in plants vernalized for 0, 4, 8, 12, 16 and 21 weeks and several weeks thereafter. Upon exposing the plants for 21 weeks to vernalization under long photoperiods, adventitious roots were predominantly produced on specific internodes of the main stem and without the application of exogenous auxin treatment (Figure 2-24A). These internodes were present below the compressed zone, similar to the internodes that produced adventitious roots when synthetic auxin was applied to plants at the end of 12 weeks of vernalization under short photoperiods.

Increase of the duration of vernalization under long days, resulted in an increase of the number of plants that produced adventitious roots on the main stem and the branches indicating a dose-dependent response (Figure 2-24B-C). None of the 4-week and 8-week vernalized plants produced adventitious roots on the main stem and branches. A few 12-week long day-vernalized plants produced adventitious roots on the main stem (as scored 2 weeks after vernalization). In Chapter 2.3, 12-week short day-vernalized plants did not produce adventitious roots on the main stem. Long day-vernalization for 16 weeks promoted earlier adventitious rooting on branches, whereas adventitious root formation on the main stem showed a response similar to 12 week long day-vernalized plants. Interestingly, adventitious roots were present on the branches of plants vernalized for 21 weeks at the end of vernalization, but roots on the main stem were observed one week after transfer to the greenhouse. The duration of vernalization also affected the number of internodes and branches that produced adventitious roots such that the longer the vernalization the higher the number of internodes and branches with roots (Figure 2-24D-E). Plants vernalized for 4 week and 8 week in long days did not produce adventitious roots, whereas more than 16 weeks of vernalization promoted adventitious root formation. Overall these results suggest that

vernalization induces the induction of adventitious rooting, day length affects the initiation and adventitious root development in *A. alpina*.

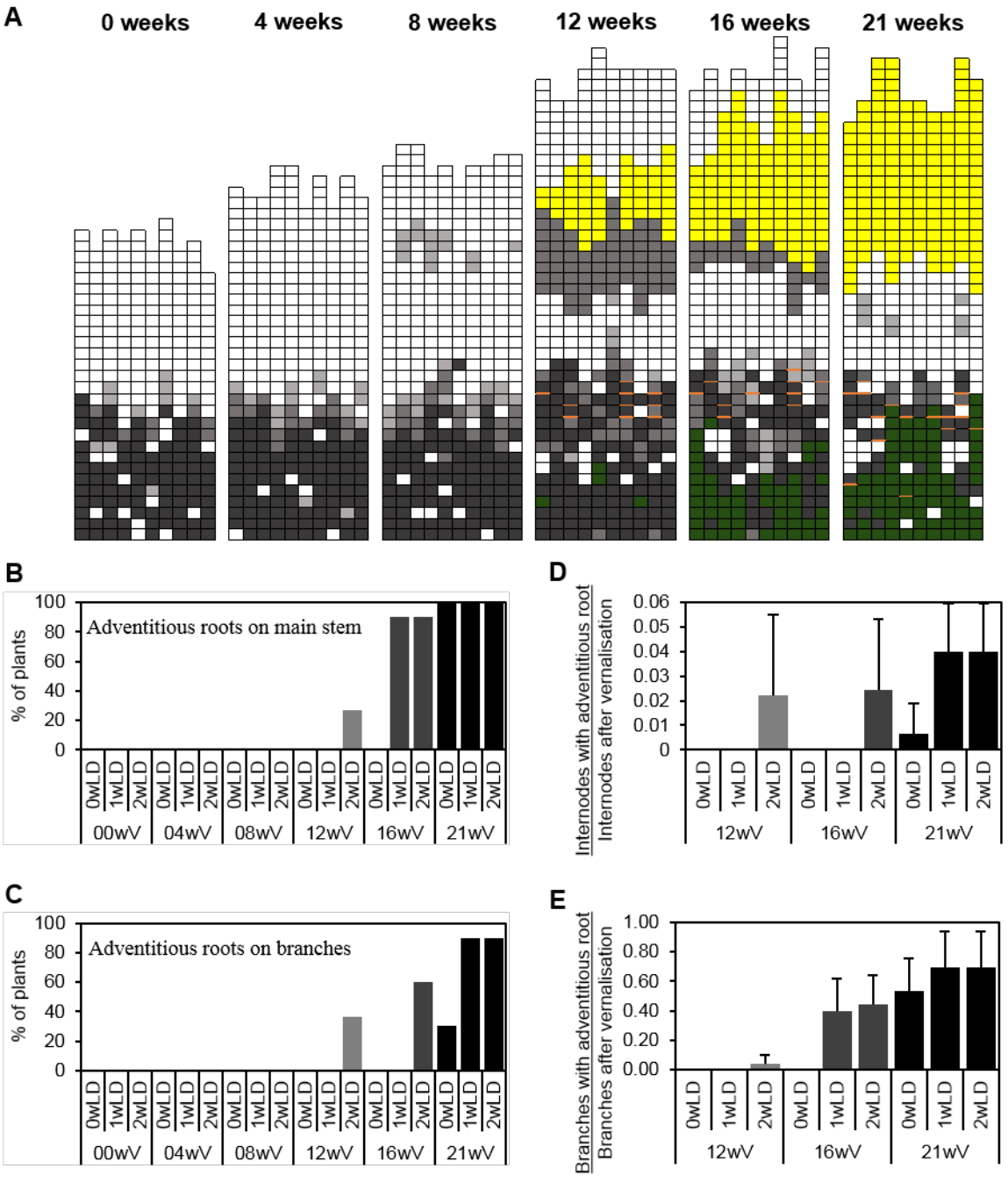

Figure 2-24. **Extended vernalization promotes adventitious root production.**
(A) Schematic representation of *A. alpina* plants showing adventitious roots before vernalization, and 4, 8, 12, 16 and 21 weeks after vernalization**.** Schematic representation of *A. alpina* plants (n=10), 2 weeks after vernalization, showing presence and absence of branches and adventitious roots in different zones of the plant. Each box represents a leaf node. Grey boxes represent leaf axils filled with branches such that the

newer the branch, the lighter the shade of grey. Orange lines represent adventitious roots on the main stem. Percentage of plants with adventitious roots on the **(B)** main stem and the **(C)** branches after exposure to different durations of vernalization (weeks in vernalization, wV) and 2 weeks in long days (wLD). Frequencies of **(D)** internodes and **(E)** branches occupied by adventitious roots after exposure to different durations of vernalization.

2.4.2. Free endogenous auxin levels during vernalization in *A. alpina*

Since auxin is a major regulator of adventitious rooting in several species, the distribution of free endogenous auxin (IAA) levels was measured in the rooting zone of plants vernalized for different durations and 5 days after vernalization. At the end of 4 weeks of vernalization auxin abundance was reduced (~ 2 times) in the rooting zone, followed by a gradual upregulation of auxin levels as the duration of vernalization increased (Figure 2-25A; Supplementary Table 12). The free endogenous IAA levels at 21 weeks of long day-vernalization was similar to Paj plants grown for 8-week in long day greenhouse conditions. While cold reduced overall auxin abundance in the beginning, there was an upregulation as the vernalization period was prolonged suggestive of a dose-dependent response.

Adventitious roots on the main stem were observed after transfer to the long day greenhouse. Therefore, auxin levels on stems were measured after the return to greenhouse conditions but before the emergence of the adventitious roots. The duration of vernalization and transfer to greenhouse conditions significantly affected the auxin abundance on the stem. The free auxin abundance showed a significant increase relative to during vernalization only in 21 weeks vernalized plants (Figure 2-25B; Supplementary Table 13). Evidently, higher auxin levels might aid the adventitious root formation in the zone upon extending the duration of vernalization, however the induction of adventitious root formation requires factors other than the free auxin level.

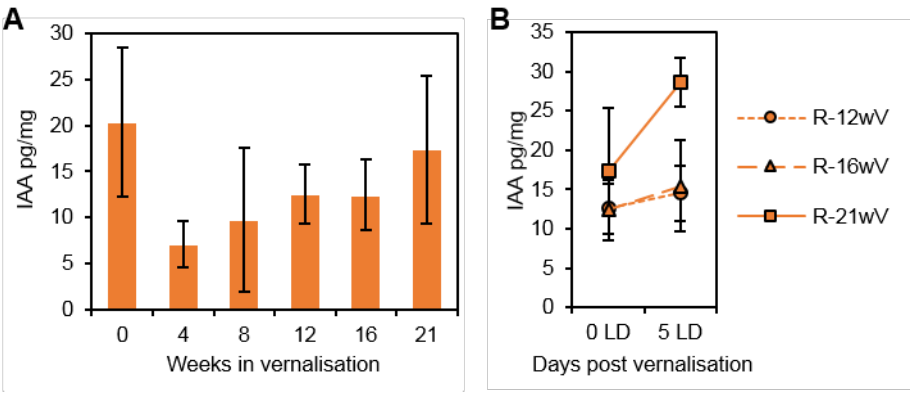

Figure 2-25. **Auxin abundance during extended vernalization and adventitious root formation.** **(A)** Quantification of auxin (IAA pg/mg) in the rooting zone before vernalization, and 4, 8, 12, 16 and 21 weeks after vernalization. **(B)** Quantification of auxin (IAA pg/mg) in the rooting zone at the end of 12, 16 and 21 weeks of vernalization, and 5 days after transfer to long day conditions. The mean and the standard deviation represent three biological replicates.

2.4.3. Regulation of hormonal response during vernalization in *A. alpina*

To get an insight on the role of vernalization during adventitious root formation, the transcriptome of the rooting zone was investigated during the course of vernalization for 21 weeks. The internodes were collected before exposure to vernalization, and then after 4, 8, 12, 16 and 21 weeks of long day-vernalization. Since the internodes before vernalization did not produce adventitious roots, these samples were considered as the control samples. Nearly 73.6% of the genes identified in this study had homologues in *A. thaliana* (Table 2-3). The number of downregulated genes during the vernalization was always more than upregulated genes (Figure 2-26). Clustering the expression profiles generated two sub-clusters representing the samples collected during vernalization and the ones collected from plants growing in long day conditions. The latter consisted of samples harvested from plants that have been vernalized for 12, 16 and 21 weeks. The rooting zone vernalized for 16 and 21 weeks promoted the formation of adventitious roots in more than 50% plants and clustered together indicating a similar transcriptome.

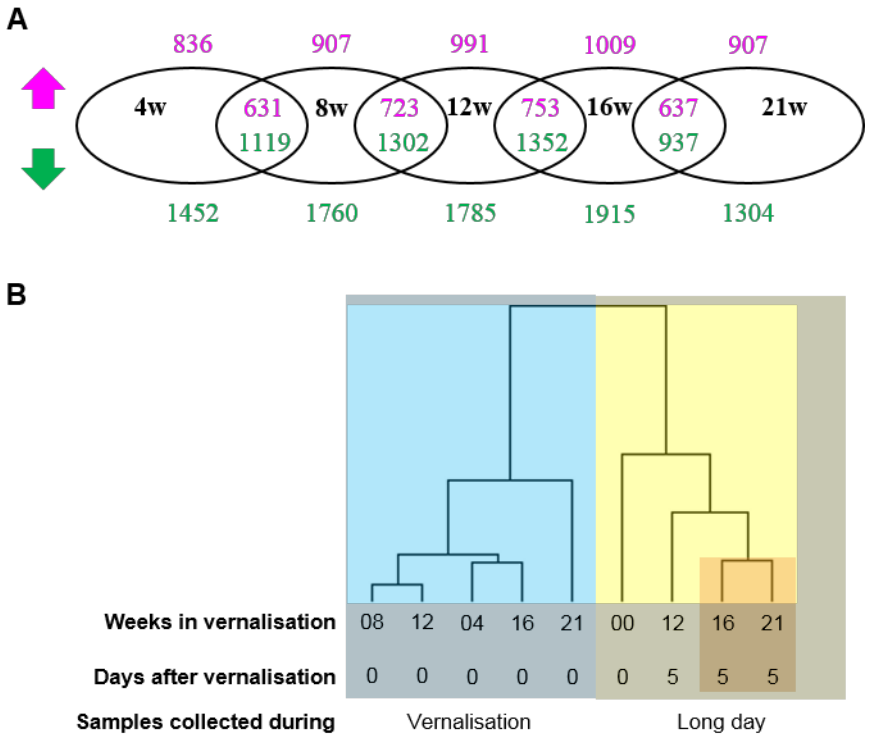

Figure 2-26. **Overview of transcriptome during extended vernalization.**
(A) Venn diagram depicting the number of genes regulated during vernalization. The number of genes upregulated (magenta) and downregulated (green) are represented above and below each set, and genes that are similarly regulated between the increasing duration of vernalization are shown as intersection. **(B)** The tree shows the distribution of the transcriptome in the rooting zones at the end of vernalization (0) and 5 days after vernalization (5). The samples were collected before vernalization (00), and from plants vernalized for 4, 8, 12, 16 and 21 weeks. The cluster is divided into two sub-clusters representing the samples collected during vernalization (blue highlight) and samples collected 5 days after transfer to long day greenhouse (yellow highlight). 16 and 21 weeks vernalized samples are highlighted in orange. The cluster was generated to determine the relationship between duration of vernalization and adventitious root formation.

Table 2-3. Genes differentially regulated during extended vernalization

Vernalization period (week)	*A. alpina* genes		*A. thaliana* genes		Percentage of genes with At homologs	
	Up	Down	Up	Down	Up	Down
4	1271	1784	836	1452	65.8	81.4
8	1407	2148	907	1760	64.5	81.9
12	1562	2178	991	1785	63.4	82.0
16	1547	2364	1009	1915	65.2	81.0
21	1266	1645	907	1304	71.6	79.3

Table 2-3. **Genes regulated during extended vernalization.**
The table presents number of genes differentially regulated in the samples analysed in this study relative to plants that were not vernalized. The plants were vernalized for 4, 8, 12, 16 and 21 weeks in long day conditions and the samples were collected during vernalization. In addition, the number of genes with *A. thaliana* homologs for further analysis are also included.

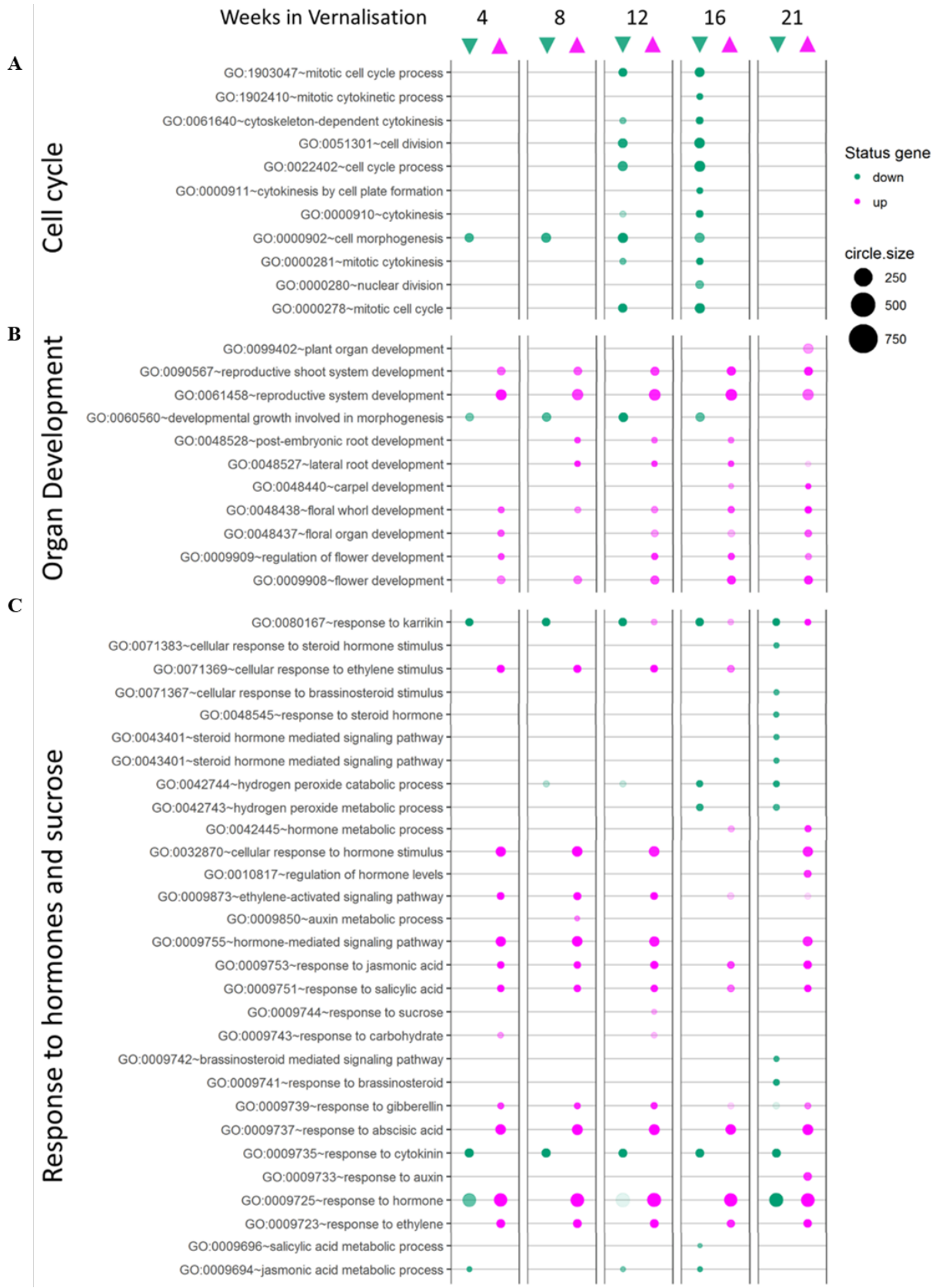
A
B
C
Weeks in Vernalisation
4
8
12
16
21
Cell cycle
GO:1903047~mitotic cell cycle process
GO:1902410~mitotic cytokinetic process
GO:0061640~cytoskeleton-dependent cytokinesis
GO:0051301~cell division
GO:0022402~cell cycle process
GO:0000911~cytokinesis by cell plate formation
GO:0000910~cytokinesis
GO:0000902~cell morphogenesis
GO:0000281~mitotic cytokinesis
GO:0000280~nuclear division
GO:0000278~mitotic cell cycle
Organ Development
GO:0099402~plant organ development
GO:0090567~reproductive shoot system development
GO:0061458~reproductive system development
GO:0060560~developmental growth involved in morphogenesis
GO:0048528~post-embryonic root development
GO:0048527~lateral root development
GO:0048440~carpel development
GO:0048438~floral whorl development
GO:0048437~floral organ development
GO:0009909~regulation of flower development
GO:0009908~flower development
Response to hormones and sucrose
GO:0080167~response to karrikin
GO:0071383~cellular response to steroid hormone stimulus
GO:0071369~cellular response to ethylene stimulus
GO:0071367~cellular response to brassinosteroid stimulus
GO:0048545~response to steroid hormone
GO:0043401~steroid hormone mediated signaling pathway
GO:0043401~steroid hormone mediated signaling pathway
GO:0042744~hydrogen peroxide catabolic process
GO:0042743~hydrogen peroxide metabolic process
GO:0042445~hormone metabolic process
GO:0032870~cellular response to hormone stimulus
GO:0010817~regulation of hormone levels
GO:0009873~ethylene-activated signaling pathway
GO:0009850~auxin metabolic process
GO:0009755~hormone-mediated signaling pathway
GO:0009753~response to jasmonic acid
GO:0009751~response to salicylic acid
GO:0009744~response to sucrose
GO:0009743~response to carbohydrate
GO:0009742~brassinosteroid mediated signaling pathway
GO:0009741~response to brassinosteroid
GO:0009739~response to gibberellin
GO:0009737~response to abscisic acid
GO:0009735~response to cytokinin
GO:0009733~response to auxin
GO:0009725~response to hormone
GO:0009723~response to ethylene
GO:0009696~salicylic acid metabolic process
GO:0009694~jasmonic acid metabolic process
Status gene
down
up
circle.size
250
500
750

Figure 2-27. **Bubble chart showing the enriched GO terms during extended vernalization.**
The upregulated (magenta) and downregulated (green) genes in the rooting zone at the end of 4, 8, 12, 16 and 21 weeks of vernalization relative to before vernalization were analysed. The size of the circle denotes the number of genes participating in each category. The GO terms selected here are differentially enriched through vernalization. The transparency of the circle represents the confidence with respect to the set p-value of 0.01.

Cell cycle and division regulating genes were downregulated at 12 and 16 weeks of vernalization and were not differentially at early and late stages of vernalization (e.g. at 4, 8 and at 21 weeks of vernalization) (Figure 2-27). Gene ontologies (GO) for post-embryonic (GO:0048528) and lateral root development (GO:0048527) were enriched after 8 weeks of vernalization. GO terms for development of floral organs including carpel development (GO:0048440), floral whorl and organ development (GO:0048438 and GO:0048437) and flower development (GO:0009909 and GO:0009908) were enriched among the upregulated genes at all time-points during vernalization (Figure 2-27).

Apart from genes involved in developmental processes, we found genes regulating hydrogen peroxide catabolism downregulated as the vernalization duration increased. Hormone mediated signalling pathway (GO:0009755) and response to hormones (GO:0009725 and GO:0032870) was upregulated at all time points during vernalization. GO terms associated with hormone metabolic processes (GO:0042445) and regulation of hormone levels (GO:0010817) were upregulated specifically at the end of 21 weeks of vernalization.

The enrichment of genes regulating hormonal response was followed during the promotion of adventitious root development to identify the role of hormones. We focused on the regulation of genes responding to nine hormones: abscisic acid, auxin, brassinosteroid, cytokinin, ethylene, gibberellic acid, jasmonic acid, salicylic acid and strigolactone. The enrichment of the genes responding to specific hormones were calculated for the rooting zone treated to different durations of vernalization compared to the rooting zone in long day grown plants. I found only one strigolactone-responsive gene, *SMAX1-LIKE 8* (*SMXL8*), upregulated in 21 week vernalized samples and therefore did not consider strigolactone for further studies. Genes responding to abscisic acid and salicylic acid were highly enriched in the pool of upregulated genes, while cytokinin-responsive genes were continuously enriched in the set of the genes downregulated in the internodes, irrespective of the duration of vernalization treatment (Figure 2-28).

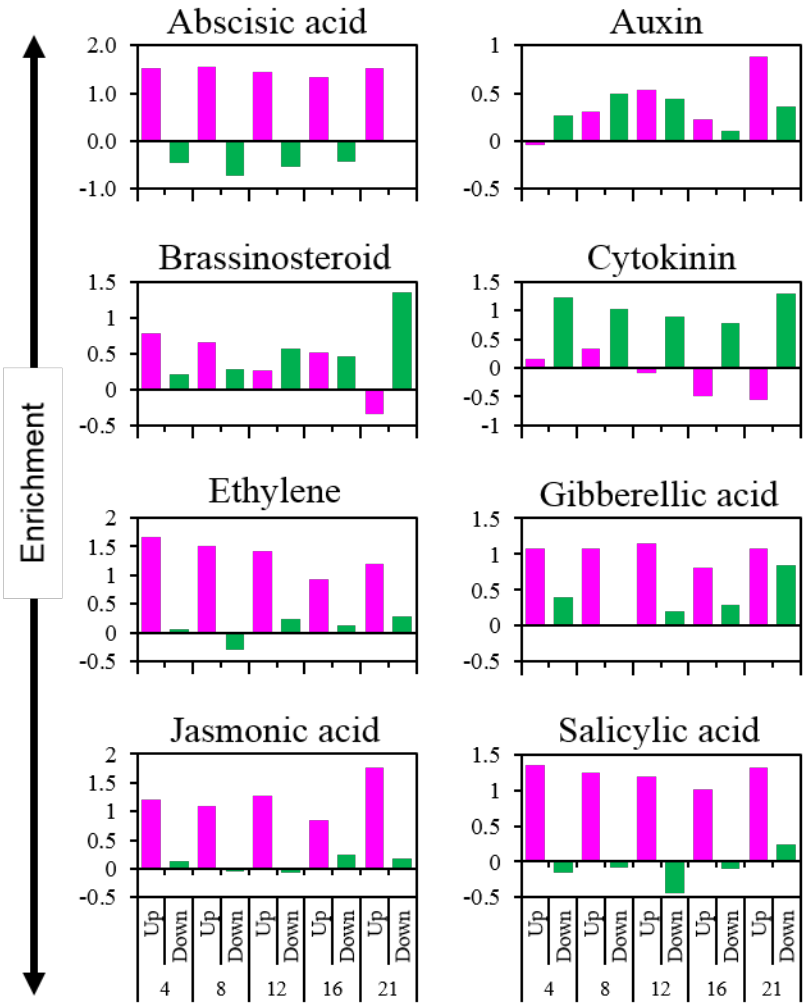

Figure 2-28. **Enrichment of genes responsive to hormones.**
(A) Abscisic acid **(B)** auxin, **(C)** brassinosteroid, **(D)** cytokinin, **(E)** ethylene, **(F)** gibberellic acid, **(G)** jasmonic acid and **(H)** salicylic acid responsive genes are represented. The upregulated (magenta) and downregulated (green) genes in the rooting zone at the end of 4, 8, 12, 16 and 21 weeks of vernalization relative to before vernalization were analysed. The enrichment value was calculated as mentioned in the Materials and Methods.

Auxin-responsive genes showed similar enrichment among the upregulated and downregulated genes until 16 weeks of vernalization. The number of upregulated auxin-responsive genes increased at vernalization of 21 weeks. The homolog of *ABCB19* was downregulated at all durations of vernalization, whereas, until 16 weeks of vernalization at least one PIN encoding gene was downregulated. Four weeks after vernalization, an auxin influx carrier was detected among the downregulated genes. Throughout the 21 weeks of vernalization, most members of the SAUR family were found downregulated, whereas we also found 3-6 *SAUR* genes upregulated. AUX/IAA

encoding genes were mostly downregulated: homolog of *IAA19* at 4, 8, 12 and 16 weeks, *IAA7* at 8, 12, 16 and 21 weeks, and *IAA14* and *IAA28* at 21 weeks of vernalization. Genes responding to jasmonic acid showed a similar response with enrichment in the upregulated genes at 21 week vernalization (Figure 2-28). Ethylene-responsive genes in the pool of upregulated genes showed a decreasing trend with the increase in the duration of vernalization.

Brassinosteroid-responsive genes were enriched among the downregulated genes at 21 weeks of vernalization. The homolog of *ATBS1-(ACTIVATION-TAGGED BRI1 SUPPRESSOR 1)-INTERACTING FACTOR 1* (*AIF1*) remained downregulated after 12 weeks of vernalization until 21 weeks. On the other hand, cytokinin-responsive genes were enriched throughout vernalization in the set of downregulated genes. The genes responsive to gibberellic acid showed an increasing trend among the downregulated genes as the ability to produce adventitious roots increases with increasing vernalization periods. A *XYLOGLUCAN ENDOTRANSGLUCOSYLASE* (*XTH*) and an *EXPANSIN* responding to gibberellic acid were found downregulated and upregulated, respectively, at the longer durations of vernalization.

2.4.4. Transcriptional changes regulating adventitious rooting during vernalization

The transcriptomic changes taking place during vernalization was investigated in samples collected at different durations of vernalization. GO terms related to cell wall organisation and regulation of cell wall components were found downregulated during the whole duration of long day-vernalization. Out of all hormonal responses, GO terms associated with only cytokinin was enriched in the downregulated genes, whereas GO terms related to abscisic acid, ethylene and jasmonic acid were significantly enriched in the upregulated gene list. Physiological studies indicated that adventitious roots are not produced after 4 and 8 weeks of long day-vernalization, and plants vernalized for 12, 16 and 21 weeks in long days produce adventitious roots on the main stem although at different frequencies. Homologs of genes involved in auxin metabolism (*GH3.6*), signalling (*SAUR1*, *SAUR30*, *SAUR50*, *SAUR51*, *SAUR52* and *SAUR53*) and transport (*ABCB11*); ethylene biosynthesis (*ACO2*); brassinosteroid signalling (*BEE2*); gibberellic acid signalling (*GASA5* and *GASA6*); and sugar biosynthesis (*SUS4*) and transport (*STP9* and *SUC1*) were downregulated throughout vernalization. However, homologs of genes involved in abscisic acid biosynthesis (*NCED4*); auxin signalling (*SAUR36*); cytokinin signalling (*CRF11*); gibberellic acid

receptor (*GID1B*); sugar biosynthesis (*SUS3*); and hormone metabolism (*MES4*) were upregulated throughout vernalization. These genes which were attenuated in a similar way throughout vernalization seem to be cold-responsive in *A. alpina*.

In this study, the samples that did not produce adventitious roots i.e. the LD-grown plants and the 4 and 8 weeks vernalized plants are the non-rooting samples, whereas the 12, 16 and 21 week vernalized samples were considered as rooting samples. We looked for candidates that were upregulated in non-rooting or downregulated in the rooting samples indicative of negative regulators, and positive regulators that were upregulated in rooting samples or downregulated in the non-rooting samples (Figure 2-29). Until 12 weeks of long-day vernalization, homologues of auxin transporter, *ABCB11*, jasmonic acid biosynthesis gene, *LOX2* and gibberellic acid signalling gene, *GASA4* were downregulated, and could be negative regulators for establishment of root primordium. Whereas the homologue of *ERF5* was upregulated until 12 weeks vernalization suggesting it participates during the establishment of the root primordium. On the other hand, homologues of development-related gene, *CUC1*; cytokinin degrading gene, *CKX1*; strigolactone biosynthesis gene, *D27*; auxin-conjugating gene, *IAGLU*, and signalling genes, *SAUR37* and *SAUR70* were upregulated in plants vernalized for 12 weeks or more indicating that the upregulated genes might promote root primordium development. Similarly, homologues of *AGL20*, *AGL44* and *AGL65* were upregulated, and *ERF38* and *ARR6* were downregulated in 16 and 21 weeks vernalized plants. Homologues of genes participating in auxin biosynthesis, *CYP79B2*, and signalling, *SAUR76*; ethylene biosynthesis, *ACS6*; gibberellic acid degrading, *GA2OX6*; jasmonic acid biosynthesis, *JOX2* and *LOX6*, and signalling, *JAZ1*; and cytokinin degrading, *CKX5* were upregulated, whereas homologues of genes involved in auxin transport, *ABCB2*, and signalling, *IAA14*, *IAA28*, *SAUR15*, *SAUR35* and *SAUR41*; brassinosteroid signalling, *BEE3*; ethylene signalling, *ERF15*; cytokinin signalling, *KMD4*; developmental processes, *LBD37*; and root developmental process, *RGF9* were downregulated specifically in 21 weeks long-day vernalized plants. Overall, this is suggestive of upregulation of cytokinin degradation and higher auxin signalling, along with downregulation of ethylene signalling during adventitious root primordia development in vernalization.

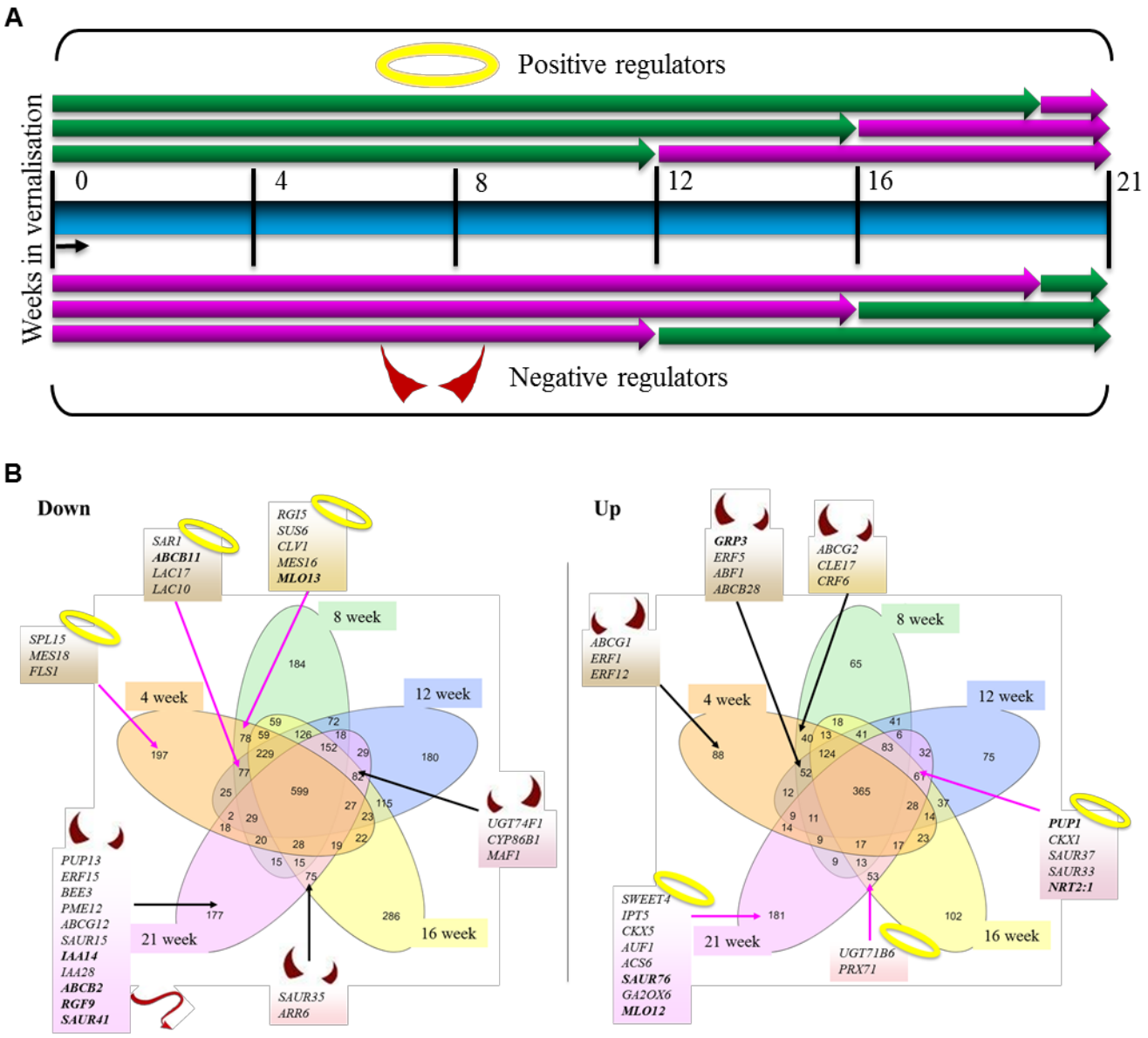

Figure 2-29. **Adventitious rooting regulation in vernalization.**
(A) The expected expression pattern of positive and negative regulators of adventitious root development. The upregulation (magenta) and downregulation (green) of genes in the rooting zone at the end of 4, 8, 12, 16 and 21 weeks of vernalization relative to LD-grown plants. **(B)** The Venn-diagrams show the number of genes and selective genes showing the expected expression pattern. Putative positive and negative regulators are represented by halos and horns. The regulation is relative to the expression of the genes in LD-grown plants.

2.4.5. Co-expression analysis

A heat map was constructed with selected genes in order to identify novel regulators of adventitious root development during extended vernalization in *A. alpina*. Genes selected for co-expression analyses showed a fold change of 8 or more between the highest and the lowest expression values. The heat map was divided into 57 clusters characterised by the expression profile of the selected genes (Figure 2-30A). Based on the interesting expression pattern, 20 clusters were examined for the genes that might regulate adventitious root development (Figure 2-30B). The clusters could further be grouped according to the overall expression profile: downregulated (Group I; green highlighted; Clusters 3, 4, 5, 8, 9 and 11) and upregulated (Group II; red highlighted; Clusters 12, 13, 14, 15, 16, 17 and 20) during the extended vernalization, upregulated in long-day plants followed by downregulation and then upregulation during vernalization (Group III; purple highlighted; Clusters 1, 2, 6, 7, 18 and 19), and genes upregulated during early vernalization followed by downregulation (Group IV; yellow highlighted; Cluster 10).

Genes that were downregulated during vernalization (Group I) are involved in auxin conjugation (*GH3.9*), signalling (*IAA7*, *IAA14*, *SPL3*, *SAUR1*, *SAUR11*, *SAUR16*, *SAUR27*, *SAUR28*, *SAUR29*, *SAUR35*, *SAUR51*, *SAUR54*, *SAUR66* and *SAUR67*) and transport (*ABCB2* and *PID*); gibberellic acid biosynthesis (*GA20OX1*), degradation (*GA2OX1* and *GA2OX4*), and signalling (*GASA6* and *GASA14*); brassinosteroid degradation (*BR6OX1* and *BR6OX2*); cytokinin signalling (*HK5*); and sugar transport (*STP14*). Genes participating in auxin metabolism (*IAGLU*) and transport (*PILS3*); cytokinin degradation (*CKX1* and *CKX5*); and gibberellic acid biosynthesis (*GA20OX2*) and receptor (*GID1B*) were upregulated during vernalization (Group II). Genes associated with Group III are involved in auxin transport (*ABCB11* and *PIN5*), conjugation (*GH3.3*) and signalling (*IAA29*, *SAR1* and *SAUR76*); cell cycle (*CYCB1;1*); jasmonic acid signalling (*JRG1*); cell expansion (*EXPA9*); cytokinin signalling (*HK2*); ethylene signalling (*ERF71*); and sugar transport (*STP9*). Group IV had only 32 *A. alpina* genes, of which only half had homologous genes in the *A. thaliana* genome. Only 8 characterised genes were found associated with Group IV including a gene encoding a fatty acid reductase (FAR5). Genes encoding MADS box transcription factors, such as *MAF1*, *AGL15*, *AGL20*, *AGL44* and *AGL65*, except *MAF3*, were upregulated during the later phases of vernalization (16 and 21 weeks).

2.4.6. Adventitious root primordium development during vernalization

GO terms associated with lateral root and post-embryonic root development (GO:0048527 and GO:0048528) were upregulated by 8 weeks of vernalization until 21 weeks of vernalization. Genes linked to these GO terms found differentially regulated include Interactor/inhibitor 1 of Cdc2 Kinase (*ICK1*), *PUCHI*, *AGL44*, HAESA (*HAE*), *PILS5*, Related to ABI3/VP1 (*RAV1*), plantUbox/armadillo repeat-containing E3 ligase9 (*PUB9*) and polyol/monosaccharide transporter 5 (*PMT5*), which showed upregulation around 4, 8, 12 and 16 weeks of vernalization but were downregulated 21 weeks after vernalization (Figure 2-31). Another group of genes including *CUC1*, *WRKY75*, *EXPA17*, *Translationally Controlled Tumor Protein* (*TCTP1*), NUCLEOSOME ASSEMBLY PROTEIN 1 (*NRP2*) and *NRT2:1* were upregulated throughout the vernalization. Overall, the presence of root associated genes during vernalization indicates the establishment of adventitious root initiator cells during vernalization.

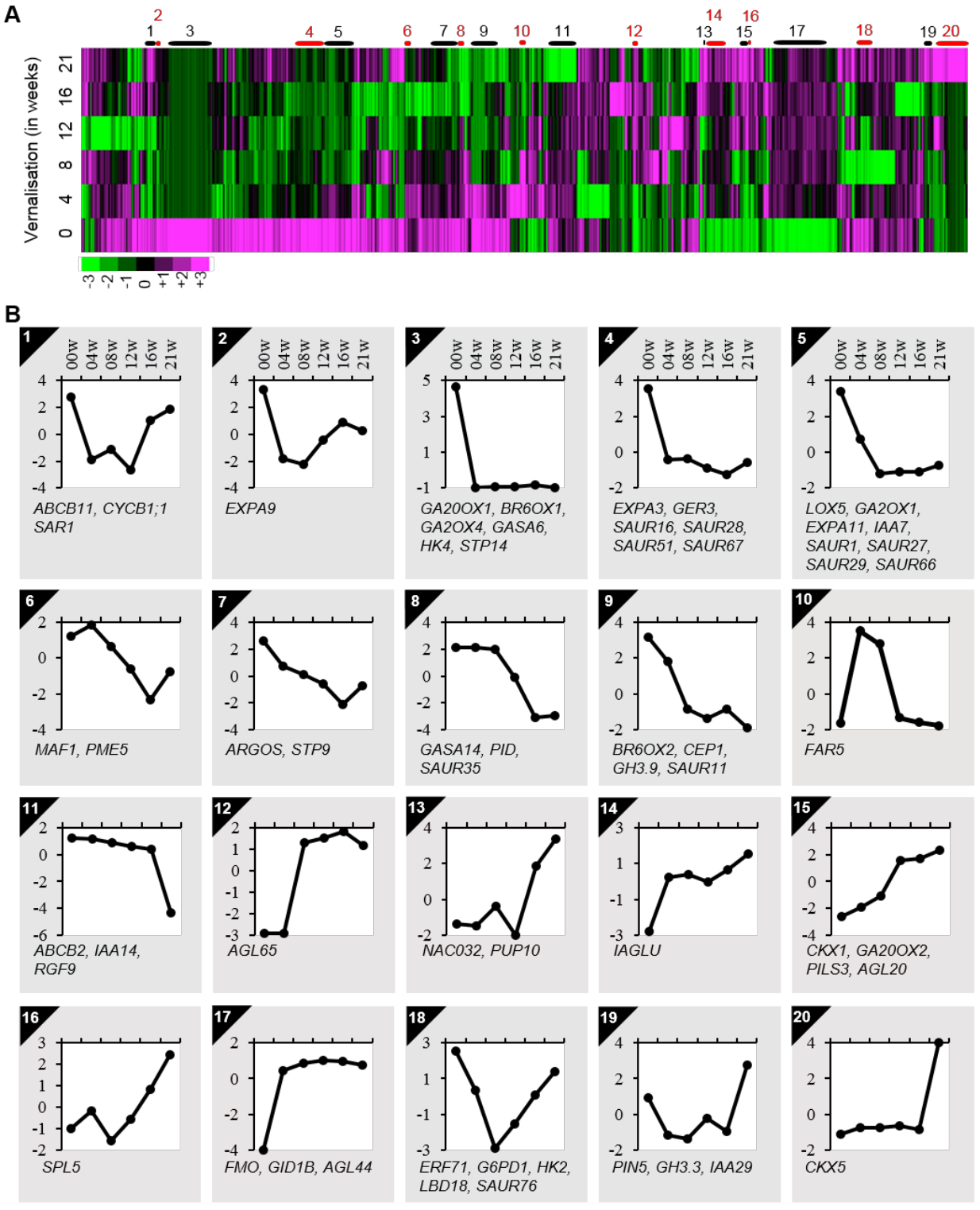

Figure 2-30. **Co-expression clustering of the differentially expressed genes during vernalization.** **(A)** Heat map of the expression pattern of 3384 genes out of the 30690 identified in *A. alpina* with $\log_2$Fold-change ≥ 2 and the difference between the maximum and minimum value ≥ 3. The heat map was generated with CLUSTER3.0 and was analysed with TreeView. Changes in the expression pattern are depicted as shown in the scale below the heat map. Green represents downregulation and magenta represents

upregulation of expression levels. The heat map shows the expression levels of selected genes before vernalization, and 4, 8, 12, 16 and 21 weeks after vernalization. **(B)** The average normalized expression pattern of genes in selected sub-clusters shown with black and red lines above the heat map. The expression levels are normalized with Cluster3.0. The sub/clusters showing similar expression pattern are highlighted in similar colour: Group I-Green, Group II-Red, Group III-Purple and Group IV-Yellow.

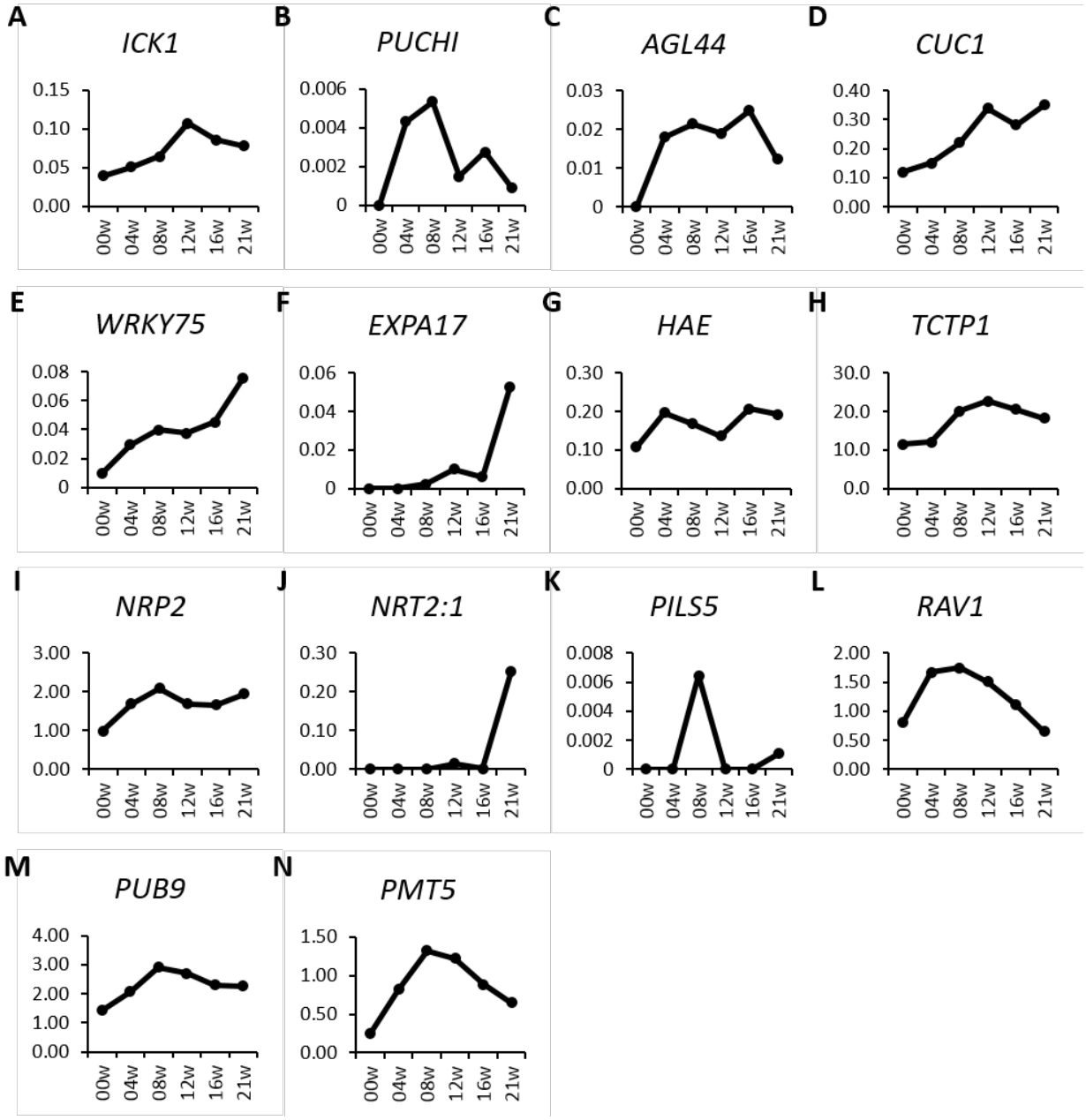

Figure 2-31. **Expression pattern of root associated genes during vernalization.**
The relative expression levels of homologs of root associated genes, **(A)** *ICK1*, **(B)** *PUCHI*, **(C)** *AGL44*, **(D)** *CUC1*, **(E)** *WRKY75*, **(F)** *EXPA17*, **(G)** *HAE*, **(H)** *TCPT1*, **(I)** *NRP2*, **(J)** *NRT2:1*, **(K)** *PILS5*, **(L)** *RAV1*, **(M)** *PUB9* **and (N)** *PMT5*, differentially regulated during the course of vernalization of 4, 8, 12, 16 and 21 weeks. The expression values are the average counts relative to the housekeeping gene, *AaPP2A*.

3. DISCUSSION

3.1. Auxin spray promotes adventitious rooting in *Arabis alpina*

Auxin spray in this study induced adventitious roots on the branches and the main stem in *A. alpina*. The effect of auxin analogues on adventitious rooting in cuttings has been shown to be dose-responsive [102,122,162]. A similar distribution is shown in *A. alpina* as the number of plants, internodes and branches developing adventitious roots post auxin spray increases with increase in auxin concentration.

A recent study suggested that the competence to root was regulated by the vegetative to reproductive transition supported by early flowering mutants producing fewer adventitious roots [163]. However, in this study, *pep1-1*, an early flowering mutant, is more responsive to auxin spray than Paj suggesting that the regulation of adventitious rooting might follow a different mechanism upon auxin spray in *A. alpina*. In many plant species, adventitious root formation declines with maturation, an age-related developmental process [35,120,164–168]. Different plant species have an optimal age for rooting, and the onset and the rate of the deterioration of rooting competence shows a varied response [169,170]. An age dependent response is also shown by both Paj and *pep1-1*. In both cases, the older eight-week plants had higher number of internodes and branches occupied by adventitious roots than their six-week-old counterparts. Surprisingly, a study from the 1960s on the effect of age on adventitious root development in the cuttings of *Populus trichocarpa* showed a similar trend; older cuttings were capable of producing more adventitious roots that younger cuttings [171]. Interestingly, six- and eight-week old *pep1-1* plants respond similarly to higher concentrations of auxin, whereas *s*uch a response was not observed for Paj. Developmentally similar, Paj plants of six and eight weeks are both adult plants capable of flowering in response to vernalization. Whereas for *pep1-1*, while six-week-old plants are vegetative, eight-week-old plants were undergoing the transition to flowering. The requirement of specific cellular pathways and stimulus for the induction and establishment of the adventitious root seem to be age-dependent in *A. alpina*. Additionally, *pep1-1* promotes adventitious rooting suggesting that the *FLC* orthologue is an inhibitor of adventitious rooting in *A. alpina*.

Unexpectedly, the branches did not behave the same as the main stem. Adventitious rooting on the branches post auxin spray only showed age-dependent effect, wherein, eight-week-old plants had more branches with adventitious roots compared to six-week-old plants for both Paj and *pep1-1*. Therefore, developmental stage of the whole plant at the time of auxin spray does not affect adventitious rooting on branches.

Adventitious root production in response to auxin spray was enhanced in *pep1-1* relative to Paj. Apart from the difference in life-history strategy between Paj and *pep1-1*, genes related to response to hormone stimulus (GO:0009725) were found to be affected between Paj and *pep1-1* but in the apices [150]. It is possible that the transcriptome of internodes would be significantly different from apices due to different functions of these tissues. The internodes that have the adventitious roots are, fascinatingly, the closest to the shoot apical meristem at the time of auxin spray in both Paj and *pep1-1*. Remarkably, in the *A. alpina* apices while *PEP1* targets genes responding to gibberellic acid only, FLC targets genes involved in response to abscisic acid, gibberellic acid, jasmonic acid and salicylic acid [150]. Interestingly, gibberellic acid is a negative regulator of adventitious rooting in *A. thaliana* [172]. In the mutant, the absence of a functional *PEP1*, thereby resulting in the decrease in response to gibberellic acid might lead to *pep1-1* being more competent to produce adventitious roots in comparison to Paj. This might also lead to the spontaneous adventitious root production in the *pep1-1* hypocotyl. A detailed study involving the examination of the role of *FLC* in *A. thaliana* during adventitious root formation would shed light on the rooting competence of *pep1-1*.

3.1.1. Adventitious root development in *A. alpina* is genotype-specific

Plants show both interspecific and intraspecific variations, a feature that has been investigated to analyse traits like flowering time regulation and circadian clock regulation to name a few. Nevertheless, understanding clonal propagation by adventitious rooting using natural variation has been utilized rarely [48,173]. It is evident from the results that auxin spray can induce adventitious roots on intact plants although the effect is ecotype specific. In *A. thaliana*, a study of adventitious rooting on the hypocotyl with several ecotypes indicated a genetic intraspecific diversity combined with high plasticity in all ecotypes [48]. In our case, Dor, Tot and Wca are non-responsive to auxin spray-dependent adventitious rooting, but in a contrasting manner. While Dor and Tot are unable

to produce adventitious roots in these conditions, the presence of auxin does not affect the already extreme production of adventitious roots in Wca. It could be assumed that Dor corresponds to an auxin insensitive variant of *A. alpina*. Nevertheless, the curling of leaves upon auxin spray probably a result of increased cell division and elongation was observed in these auxin-treated-plants. Alternatively, Dor might require higher concentrations of auxin to produce adventitious roots. Fascinatingly, in Tot, the branches and the main stem showed unexpected variation in response to adventitious rooting at all auxin concentrations in this study. While the Tot main stem remained non-responsive to auxin spray comparable to Dor, the Tot branches behaved similar to the branches of Paj, *pep1-1* and Wca. No concentration dependent effects were observed in adventitious rooting in the branches of Paj, *pep1-1*, Tot and Wca since auxin applied at any concentration showed a similar response trend. Overall, it indicates that the variation in response to auxin spray in the main stem and branches could likely be the result of difference at either cellular arrangement or in the abundance of adventitious rooting regulators. In many species, genotypes can be categorized as easy- and difficult-to-root [124,174–177]. In this study, we can conclude that among the ecotypes used in this study, Paj and Wca are easy-to-root genotypes while Dor and Tot are difficult-to-root genotypes.

Interestingly, the ecotypes showed a variation in adventitious rooting even in the absence of auxin spray. Architecturally, Dor, Tot and Wca are rosette-like with nearly non-existent internodes like *A. thaliana*. The rosette architecture of the *A. thaliana* is believed to restrict adventitious root formation on the main stem [178]. Thereby, it was assumed that a similar structural incompetence would lead to absence of adventitious roots on the main stem of Dor, Tot and Wca plants. Surprisingly, all six-week old Wca plants produced adventitious roots on the same internode suggesting that the specific rosette architecture does not affect adventitious root formation in *A. alpina*.

A. thaliana shows natural variation in adventitious rooting in hypocotyl [48,179]. Interestingly, a variation in the frequency of plants with adventitious roots on the hypocotyl was observed among the ecotypes studied in *A. alpina*. Among the perpetually flowering accessions and the *pep1-1* mutant, Tot produced no adventitious roots on the hypocotyl, whereas a few Paj and Dor plants did produce adventitious roots on hypocotyl and *pep1-1* showed the highest frequency. The hypocotyl tissue, considered as an easy-to-root tissue, has been used to study adventitious rooting,

in the presence of stimulators in both rooted and de-rooted plants [50,162,180,181]. Prior studies displayed the decline in the competence to form adventitious roots on the hypocotyl in *A. thaliana* plants to correlate to the 'age' of the hypocotyl [182]. Whereas in the ecotypes of *A. alpina*, the plants produced adventitious roots in our greenhouse conditions and external factors such as auxin spray did not affect the occurrence of adventitious roots on the hypocotyl. The adventitious roots on the hypocotyls were found in six-week-old plants and their frequency remained unaffected as the plants aged. Likewise, the presence of adventitious roots on the hypocotyl can be again associated to specific cellular changes in the hypocotyl at specific developmental stages and age-specific inherent chemical changes rather than environmental variations.

3.1.2. Spontaneous adventitious root production in Wca

In several clonal species, trade-offs between sexual and vegetative reproduction have been evaluated [183–189]. Predictions point out that trade-offs can be displayed only when a critical resource is a limiting factor or the functions are competing for the same resources at the same time [190]. In nature, unpredictable environmental conditions manipulate the balance between both the forms of reproduction. In most plant species, the timing and, as such, the investment towards the different life-history strategies is staggered to minimize the trade-offs and costs [184,186,190–193]. Therefore, in a greenhouse with optimal conditions for plant growth, in terms of nutrient availability and other abiotic factors, plants can undergo both sexual and clonal propagation simultaneously. This probably explains the phenomenon generously displayed in the accession Wca, which produces adventitious roots and undergoes flowering at the same time. Eleven-week old Wca plants, unlike other genotypes, produced adventitious roots on almost all lower internodes without external stimuli.

Wca plants behaved like the *A. thaliana superroot* (*sur*) mutants with surplus adventitious roots on most part of the main stem excluding the inflorescence stem [24]. However, unlike *sur1* mutant plants, which have elongated hypocotyl due to the excessive auxin levels, Wca plants have a rosette-like architecture with compressed internodes in comparison to Paj and *pep1-1*. The free endogenous IAA levels was unaffected in all *A. alpina* genotypes in this study suggesting that Wca plants might be affected in their ability to sense auxin or downstream regulators of adventitious root development. Unlike the auxin abundance in the main stem, the relative expression of both

the homologs of the auxin responsive genes *GH3.3* and *GH3.6, which* are known to positively regulate adventitious rooting in the etiolated hypocotyls of *A. thaliana* [52], were upregulated in Wca plants. In *A. alpina*, both the genes have Single Nucleotide Polymorphisms (SNPs) in all the ecotypes. The SNPs combined with the differences in the expression of these genes suggest changes at functional level.

Apart from auxin, ethylene also regulates adventitious root formation in several species [18,81,82,84,89,194]. A tomato abscisic acid deficient mutant, *not*, characterized by excessive ethylene production, shows dense outgrowth of adventitious roots are observed on the main stem [89]. Characterizing these genes further by generating transgenic plants would shed light on their function during adventitious root formation in *A. alpina* and the peculiar phenotype of Wca plants.

3.2. Adventitious rooting in intact vernalized *A. alpina* plants post auxin spray

The effect of auxin spray application on vernalized Paj plants having undergone vegetative-to-flowering transition was also addressed in this study. The vernalized flowering Paj plants produced adventitious roots on the main stem and the branches following the application of auxin, in a similar manner to the vegetative Paj plants. Likewise, the flowering Paj plants showed a dose-dependent response to auxin, with higher doses causing more plants to develop adventitious roots. However, this study shows that intact flowering Paj plants behaved similarly to vegetative Paj plants when sprayed with auxin. Overall, the vegetative to reproductive transition does not negatively regulate adventitious rooting in *A. alpina*.

A spatial pattern indicating auxin spray application induced adventitious roots selectively on the internodes closest to the shoot apical meristem was observed. Due to the continuous cold temperature during the 12 weeks of vernalization, the elongation of internodes is restricted leading to a rosette-like ‘compact zone’. The internodes that are competent to form adventitious roots in response to the auxin application were elongated before the plants experienced vernalization, and are present below the compact zone [161]. The position of adventitious roots on the main stem was strictly restricted to the 2–3 uppermost elongated internodes in flowering Paj plants whereas in the vegetative plants several elongated internodes below the shoot apical meristem produced adventitious roots. The spatial pattern of adventitious roots in adult intact plants has received little attention in the past [195]. A study in the Puerto Rican forest found that aerial roots in trees and

shrubs were produced beneath the terminal leafy zone and the distance from the base was species dependent. However, for vines in the same forest, variations were observed among species, with some generating adventitious roots throughout the stem and others producing nodes dedicated to rooting. Furthermore, in some species, primordia can develop on pre-determined internodes [196]. Understanding the mechanism used to determine the position of primordia would be useful in an agricultural context, allowing the induction of adventitious roots in non-rooting cuttings.

Juvenility is one of the competence factors for adventitious root formation, hence the preferred use of juvenile cuttings for the clonal propagation of crops. Most studies showed that cuttings obtained from the juvenile phase were more competent than the ones obtained from mature adult plants [33,139,164,197–201]. Factors regulating the continuous physiological and molecular changes during the aging of plants might play a role in the regulation of adventitious rooting. Among these factors, auxin homeostasis and signalling, microtubule remodelling and nitric oxide signalling differentiate juvenile cuttings from the mature ones. However, it is unclear how these factors may regulate adventitious rooting on the different internodes of an intact plant. The rooting and non-rooting zones differ in terms of distance from the shoot apical meristem, but both the zones are produced during the mature phase of the plant development. In this study, the elongated internodes closest to the shoot apical meristem produce adventitious roots in response to the auxin spray application. The internodes undergoing elongation after auxin spray do not produce adventitious roots most likely because these internodes were not treated to auxin. The presence of both the rooting and non-rooting internodes in the same intact plant indicates that the whole plant is not equally competent to produce adventitious roots. Further studies should focus on understanding the correlation between adventitious rooting and the plant architecture.

MicroRNA156 (*miR156*) is a well-studied juvenility marker whose expression declines as the plant ages. In tobacco, maize and *A. thaliana*, the overexpression of *miR156* causes an extended juvenile period and promotes adventitious root formation [202–204]. However, there was no correlation between the abundance of *miR156* and the ability of *Eucalyptus grandis* plants to produce roots [200]. No difference was observed in the abundance of miR156 targets between the rooting and non-rooting zones in this study.

Likewise, nitric oxide (NO) is an indicator and a positive regulator of adventitious root development in several species [205–210]. Plants produce nitrate reductases to synthesize NO from nitrate and nitrite, and a nitrate reductase gene was shown to be upregulated in the juvenile cuttings of *E. grandis* [199]. Genes encoding nitrate transporters (NRT) and enzymes involved in nitrogen metabolism (GDH3) were upregulated in the rooting zone of *A. alpina* at the end of vernalization and also during later phases of adventitious root development. In the rooting zone, the accumulated nitrate probably, upon conversion into NO, stimulates the MAPK and cGMP signalling pathways to promote adventitious root formation [206,207]. Overall, the position of the rooting zone is already defined before auxin spray in *A. alpina* structurally, indicating that there might be differences in the transcriptomic make-up of the two zones.

3.3. Enhanced auxin responsiveness induces competence for adventitious root development at specific internodes before auxin spray

At the end of 12 weeks of vernalization, auxin-responsive genes are preferentially expressed in the rooting zone of *A. alpina* correlating with the ability to produce adventitious roots following auxin application. This indicates that the rooting zone is already competent to produce adventitious roots at the end of vernalization. This hypothesis was examined by analysing the rooting zone transcriptome to identify competence factors. At the end of vernalization, the non-rooting zone was competent to respond to abscisic acid, ethylene, jasmonic acid and salicylic acid, as indicated by the upregulation of genes responding to these hormones. Abscisic acid inhibits cell-cycle progression, in turn suppressing auxin-dependent lateral root initiation in peanut (*Arachis hypogea*) and adventitious root growth in rice and tomato [85,89,211–214]. Contrarily, exogenously applied abscisic acid promotes adventitious root formation in stem cuttings, however the mechanism remains unexplained [87,88]. The presence of abscisic acid-responsive genes suggests that auxin transport is inhibited, thereby preventing root initiation at the non-rooting zone [212]. Abscisic acid also affects ethylene signalling, a known stimulator of adventitious root development [85]. Transcriptomic analysis indicated the modulation of ERF encoding genes during adventitious root development [18]. However, ethylene can also inhibit lateral root formation by suppressing auxin transport [215]. ERF encoding genes were specifically downregulated in the rooting zone, indicating an antagonistic role during adventitious root induction in *A. alpina*. Similarly, jasmonic acid is known to inhibit adventitious root formation, although some studies have concluded the opposite

[52,216]. The jasmonic acid biosynthesis genes of the *LOX* family were upregulated in the non-rooting zone, suggesting that higher jasmonic acid levels suppress adventitious rooting in *A. alpina*. In contrast, salicylic acid is a positive regulator of adventitious rooting but the salicylic acid responsive genes we analysed were downregulated in the rooting zone, hinting at a species-dependent role [76]. Finally, the strigolactone biosynthesis genes *CCD7* and *CCD8* were downregulated in the rooting zone. Previous studies have shown that *ccd7* and *ccd8* mutants produce more adventitious roots than the wild-type in *A. thaliana*, suggesting the CCD7 and CCD8 proteins inhibit adventitious root induction in the non-rooting zone [77].

Although genes related to several hormones were strongly expressed in the rooting zone, auxin-response genes were expressed predominantly in the rooting zone at the end of vernalization. The auxin efflux carrier gene, *ABCB19*, was upregulated in the rooting zone, indicating local auxin accumulation at the end of vernalization [26]. Auxin transport can also be inhibited by flavonoids, reflected by the higher expression of flavonol synthase *FLS1* in the rooting zone [217,218]. Among other factors regulating auxin signalling, an upregulation of *IAA7* and the downregulation of *IAA1* in the rooting zone was observed. In *A. thaliana*, the gain-of-function mutation of *IAA7* (*axr2*) reduces the number of adventitious roots whereas the corresponding mutation of *IAA1* (*axr5*) affects lateral root formation, but the precise function of these genes in adventitious root development remains unknown [24,219]. Interestingly, in *A. thaliana* both IAA7 and IAA1 interact with the ARF proteins (ARF6, ARF7, ARF8 and ARF19) that promote adventitious rooting [51,73,220]. These *AUX/IAA* genes may therefore suppress the positive regulators of adventitious rooting in the absence of ARF proteins in the rooting and non-rooting zones. None of the *SAUR* genes expressed at the end of the vernalization have been characterized for their role in root development. Interestingly, although *SAUR* gene expression is known to be regulated by auxin, in this case the level of free endogenous auxin does not differ significantly between the rooting and non-rooting zones [221]. The presence of other factors during development and vernalization would make the rooting zone competent to respond to auxin and produce adventitious roots. Overall, the data suggest that although the rooting and the non-rooting zones are successive sections of the main stem, they already differ in competence at the end of vernalization.

3.4. Hormonal regulation during vernalization

The difference in the transcriptomes of the rooting and the non-rooting zone at the end of 12 weeks of vernalization could be a remnant of differences created during vernalization. Auxin signalling was repressed during vernalization with SAUR encoding genes affected the most. None of the downregulated SAURs have been characterised for their role in adventitious root formation. While most of the downregulated SAURs were upregulated throughout adventitious root development in the rooting zone following auxin application, they did not play a role in adventitious root development in vernalization. A few SAURs including *SAUR11* and *SAUR35* were expressed in the early vernalization and then repressed. Among the AUX/IAA encoding genes, the gain-of-function mutant of *IAA14* is known to promote adventitious root formation, while *IAA7* has not yet been characterised. *SPL3* responds to auxin signalling and is responsible for inhibiting root primordium development and emergence in *A. thaliana*, although its role in adventitious root development is yet to be established [222]. Therefore, inhibition of SPL3 might promote the formation of root primordium during vernalization. Even auxin transport genes were affected during vernalization pointing to the changes in free endogenous auxin levels. While *ABCB2* and *PID* were downregulated in vernalization, *ABCB11*, *PIN5* and *PILS3* were enriched at 21 week vernalization suggesting an increase auxin transport activity correlating with the formation of the primordium. *SAR1*, a nucleoporin encoding gene, was upregulated 21 weeks after vernalization suggesting the importance of nuclear export of genes participating in hormone signalling in adventitious root development [223].

Genes participating in gibberellic acid degradation were downregulated during vernalization, whereas biosynthesis gene *GA20OX2* and the receptor *GID1B* were enriched in the 21 week vernalized samples probably as a response to the downregulation of gibberellic acid response genes such as *GASA6* and *GASA14*. Interestingly, during adventitious root induction in vernalization cytokinin degrading genes were upregulated, whereas brassinosteroid degrading genes were downregulated. It indicates the role of cytokinin as a negative regulator and brassinosteroid as a positive regulator of adventitious rooting during vernalization. Interestingly, brassinosteroid responsive genes are downregulated in 21 week vernalized rooting zone.

Members of MADS box gene family have been found to be expressed in roots and implicated in root development [224,225]. *PtAGL16* regulates adventitious root primordium formation in Poplar and *AGL21* enhances lateral root production in an auxin-dependent manner [226,227]. Most AGL encoding genes have not well characterised during root development. Among the MADS-box genes found in the study, MAF1, AGL15 and AGL44 were found to be expressed in root and root primordium [228–230]. Overall, MADS-box genes are required for the proper development of root primordium during vernalization. Further characterisation of these AGLs would help our understanding of MADS-box gene regulated root development. Largely, the transcriptomic differences between the zones seem to be regulating the competence to root in *A. alpina*.

3.5. Establishment of adventitious root initiator cells during vernalization

Surprisingly, the expression of several meristem-associated genes was upregulated in the rooting zone at the end of 12 weeks short-day vernalization. In previous studies, *CLE16* and *RGF9* were shown to be expressed in both the shoot and root, *CEP1* was characterized in the root, and *WOX1* and *PDF1* were characterized in the shoot meristem [231–235]. *JKD*, *LOB* and *MGP* show meristem specific expression in the root and shoot [236–239]. The expression of meristem-associated genes in the rooting zone at the end of vernalization suggested that the rooting zone might contain pre-formed primordia, as found in the stems of some species of apple, *Jasmonium*, *Populus*, *Salix* and *Solanum* [240,241]. In response to stimulation, the primordia develop into adventitious roots under optimal environmental conditions, and are believed to initiate from vascular cells. In *A. alpina*, the primordia emerged from the vascular cambium, but preformed primordia could not be found histologically at the end of vernalization, perhaps because they are composed of only a few initiator cells which would be difficult to detect. The short life cycle of *A. alpina* allows the source of these cells to be traced throughout development.

Following the rooting zone during vernalization points to the presence of root development associated genes including the positive and the negative regulators. *WRKY75* and *ICK1*, negative regulators of root development, showed a gradual upregulation in their expression pattern [242,243]. Other negative regulators of root development including *PILS5* was upregulated 8 weeks into vernalization, whereas *RAV1* showed highest expression 4 and 8 weeks into vernalization and thereafter a gradual decline [244,245]. Among the positive regulators, *EXPA17* and *NRT2:1* were

upregulated only after 21 weeks of vernalization [246,247]. Other positive regulators of root development including *CUC1*, *TCTP1*, *NRP2*, *PUCHI*, *AGL44*, *HAE*, *PUB9* and *PMT5* showed gradual upregulation during vernalization [230,248–253]. The presence of the positive regulators indicates the formation and presence of adventitious root initiator cells. Negative regulators of root development probably inhibit the differentiation of the initiator cells into root primordia during vernalization until the roots are required. Adventitious root development in plants that would flower suggests a correlation between sexual and clonal propagation in the perennial *A. alpina*.

3.6. Cell wall remodelling is a vital process during adventitious rooting

Proteoglycans are implicated in many plant growth processes including root development [254]. Classical *AGP* encoding genes, as well as the chimeric *FLA* genes were found regulated in the transcriptome. Homologs of *AGP* encoding genes, *AGP12*, *AGP18* and *AGP26*, and the *FASCILIN-like ARABINOOGALACTAN* encoding genes *FLA1*, *FLA2*, *FLA7*, *FLA8* and *FLA17*, are upregulated in the rooting zone until the late phase of adventitious rooting. CASPs are another group of proteins directing local modifications of the cell wall. Homologs of *CASP* encoding genes were upregulated in the rooting zone in the later phases of adventitious root formation. Related to CASPs are CASP-Like proteins, which were enriched in the non-rooting zone, also suggested to participate in tissue-specific cell wall modification machinery [255]. Along with AGPs and CASPs, EXPANSINs have been characterized to regulate cell expansion by stimulating pH dependent cell wall loosening. They are known to influence root initiation and emergence supposedly by regulating cell division and expansion [256]. In this transcriptome data, homologs of *EXP* coding genes were enriched in the mid- and late-phase of adventitious root development.

Xyloglucan, an additional structural components of the cell wall, are metabolized by XTH proteins [257]. Homologs of *XTH* genes were upregulated in the rooting zone at mid-phase and the later phases of adventitious root development. XTH activity has been reported at the site of lateral root emergence probably aiding cell wall remodelling as the primordium grows [257]. Another component in the cell wall, lignin, are regulated by laccases [258]. Laccases have been found to have anti-oxidative properties that help remove stress induced Reactive-Oxygen Species (ROS) which inhibit root growth [259]. Laccases were found in the petunia stem base during adventitious root development [18]. Whereas, in tea, *Camellia sinensis*, and mung bean seedlings, the stem cuttings had laccase downregulated upon extended auxin treatment [17,42]. We found laccases to be mostly

downregulated in the rooting zone suggesting an inhibitory role. Further studies regarding the involvement of laccases would help understand the role they play during adventitious rooting. Cell wall remodelling and cell expansion associated genes seem to be regulated during auxin-induced adventitious rooting. Altogether, it elucidates that auxin spray transforms cell wall and possibly aids adventitious root primordium growth in the stem.

3.7. Precise auxin homeostasis supports adventitious rooting in response to the auxin spray

Auxin signalling and transport affect adventitious root formation in several plant species and were followed throughout the study. The upregulation of homologs of auxin transporters and their regulators (e.g. *ABCB19*, *PILS5*, *PID* and *PIN7*) 24 h after auxin spray, and the persistent upregulation of *ABCB19*, *PID* and *WAG2* during later phases, suggests auxin efflux is an important factor required during the mid-phase of adventitious rooting in *A. alpina*. Incidentally, no auxin influx carriers were differentially expressed between the rooting and non-rooting zones in this study. Auxin transport is also affected by the presence of flavonoids and accordingly *TT4*, a gene involved in flavonoid biosynthesis, was found upregulated in the rooting zone of *A. alpina* during the mid-phase of adventitious rooting [218,260]. Homologs of CYP genes implicated in auxin biosynthesis (*CYP83B1*, *CYP79B2* and *CYP79B3*) were found downregulated in the rooting zone 120 h after auxin spray. Bearing in mind that auxin can act as an inhibitor during the later stages of root growth, it might be necessary to downregulate auxin biosynthesis at this stage. Auxin definitely affects the expression of these cytochromes in *A. alpina*.

GERMIN genes are strongly repressed during adventitious root formation in the stem cutting base in poplar and mung bean seedlings [18,42]. Homologs of auxin-binding GERMIN and GLP encoding genes were downregulated in the rooting zone 6 and 24 h after auxin spray application suggesting that *GERMIN* genes inhibit adventitious rooting. In *A. alpina*, 47 *SAUR* genes with homologs in *A. thaliana* were identified. Several of these genes were induced or repressed in response to auxin treatment specifically in the rooting zone. Indeed 17 auxin-induced *SAUR* genes remained upregulated even 120 h after the auxin spray, although they were also slightly upregulated in the non-rooting zone until 24 h after auxin treatment. In tomato, *SAUR-like* genes respond to ethylene and regulate adventitious rooting [261]. Several *SAUR* genes were also downregulated following the

auxin spray, particularly in the rooting zone. In *A. alpina*, *SAUR* genes might play a role during the early phase of adventitious root development, given the strong regulation of these genes at 6 and 24 h after spraying. This hypothesis is supported by previous studies showing that *SAUR41* and *SAUR76* positively regulate root growth and development, and that *SAUR41* also regulates auxin transport [221].

Another group of auxin-inducible genes is the *AUX/IAA* family, which primarily encode negative regulators of auxin signalling. Some of these were upregulated 6 and 24 h after applying the auxin spray, but most strongly in the rooting zone. A few *AUX/IAA* genes are known to participate in adventitious rooting based on the phenotypes of the gain-of-function mutants *iaa3/shy2*, *iaa14/slr1*, *iaa17/axr3* and *iaa28-1* [54,72,137]. The upregulation of these genes upon auxin treatment in *A. alpina*, probably facilitates the regulation of the transcriptional machinery following the sudden increase in auxin levels, leading to auxin signalling homeostasis. The sustained upregulation of the *AUX/IAA* genes specifically in the rooting zone 72 h after spraying might be responsible for the transient auxin-induced responses necessary during root formation. Apart from *MP/ARF5*, *ARF10*, *ARF11* and *ARF19*, none of the other *ARF* genes were regulated by the auxin spray, emphasizing the role of post-transcriptional regulation during adventitious root development in *A. alpina*. Acyl-acid-amido-synthetase encoding genes were also found among the auxin responsive genes expressed at different levels in the rooting and non-rooting zones, among which *GH3.3* and *GH3.6* control adventitious rooting in *A. thaliana* by modulating jasmonic acid homeostasis [52]. Overall, the ability to respond to auxin distinguishes the rooting and non-rooting zones in *A. alpina*. The importance of the *SAUR* and *AUX/IAA* genes during adventitious rooting should be explored further.

3.8. Auxin interacts with other hormones to stimulate adventitious rooting in the rooting zone

Hormonal crosstalk plays a key role during adventitious root formation with auxin purported to play a central role [52,85,172]. However, little is known about these crosstalk events and how they may influence the different phases of adventitious root development. The loss of equilibrium among these interactions may constrain adventitious root formation.

The NCED family genes related to abscisic acid biosynthesis were upregulated in the auxin-sprayed rooting zone after 24, 72 and 120 h after treatment. The expression of *NCED1* was shown to suppress excessive adventitious root production in the tomato mutant *notabilis* [89]. During adventitious root development, the expanding root primordium and the surrounding tissue might experience stress, leading to the upregulation of abscisic acid synthesis. The effect of abscisic acid on adventitious root formation probably depends on the developmental phase.

Brassinosteroid signalling including genes encoding BEE transcription factors and brassinosteroid-related kinases were upregulated 24 and 72 h after auxin application. The role of brassinosteroids in adventitious root formation and the auxin-brassinosteroid crosstalk during adventitious root development remains to be investigated. In *A. alpina*, brassinosteroid signalling seems to be a requirement during the mid-phase of adventitious root development.

In this study, auxin affected ethylene signalling and responses similarly in the rooting and non-rooting zones. Some genes encoding members of the ERF transcription factor family were upregulated, particularly in the rooting zone 24 and 72 h after auxin application. In rice, ERF transcription factors may be responsible for the initiation of crown roots, and in poplar they trigger excessive adventitious root production [74,262]. Ethylene biosynthesis genes were also induced in the rooting zone, with the same profile as the *ERF* genes. These data suggests that the auxin-dependent regulation of ethylene biosynthesis and signalling is important during the formation of the adventitious root primordium.

The induction of cytokinin homeostasis genes was observed 24 h after auxin spraying, including *CKX5* and *SOFL2*, which may regulate the levels of the endogenous cytokinin levels in the rooting zone. Surprisingly, two members of the cytokinin-activating LOG enzyme family were also enriched in the rooting zone. A *LOG* genes triple mutant stimulated adventitious rooting in *A. thaliana* [263]. Cytokinin is probably required at very low levels to regulate cell division and differentiation.

Hormones can undergo activation or deactivation to regulate their activity and abundance in a developmental phase and tissue dependent manner. Post auxin synthesis auxin can be conjugated to amino acids, peptides and glucose to fulfil different functions, as well as provide a steady-state source of free endogenous auxin [61]. *UGT74E2* was upregulated in the early phase of adventitious rooting in the rooting zone. Among the differentially expressed *UGT* genes we identified, the

homolog of *UGT72B3* was downregulated in the rooting zone 24 and 72 h after auxin treatment, whereas a homolog of *UGT76B1* (which mediates crosstalk between salicylic acid and jasmonic acid) was upregulated in the rooting zone by the auxin spray, until 120 h after auxin spray when it was found suppressed. In contrast, *UGT73C7* was upregulated only 6 h after auxin spraying specifically in the rooting zone. These data suggest that the differential regulation of hormonal signalling (especially auxin), their crosstalk, and the dynamic behaviour of the transcriptome and proteome during the early stage of adventitious root development set the stage for the initiation and induction of adventitious roots in the auxin-treated rooting zone.

4. PERSPECTIVE

Auxin application using spray proves to be an effective protocol for hormone delivery in *A. alpina*. Auxin enhances adventitious root formation in *A. alpina* and more importantly can be used to study natural variation and identify factors regulating adventitious rooting. In the aspect of adventitious rooting, four *A. alpina* ecotypes that behave unlike each other have been discovered. Considering that the ecotypes were collected at different locations, it gives an opportunity to understand how adventitious rooting might help *A. alpina* adapt to their environment, and which habitats would require and therefore induce adventitious root formation. These ecotypes provide the prospect of finding genes that would manage adventitious root formation to improve resource allocation, do better in stressful habitats or regenerate roots after de-rooting accidents and flooding [264,265]. The presence of a natural variation makes *A. alpina* a valuable source for quantitative trait loci analyses; genome-wide association studies (GWAS) and the identification of useful allelic variations. The establishment of a mapping population using these ecotypes can be explored to identify the competence factor that commits the plants to produce adventitious roots.

Auxin application on vernalized Paj plants exposed zones on the main stem with (rooting zone) and without (non-rooting zone) the ability to produce adventitious roots. The presence of meristematic mass and root primordia was observed following the application of auxin spray, predominantly in the rooting zone. Auxin might induce cell division in juvenile as well as adult tissues which probably explains why no cell cycle genes were differentially expressed in the rooting and non-rooting zones [33]. However, the dividing cells of juvenile tissues have the ability to respond further to the auxin stimulus and undergo differentiation into a meristematic mass followed by root primordium establishment, which is not the case for adult tissues or the non-rooting zone. In the rooting zone, upregulation of meristem-associated genes was observed during the later developmental time points post auxin spray. Importantly, meristem associated genes were also found enriched even before auxin application, hinting at the existence of adventitious rooting promoting factor(s) specifically in juvenile tissue and suggesting the presence of initiator cells during vernalization.

Meristem associated genes, both positive and negative regulators, were found expressed during vernalization. Extending the duration of vernalization promotes adventitious root formation in *A. alpina* indicating the development of the adventitious root primordia during vernalization. Upregulation of auxin response and downregulation of brassinosteroid response is required for the induction and emergence of adventitious root primordium during vernalization. Upregulated auxin signalling supports the establishment of the initiator cells in the early stages of vernalization.

5. MATERIALS and METHODS

Standard molecular biology techniques such as Polymerase Chain Reaction (PCR) and agarose gel electrophoresis were conducted as described in [266], unless otherwise stated.

5.1. Plant materials and growth condition

5.1.1. Natural variation for adventitious rooting

In the study aimed at understanding the presence of natural variation in adventitious rooting, the ecotypes Pajares (Paj), Dorfertal (Dor), Totes Gebirge (Tot) and West Carpathians (Wca) described earlier were used [111]. The *perpetually flowering 1-1* (*pep1-1*) has been characterized earlier for flowering time regulation [107]. The plants were grown on soil in long-day (LD) greenhouse at 20°C/18°C day/night temperatures with 16 hour light and 8 hours dark during the whole experiment. The age of the plants during the physiological experiments are mentioned in the figure legends.

5.1.2. Effect of auxin and extended vernalization on adventitious rooting

Only the Paj accession, collected in the Coedillera Cantábrica mountains in Spain, was used for the physiological and transcriptomic analyses to understand the effect of auxin and extended vernalization during adventitious root development [107]. The plants were grown in soil in a greenhouse under LD conditions (16-h photoperiod) at 20°C/18°C day/night temperatures until they were 8 weeks old to begin with. To understand the effect of auxin on adventitious rooting, the plants were then moved to vernalization chambers maintained at 4°C under SD conditions (8-h photoperiod) for 12 weeks. Whereas to understand the effect of vernalization on adventitious rooting, the 8 week-old plants were transferred to vernalization chambers maintained at 4°C under LD conditions for 4, 8, 12, 16 and 21 weeks. The plants were returned to the LD greenhouse after the required vernalization period as mentioned in the respective figure legends.

5.1.3. EMS mutagenesis screen

The mutagenized *pep1-1* seeds were obtained from Jun-Prof. Dr. Maria Albani (University of Cologne, Cologne, Germany). The mutagenized seeds along with *pep1-1* were grown on soil with nearly 48 plants screened for each EMS population (Four plants/ pot). The plants were grown in controlled LD greenhouse. The screen was carried out in three batches with overall 1770 six-week old M1 plants. The plants were screened for phenotypic differences relative to *pep1-1* before and 2 weeks after spraying. Seeds from plants showing interesting phenotypes were collected for further processing and analysis.

5.2. Application of auxin using spray

To induce adventitious roots, plants were sprayed with auxin solutions of different concentrations. 1-Naphthaleneacetic acid (1-NAA, Sigma Aldrich), an auxin analog, was first dissolved in DMSO to prepare a stock solution of 1 M, which was further diluted with water to obtain 10, 20, 50 and 100 µM solutions. The quantity of DMSO was kept constant in all dilutions by adding extra DMSO to the dilutions and maintained below 0.1% (v/v). Tween-20 was added as a surfactant at 0.2% (v/v). 50 mL spray bottles were used to spray on plants with 50 mL of the solution sprayed on a group of 10 six-week and eight-week old plants. 75mL of the solution was sprayed on 12 vernalized Paj plants.

For the EMS screen, the six-week old plants were sprayed with 100 µM solution as prepared above. The plants were sprayed thrice in 3 weeks after regular intervals using spray can.

5.3. Plant phenotyping

5.3.1. Adventitious rooting scoring

The plants were scored for number of leaves, number and position of branches to follow the whole plant architecture. Internodes, hypocotyls and branches having adventitious roots before spraying with auxin solutions and every week until five weeks after the spray were recorded. Plants exposed to extended vernalization were scored in a similar way every week until two weeks after the end of vernalization. Around 9-12 plants were scored for each condition.

5.3.2. Flowering time measurements

Flowering time was measured when the whole bud could be seen and demonstrated as number of days to flower (from the time seeds were put on soil) or number of leaves at flowering. A total of 9 plants were scored.

5.4. Characterization of *GH3* genes in *A. alpina*

The *GH3* genes, *GH3.3*, *GH3.5* and *GH3.6*, were searched in the *A. alpina* genome using NCBI-BLAST. While the homologs of *GH3.3* and *GH3.6*, were found with more than 90% identity at query cover of more than 75%, *GH3.5* only showed a query cover of 60%. Additionally, the homologs of *GH3.3* and *GH3.6* were found during the sequencing of *A. alpina* genome while *GH3.5* was not found. The sequences of the GH3 genes in Paj, Dor, Tot and Wca were sequenced by GATC-Biotech with carefully designed primers (Supplementary Table 14) to maximize the quality of the sequence achieved.

The corresponding *GH3* genes from the respective ecotypes were aligned by ClustalW using MEGA5. The shade plot was created using BOXShade (https://embnet.vital-it.ch/software/BOX_form.html).

5.5. Sample collection for gene expression studies

For all qRT-PCR experiments mentioned below, samples were prepared from three biological replicates.

5.5.1. Natural variation for adventitious rooting

The whole main stem of six-week old Paj, *pep1-1*, Dor, Tot and Wca from 7 plants excluding the leaves, petioles and buds was collected in liquid nitrogen.

5.5.2. Auxin-treated 12-week vernalized Paj plants

For samples generated from auxin-treated 12-week vernalized plants, the rooting and the non-rooting zones were collected, excluding the leaves, petioles and branches, before auxin spray, 6, 24, 72 and 120 h after mock/ auxin spray. The first two internodes underneath the compact zone

were marked as the rooting zone and the two internodes adjacent to this were marked as the non-rooting zone. Tissue harvested from 10 plants per time point and treatment were collected in liquid nitrogen.

5.5.3. Extended exposure to vernalization

The rooting and the non-rooting zone samples collected from plants exposed to extended vernalization were 2 cm each. The rooting and the non-rooting zones collected from plants vernalized for 4, 8, 12, 16 and 21 weeks at the end of vernalization and 5 days after vernalization. Tissue harvested from 10 plants per time point and treatment were collected in liquid nitrogen.

5.6. RNA isolation and cDNA synthesis

The samples were ground to powder using a mortar and pestle and ~80 mg of each powdered sample was used for RNA extraction. Total mRNA was extracted using a QIAGEN RNeasy Plant Mini Kit and DNA was removed using Invitrogen DNA-*free* DNA removal kit, according to the protocol provided by the manufacturer. The RNA concentration and integrity were determined using a NanoDrop ND-1000 UV-Vis spectrophotometer (Thermo Fischer Scientific). For expression analysis, first strand cDNA was synthesized using 2µg RNA using oligo dT primer (18b) along with SuperScript III Reverse Transcriptase kit according to the manufacturer's instructions. The cDNA samples were diluted with 110 µL deionized water.

5.7. Real-Time RT-PCR experiments and data analyses

The abundance of transcripts was quantified by real-time RT-PCR based on three biological replicates, each with 3 technical replicates. Each 20-µL reaction comprised 3 µL cDNA, 10 µL iQ SYBR Green Supermix (Bio-Rad) and 125 nM forward and reverse primer. A CFX Connect Real Time PCR Detection System (Bio-Rad) was used to determine the Ct values. Each reaction was heated to 95°C for 3 min, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The samples were heated from 55°C to 95°C at a rate of 0.1°C/s in increments of 0.5°C for melt curve analysis.

The *PP2A* gene homolog from *A. alpina* did not show any changes in expression in response to auxin in *A. alpina*, and thus was used as the house-keeping gene for normalisation. Data were analysed using the ΔCt (cycle threshold) method and presented as the mean and standard deviation

of three biological replicates. The gene expression was calculated by using the following formula: $2^{((Ct_{GOI} - Ct_R)}$, where Ct_{GOI} is the Ct of the gene of interest and Ct_R is the Ct of the reference house-keeping gene. Data were normalized relative to the expression level in the non-rooting zone at the end of vernalization.

5.8. Statistical analyses

Student's t-test was used to get significant values in Figures 2-6, 2-8, 2-20 and 2-25. A one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc-test with Bonferroni correction was used to get the values significantly different ($P < 0.05$) for the qPCR and endogenous IAA quantification (Figure 2-2). These tests were carried out in R (Version 3.4.3).

Multifactorial ANOVA combined with post-hoc Bonferroni corrections were carried out for data in Figures 2-7 and 2-9 to determine significantly regulated samples (corrected $P < 0.05$) predicted by the genotype. For Figure 2-7, the statistical model included genotype, age, concentration of auxin and their interactions as fixed effects. The statistical model for Figure 2-9 consisted of the genotype, concentration of auxin and their interaction as fixed effects.

5.9. Free IAA quantification

Plant material (around 15 mg fresh weight) was purified as previously described (Andersen et al., 2008). 500 pg 13C6-IAA (Indole-3-acetic acid) internal standard was added to each sample before homogenisation and extraction. Free IAA was quantified in the purified samples using combined gas chromatography - tandem mass spectrometry. The mean and the standard deviation represent three biological replicates.

5.10. Histological analyses

The histological analysis was done by Dr. Alice Vayssières in collaboration with Dr. Ulla Neumann (MPIPZ). The samples collected were the rooting zone at the end of vernalization and 5 days after auxin spray. For light microscopy analysis, samples were fixed in 2.5 % glutaraldehyde and 2 % paraformaldehyde in 0.05 M sodium cacodylate buffer, pH 6.9, for 2 hrs at room temperature followed by an overnight incubation at 4°C. Subsequently, samples were rinsed six times for 10 minutes in 0.05 M sodium cacodylate buffer (pH 6.9, rinse 3 and 4 supplemented with

0.05 M glycine) and postfixed in 0.5% osmium tetroxide in 0.05M sodium cacodylate (pH 6.9) supplemented with 0.15% potassium ferricyanide, for 1 h on ice. After thorough rinsing in 0.05 M sodium cacodylate buffer (pH 6.9) and water, samples were further dehydrated with a series of ethanol, gradually transferred to acetone and embedded into Araldite 502/Embed 812 resin (EMS, catalog number 13940) using the ultrarapid infiltration by centrifugation method revisited by McDonald (2014) [267]. For bright field observation, transverse semithin sections (1 µm) of stem segments carrying adventitious roots were collected on glass slides, stained with 1% aqueous toluidine blue supplemented with 1% sodium tetraborate, and mounted permanently in Araldite 502/Embed 812 resin [268].

5.11. Library preparation and RNA sequencing

The purified RNA samples were sent to the Max Planck Genome Center, Cologne, Germany, for library preparation and sequencing (https://mpgc.mpipz.mpg.de/home/). Briefly, 1 µg of total RNA was enriched for polyA RNA using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs), followed by library preparation using the NEBNext Ultra Directional II RNA Library Prep kit (New England Biolabs). RNA quality and quantity were monitored throughout by capillary electrophoresis (TapeStation, Agilent Technologies) and fluorometry (Qubit, Thermo Fisher Scientific). Sequencing was performed on a HiSeq3000 device (Illumina) generating 150-bp single-end reads. Before further processing, the sequencing data quality was verified using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/).

5.12. Differential gene expression analysis

5.12.1. Effect of auxin spray on 12-week vernalized Paj plants

Reads derived from the Illumina library were aligned using TopHat with Bowtie as its algorithmic core [269]. Cuffdiff was used to identify differentially expressed genes and determine their expression levels from the mapped reads under all conditions. The expression of selected genes was validated by quantitative RT-PCR.

The differential expression among samples was analysed in more detail using the *R* package "cummeRbund" [269]. Taking all samples into account, we mapped a total of 30,690 *A. alpina* genes

to the reference genome. The quality of the samples was assessed by producing dispersion plots among replicates. Reads for 12,612 unique genes showing more than a 2-fold change in expression level, and a corrected p-value below 0.05, were selected for further analysis.

5.12.2. Effect of extended vernalization on Paj plants

Reads derived from the Illumina library were mapped and aligned to the reference genome using HISAT2 followed by assembly and quantification of expression levels in different samples using STRINGTIE. The gene counts of all samples were obtained by using a Python script. The quality of the samples was assessed by producing dispersion plots among replicates. The differentially expressed genes with more than 2-fold change and a corrected p-value below 0.05 were obtained using DESeq2 and selected for further analysis.

5.13. KEGG pathway and GO analysis

Differentially expressed *A. alpina* genes with homologs in *A. thaliana* were used as input data for the KEGG (Kyoto Encyclopedia of Genes and Genomes) Mapper (https://www.kegg.jp/kegg/tool/map_pathway1.html) [270]. For the bar chart, the KEGG pathways shown have more than 1% of the total genes identified by the KEGG Mapper. The outcome of KEGG analysis is presented Supplementary Table 10.

Gene Ontology enrichment was carried out using DAVID v6.8 online [271,272]. DAVID is a functional annotation tool that finds enriched biological terms within a list of genes. As above, *A. alpina* genes with homologs in *A. thaliana* were used as the input data. The overrepresented GO terms were selected based on fold enrichment and a p-value below 0.05. The GO terms were used to find the fold enrichment of groups of genes overrepresented in each sample or category.

5.14. Bubble chart for GO enrichment

The *R*-based graphical tool BACA (Bubble chArt to Compare Annotations) was used to visualize the GO enrichment results generated using DAVID [272,273]. A bubble chart was generated summarizing the enriched GO terms ($p < 0.05$) among the modulated genes in different samples. The size of each bubble, representing a GO term, represents the number of genes in a list of differentially expressed genes associated with the GO term, and the colour indicated the direction

of modulation (green = downregulation, magenta = upregulation. A minimum of five genes was required for the GO term to be considered for further analysis.

5.15. Venn diagram

The Venn diagrams were created using Venny v2.1.0 (http://bioinfogp.cnb.csic.es/tools/venny/) [274].

5.16. Co-expression analysis

Genes sharing similar expression profiles were identified using CLUSTER3.0 [275]. Heat-maps were generated for the genes passing this threshold using TREEVIEW based on the values obtained from CLUSTER3.0 [276]. The clusters were divided based on visual inspection of the whole heat-map showing the most similarity in distribution. The parameters of gene selection to create the heat map are mentioned in the respective figure legends. The genes and corresponding GO terms in the clusters representing the most interesting expression profiles were further analysed.

5.17. Generation of transgenic lines of *miR160*, *miR167* and *DR5::GUS*

The *DR5::GUS* plasmid was obtained from Dr. Thomas Guilfoyle (University of Missouri). It was modified by Dr. Alice Vayssières to include the BASTA selection marker. *35S::MIM160* and *35S::MIM167* mimicry plasmids were obtained from The Nottingham Arabidopsis Stock Centre (NASC), *35S::miR160c* and *35S::miR167a* plasmids were obtained from Prof. X. Y. Chen (Shanghai Institutes for Biological Sciences) and Prof. Jason W. Reed (Department of Biology, University of North Carolina at Chapel Hill), respectively. The plasmids *35S::MIM160*, *35S::MIM167* and *35S::MIR160c* were transferred into *Agrobacterium tumifaciens* strain *GV3101*. To achieve this, 250ng plasmid DNA was added to a 50 µL aliquot of competent *A. tumificiens* cells. The mix was treated to an electric pulse of 2.2 kV followed by immediate addition of 600µL LB medium. The mix was incubated for 2 hours at 28°C with shaking, followed by plating on LB agar plates with appropriate anti-biotics which were incubated at 28°C for 72 hours. The selection of colonies transformed with the plasmid of interest was verified by colony PCR.

Agrobacterium based transformation protocol optimized for *A. alpina* was used for introducing the plasmids into both Paj and *pep1-1*. Transformation was carried out by growing *Agrobacterium* carrying the desired constructs overnight at 28°C in 1 L LB medium containing required antibiotics. When the optical density (OD) at 600 nm reached 1.0, cells were harvested by centrifugation (6000rpm, 10 mins). Obtained pellets after centrifugation were re-suspended in 1L transformation buffer (50 g Sucrose, 500 µL Silwet-L77 maintained at pH5.7). Attempts to transform plants were carried out using the floral dipping method, whereby flower buds were dipped into the transformation buffer for two minutes. The plants were then placed horizontally on plastic trays and incubated for approximately 24 hours in absolute dark. Plants were subsequently grown in LD until seeds were ready for collection. Plants carrying the plasmid were selected on soil based on their resistance to BASTA (Bayer), applied by spraying.

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7. SUPPLEMENTARY INFORMATION

7.1. Figures

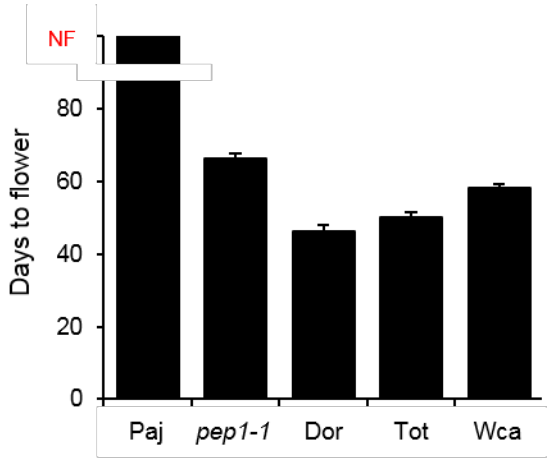

Figure 7-1. **Number of days to flower for the *A. alpina* accessions and the *pep1-1* mutant in long days**.
Paj did not flower during the course of the experiment in the absence of vernalization. NF represents 'not flowered'. Nine plants of each accession/mutant were characterized in this study and the error bar is represents the standard deviation.

A


Paj ATGACCGTTGATTTAGCTCTACACTCTCCGATGATCCACTCACCGTCCGATAAGGATTTGAAGGCTCTCAGGTTCATCGAGGAGATGACACGTAACGTCGACTTCGTTCAGAGAAAAGTC
Dor ATGACCGTTGATTTAGCTCTACACTCTCCGATGATCCACTCACCGTCCGATAAGGATTTGAAGGCTCTCAGGTTCATCGAGGAGATGACACGTAACGTCGACTTCGTTCAGAGAAAAGTC
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Wca ATTAGGGAGATACTTAGTCGTAACTCTGAGACTGAGTACCTGAAACGATACGGTCTTAAGGGATTCACTGACCGGAAAACATTTAAGATCAAAGTTCCGGTTATTACGTATGAAGATCTT
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Wca CTTCAAACCAATTGGAAAGAACTCGCCAGCAATATCTCCACCGGAACCCTAAGCTCGAGAATCTCTGATCCGGCTATTAGAGAGAGCATGTCCAAGATCTTGACCAAACCGGACCAAGAG
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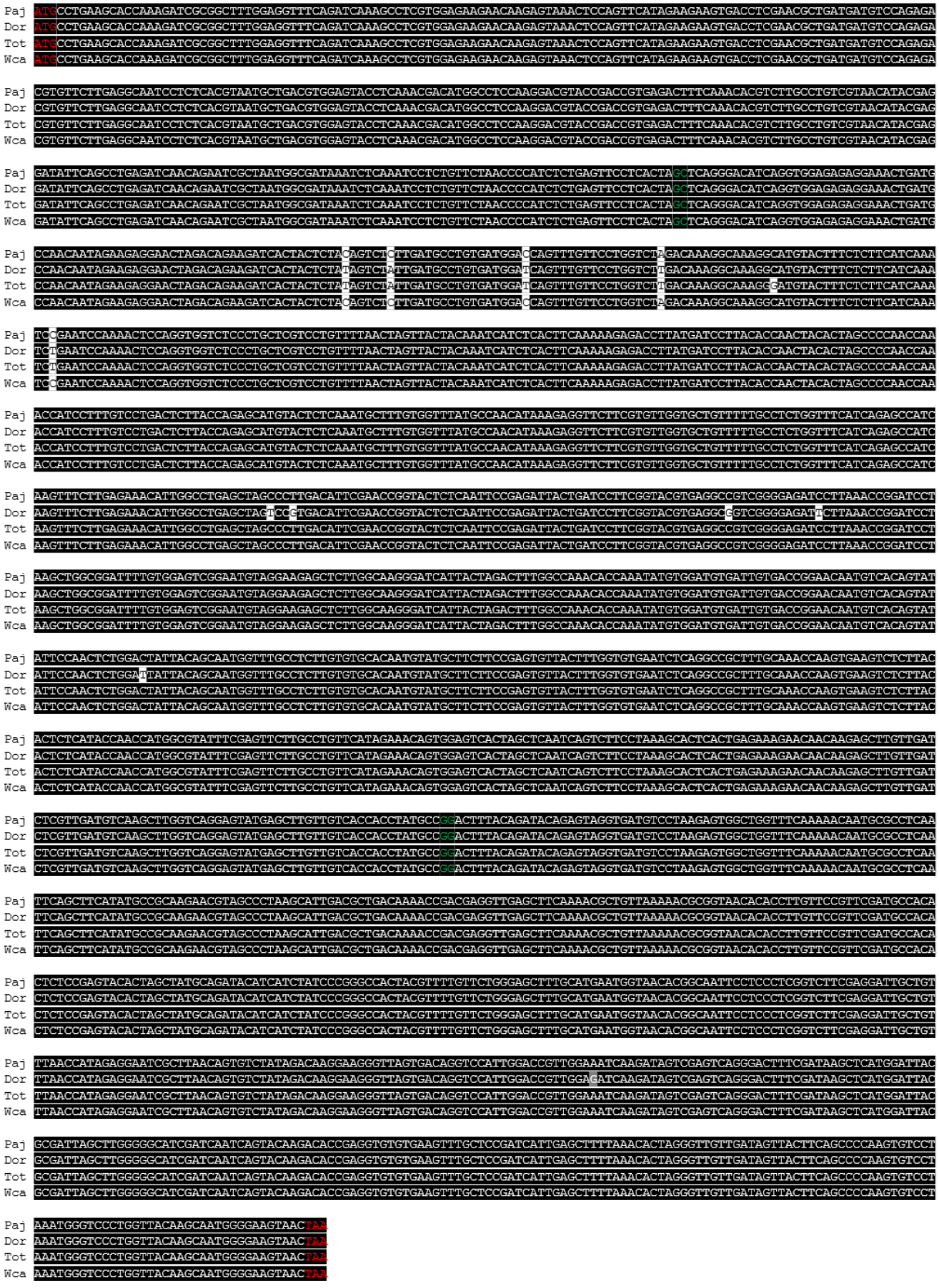
B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Tot AAATGGGTCCCTGGTTACAAGCAATGGGGAAGTAACTAA
Wca AAATGGGTCCCTGGTTACAAGCAATGGGGAAGTAACTAA

Figure 7-2. **Multiple sequence alignment of (A) *GH3.3* and (B) *GH3.6* in Paj, Dor, Tot and Wca.**
The cDNA sequences were aligned by Clustal Omega multiple alignment program with default parameters. The BOXSHADE server with default parameters was used for showing the conserved bases. Black represents conserved base in all the sequences, gray shading is for more than 50% conserved sequence and no shading represents no conserved sequence. Red font represents the start codon (ATG) and the stop codon (TAA). The splice junctions are denoted in green font colour.

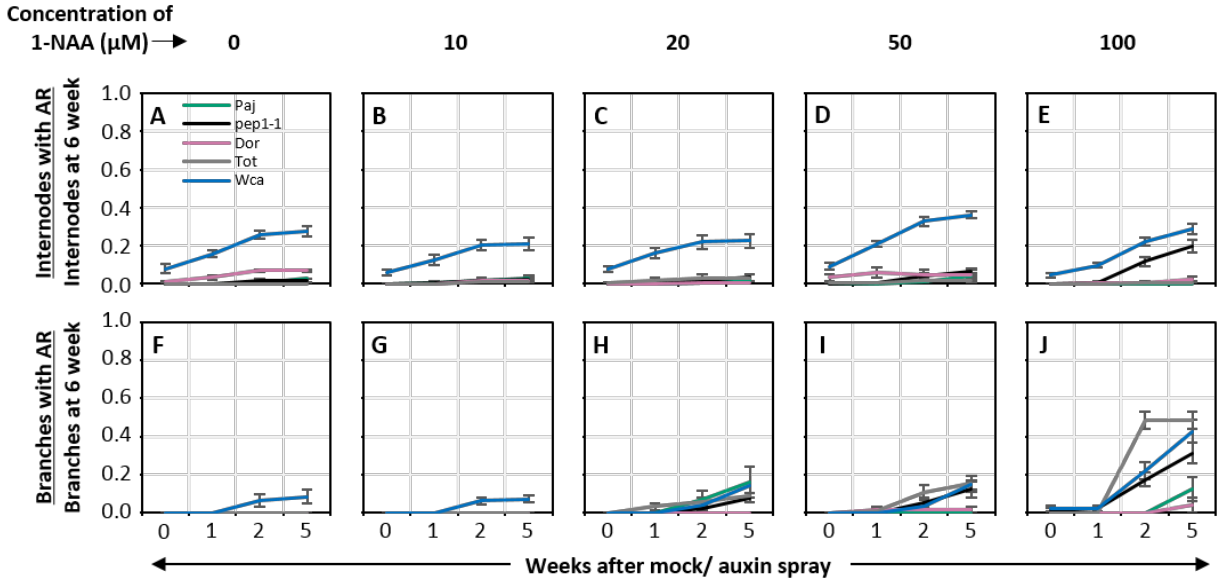

Figure 7-3. **Auxin spray induces adventitious roots in a dosage dependent manner in *A. alpina* accessions and *pep1-1*.**
Proportion of **(A-E)** internodes and **(F-J)** branches with adventitious roots after the application of 0, 10, 20, 50 and 100 µM 1-NAA relative to before spray in six-week old Paj, *pep1-1*, Dor, Tot and Wca plants. Plants were scored before spray and 1, 2 and 5 weeks after spray. Nine plants were characterized for each accession/mutant for each treatment. Statistical analyses are presented in Supplementary Table 5.

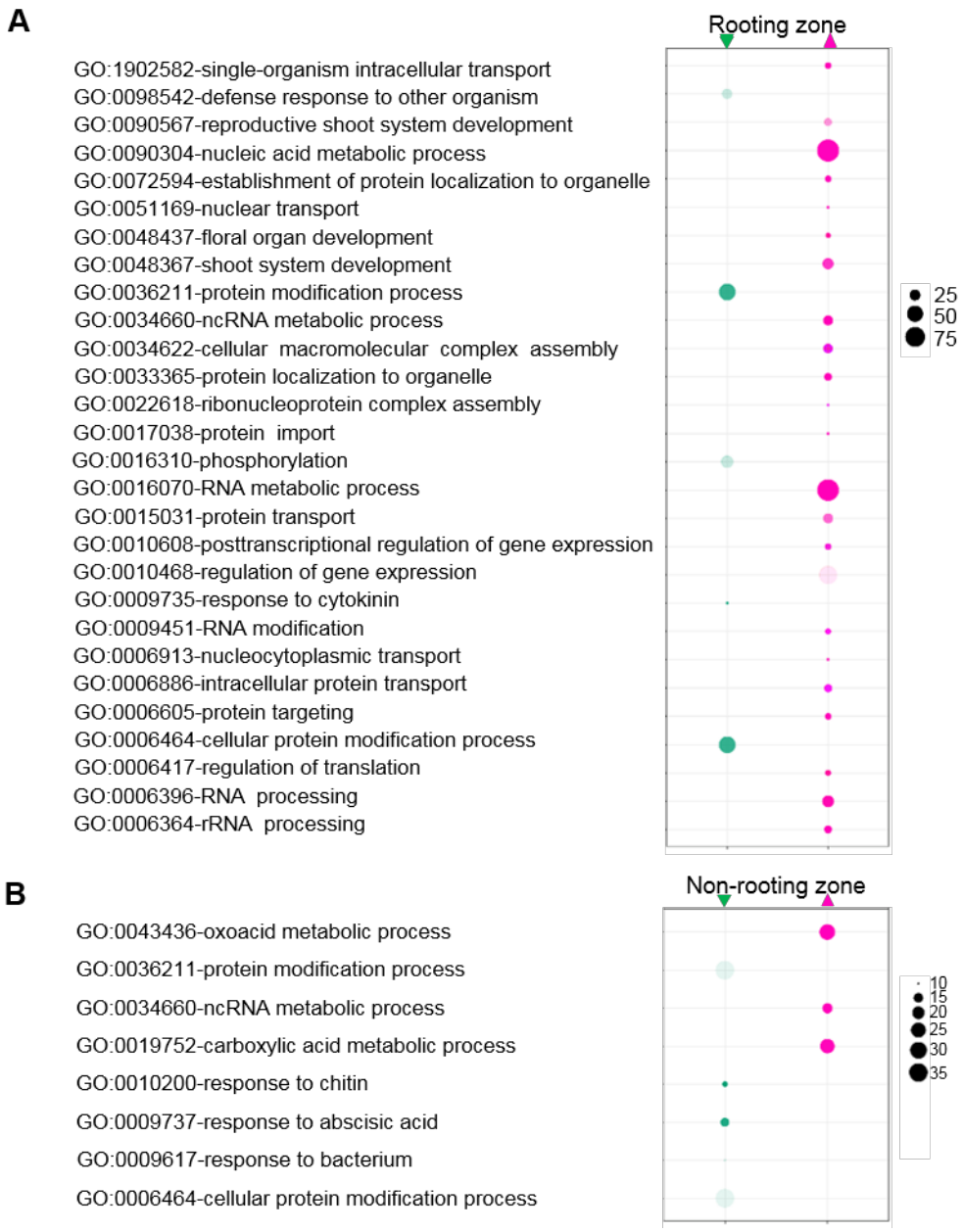

Figure 7-4. **Enriched GO terms 6 hours after auxin spray.**
GO terms enriched in the list of genes specifically regulated in the rooting (A) and the non-rooting (B) zone 6 hours after spray. The bubble chart was generated using the R package 'BACA'. A minimum of 10 genes were required for a GO term to be considered as enriched. Magenta and green represent upregulated and downregulated genes respectively.

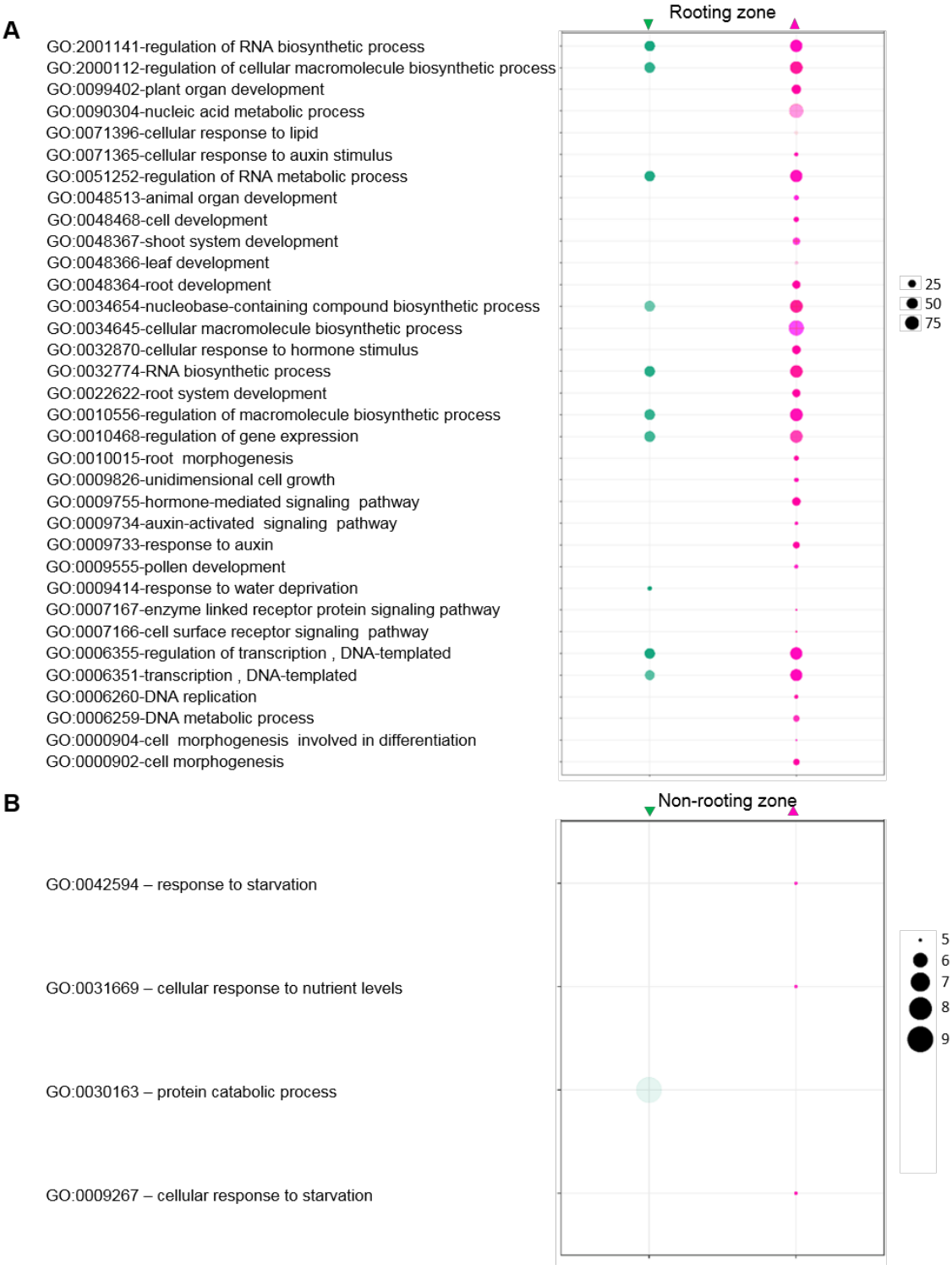

Figure 7-5. **Enriched GO terms 24 hours after auxin spray.**
GO terms enriched in the list of genes specifically regulated in the rooting (A) and the non-rooting (B) zone 24 hours after spray. The bubble chart was generated using the R package 'BACA'. A minimum of 10 and 5 genes were required for a GO term to be considered as enriched in the rooting and the non-rooting, respectively. Magenta and green represent upregulated and downregulated genes respectively.

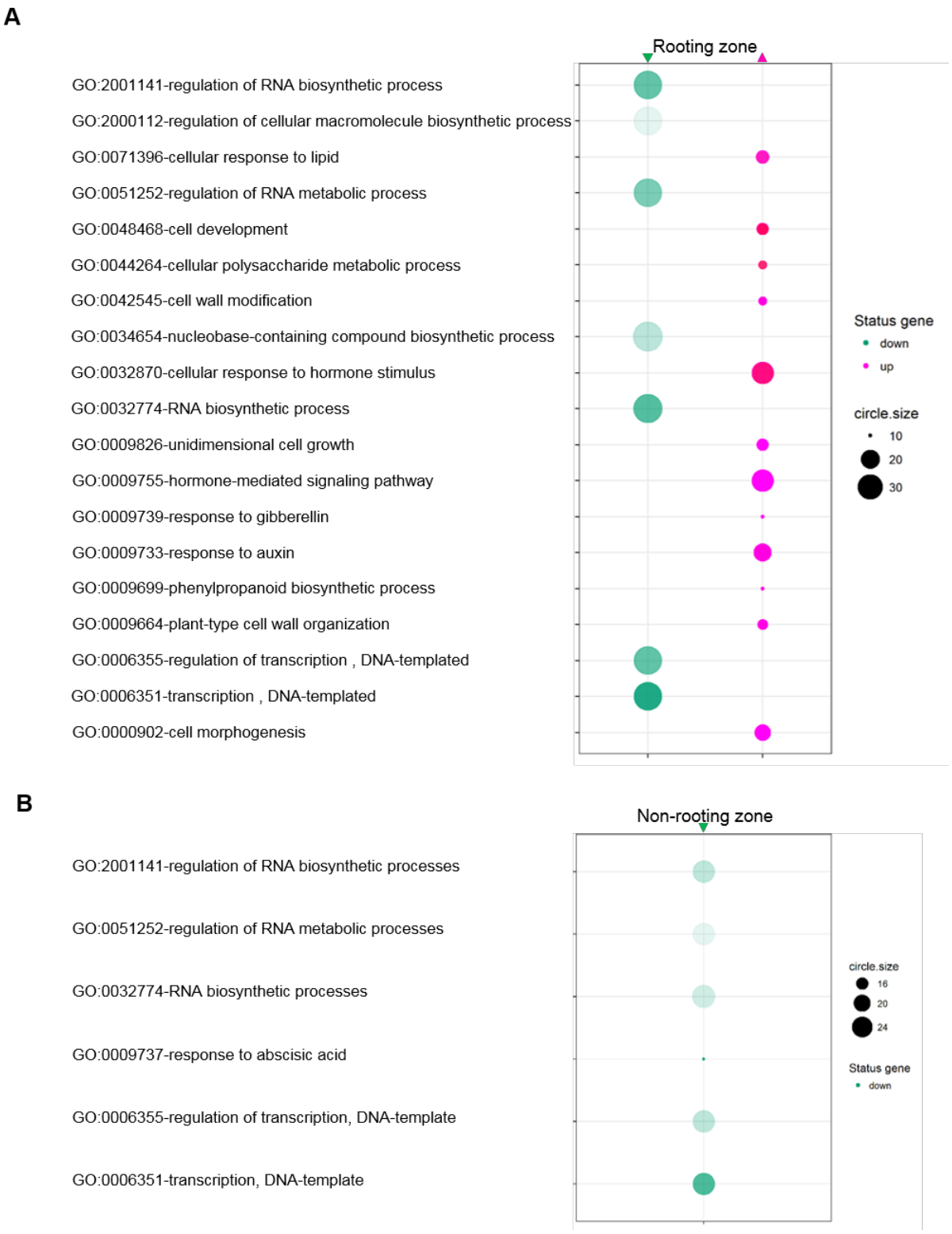

Figure 7-6. **Enriched GO terms 72 hours after auxin spray.**
GO terms enriched in the list of genes specifically regulated in the rooting (A) and the non-rooting (B) zone 72 hours after spray. The bubble chart was generated using the R package 'BACA'. A minimum of 10 genes was required for a GO term to be considered as enriched. Magenta and green represent upregulated and downregulated genes respectively.

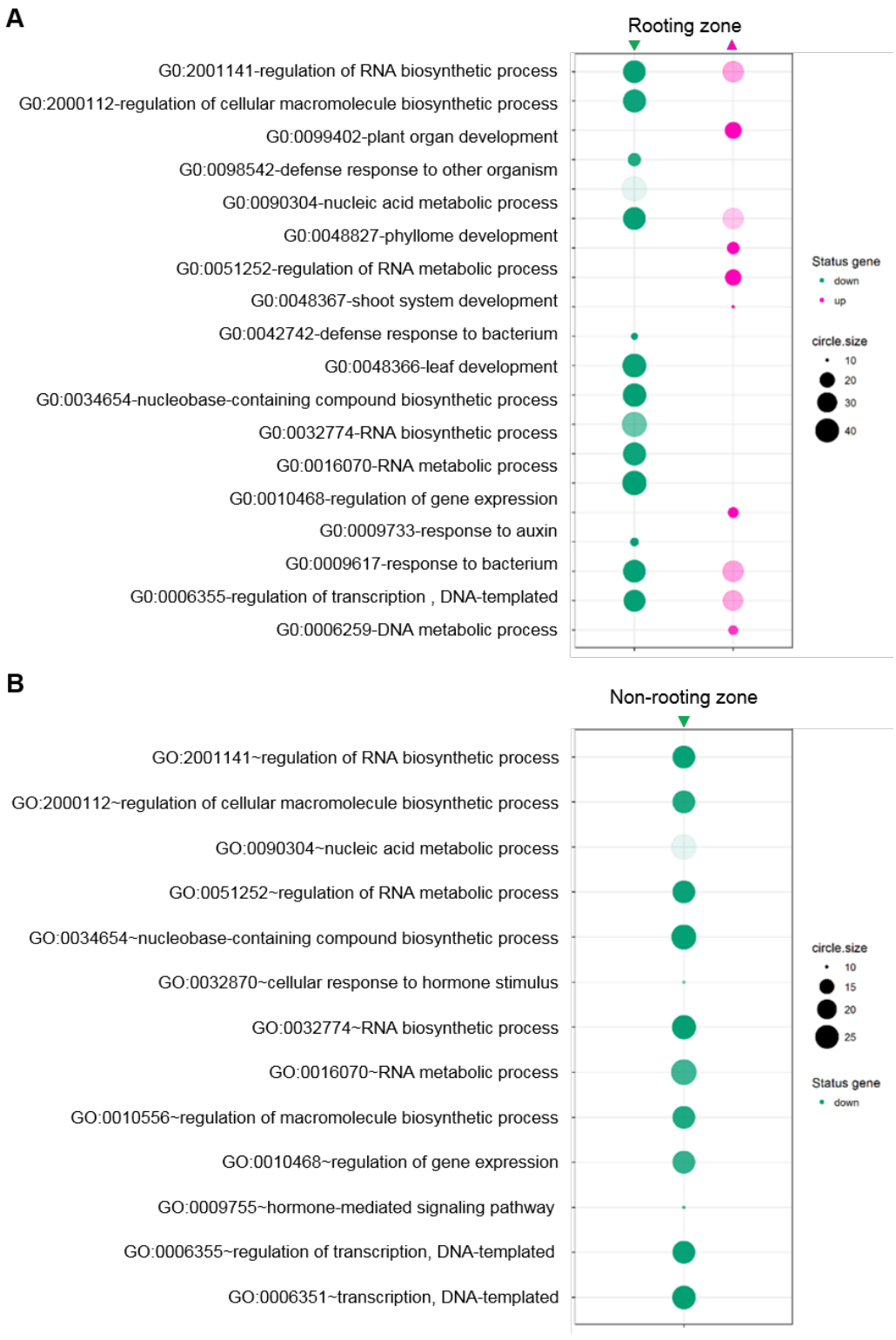

Figure 7-7. **Enriched GO terms 120 hours after auxin spray.**

GO terms enriched in the list of genes specifically regulated in the rooting (A) and the non-rooting (B) zone 120 hours after spray. The bubble chart was generated using the R package 'BACA'. A minimum of 10 genes was required for a GO term to be considered as enriched. Magenta and green represent upregulated and downregulated genes respectively.

Figure 7-8. **Co-expression profiles of all sub-clusters.**
Expression profile of DEGs clustered into different sub-clusters based on similar expression pattern. The average normalized expression pattern of genes generated by Cluster3.0 is represented in the sub-clusters of each cluster; clusters are highlighted as Cluster I – Blue, Cluster II – Orange, Cluster III – Yellow and Cluster IV – Green. The patterns depict the mock treated rooting (dashed orange line) and the non-rooting zone (Dashed black line), and the auxin treated rooting (orange line) and non-rooting zone (black line).

7.2. Tables

Supplementary Table 1. Significance test for homolog of *GH3.3* in A. alpina. Summary data for an ANOVA and the following Tukey's multiple comparison post-hoc test for pairwise comparison of the expression of the homolog of *GH3.3* in A. alpina ecotypes and the *pep1-1* mutant. The values with red font denote significant values of p-value<0.05.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Name	4	822.3	205.59	72.1	2.47E-07***
Residuals	10	28.5	2.85	-	-

***p<0.001, **p<0.01, *p<0.05

Supplementary Table 2. Significance test for homolog of *GH3.6* in *A. alpina*. Summary data for an ANOVA and the following Tukey's multiple comparison post-hoc test for pairwise comparison of the expression of the homolog of *GH3.6* in *A. alpina* ecotypes and the *pep1-1* mutant. The values with red font denote significant values of p-value<0.05.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Name	4	2.9374	0.7344	51.68	1.21E-06***
Residuals	10	0.1421	0.0142	-	-

***p<0.001, **p<0.01, *p<0.05

Supplementary Table 3. Significance test for endogenous IAA levels in *A. alpina*. Summary data for an ANOVA and the following Tukey's multiple comparison post-hoc test for pairwise comparison of the levels of free endogenous IAA levels in the main stem of 6-week-old *A. alpina* ecotypes and the *pep1-1* mutant.

	Df	**Sum Sq**	**Mean Sq**	**F value**	**Pr(>F)**
Ecotype	4	9407	2352	1.51	0.27
Residuals	10	15593	1559	-	-

***p<0.001, **p<0.01, *p<0.05

Supplementary Table 4. Statistical models (ANOVA) describing the relationship between genotype (Paj and *pep1-1*), age (6- and 8-week old) and auxin concentration (0, 10, 20, 50 and 100 μM) and response variable representing adventitious rooting on branches and the main stem in Figure 2-7.

Response Variable		df	*F*	*p*	Partial eta^2	Adjusted R^2
Adventitious rooting on branches	Model	19, 177	36.77	<0.001		0.78
	Intercept	1	761.52	<0.001	0.81	
	Genotype	1	5.33	0.02	0.03	
	Age	1	245.09	<0.001	0.58	
	Auxin	4	86.7	<0.001	0.66	
	Genotype*Age	1	9.14	0.003	0.05	
	Genotype*Auxin	4	5.26	0.001	0.11	
	Age*Auxin	4	13.11	<0.001	0.23	
	Genotype*Age*Auxin	4	3.33	0.01	0.07	
Adventitious rooting on main stem	Model	19, 177	17.84	<0.001		0.62
	Intercept	1	304.27	<0.001	0.63	
	Genotype	1	23.79	<0.001	0.12	
	Age	1	43.63	<0.001	0.2	
	Auxin	4	53.59	<0.001	0.55	
	Genotype*Age	1	2.34	0.13	0.01	
	Genotype*Auxin	4	5.75	<0.001	0.12	
	Age*Auxin	4	2.05	0.09	0.04	
	Genotype*Age*Auxin	4	5.95	<0.001	0.12	

Supplementary Table 5. Pairwise comparisons within genotypes, age and auxin concentration for adventitious rooting on branches and the main stem in Figure 2-7.

Variable	Factor	Pairwise comparison	p
Adventitious rooting on branches	Genotype	Paj - *pep1-1*	0.00
	Age	6 week - 8 week	0.00
	Auxin Concentration	0 µM - 10 µM	1.00
		0 µM - 20 µM	0.57
		0 µM - 50 µM	0.00
		0 µM - 100 µM	0.00
		10 µM - 20 µM	0.43
		10 µM - 50 µM	0.00
		10 µM - 100 µM	0.00
		20 µM - 50 µM	0.00
		20 µM - 100 µM	0.00
		50 µM - 100 µM	0.00
Adventitious rooting on main stem	Genotype	Paj - *pep1-1*	0.02
	Age	6 week - 8 week	0.00
	Auxin Concentration	0 µM - 10 µM	0.00
		0 µM - 20 µM	0.00
		0 µM - 50 µM	0.00
		0 µM - 100 µM	0.00
		10 µM - 20 µM	0.32
		10 µM - 50 µM	0.00
		10 µM - 100 µM	0.00
		20 µM - 50 µM	0.00
		20 µM - 100 µM	0.00
		50 µM - 100 µM	0.00

Supplementary Table 6. Statistical models (ANOVA) describing the relationship between genotype (Paj, Dor, Tot, Wca and *pep1-1*) and auxin concentration (0, 10, 20, 50 and 100 μM) and response variable representing adventitious rooting on branches and the main stem in Figure 2-9.

Response Variable		**df**	***F***	***p***	**Partial eta^2**	**Adjusted R^2**
Adventitious rooting on branches	Model	24, 470	11.06	<0.001		0.33
	Intercept	1	460.42	<0.001	0.5	
	Genotype	4	20.28	<0.001	0.15	
	Concentration	4	33.88	<0.001	0.22	
	Genotype*Concentration	16	2.15	0.006	0.07	
Adventitious rooting on main stem	Model	24, 470	11.06	<0.001		0.59
	Intercept	1	460.42	<0.001	0.55	
	Genotype	4	20.28	<0.001	0.57	
	Concentration	4	33.88	<0.001	0.06	
	Genotype*Concentration	16	2.15	0.006	0.13	

Supplementary Table 7. Pairwise comparisons within genotypes, age and auxin concentration for adventitious rooting on branches and the main stem in Figure 2-9.

Variable	Factor	Pairwise comparison	p
Adventitious rooting on branches	Genotype	Paj - *pep1-1*	0.67
		Paj - Dor	0.00
		Paj - Tot	0.03
		Paj - Wca	0.00
		pep1-1 - Dor	0.00
		pep1-1 - Tot	1.00
		pep1-1 - Wca	0.85
		Dor - Tot	0.00
		Dor - Wca	0.00
		Tot - Wca	1.00
	Auxin Concentration	0 µM - 10 µM	0.00
		0 µM - 20 µM	0.00
		0 µM - 50 µM	0.00
		0 µM - 100 µM	0.00
		10 µM - 20 µM	0.20
		10 µM - 50 µM	0.01
		10 µM - 100 µM	0.00
		20 µM - 50 µM	1.00
		20 µM - 100 µM	0.00
		50 µM - 100 µM	0.03
Adventitious rooting on main stem	Genotype	Paj - *pep1-1*	0.00
		Paj - Dor	0.00
		Paj - Tot	0.00
		Paj - Wca	0.00
		pep1-1 - Dor	0.00
		pep1-1 - Tot	0.00
		pep1-1 - Wca	0.00
		Dor - Tot	0.00
		Dor - Wca	0.00
		Tot - Wca	0.00
	Auxin Concentration	0 µM - 10 µM	1.00
		0 µM - 20 µM	0.59
		0 µM - 50 µM	0.01
		0 µM - 100 µM	0.00
		10 µM - 20 µM	1.00
		10 µM - 50 µM	0.11
		10 µM - 100 µM	0.00
		20 µM - 50 µM	1.00
		20 µM - 100 µM	0.03
		50 µM - 100 µM	1.00

Supplementary Table 8. Characterisation of mutants obtained from EMS mutagenesis screen. The mutants are categorised as Others (Mutants identified before auxin spray), No AR (No adventitious roots produced after auxin spray) and No AR (+) (Mutants identified before auxin spray but produced no adventitious roots after auxin spray.

Pool	Features	Group
1	Reduced growth	Others
1	Reduced growth	Others
1	Reduced growth	Others
1	Reduced growth	Others
1	Reduced growth	Others
2	Leaf shape	Others
2	Adventitious rooting only on branches	No AR (+)
8	No adventitious rooting	No AR
8	No adventitious rooting	No AR
9	Leaf shape	Others
13	Delayed adventitious rooting; Aborted adventitious rooting; Reduced growth	No AR (+)
15	Delayed branching	Others
17	Thick stem; Reduced height; No adventitious rooting; Multiple branches	No AR (+)
17	Thick stem; Reduced height; No adventitious rooting; Multiple branches	No AR (+)
17	Thick stem; Reduced height; No adventitious rooting; Multiple branches	No AR (+)
19	Dark green leaves, Reduced growth plant	Others
21	Reduced height	Others
24	Reduced height, Reduced apical dominance	Others
26	Reduced growth; Leaf shape; Leaf color	Others
34	No adventitious rooting	No AR
36	Delayed branching	Others
37	Reduced growth	Others
37	Reduced growth	Others
38	Reduced branching	Others
38	Reduced branching	Others
43	Delayed adventitious rooting; Leaf shape	No AR (+)
52	Reduced growth	Others
56	Reduced height; Leaf shape	Others
57	Reduced growth	Others
70	Reduced height	Others
71	Reduced growth; Leaf shape	Others
73	No adventitious rooting	No AR
76	Reduced growth; Early senescence	Others
76	No adventitious rooting	No AR
77	Reduced growth; compact; curled leaves	Others
78	Reduced growth	Others
78	Reduced height	Others
79	Reduced growth	Others
79	Reduced height; Senescence	Others
79	No adventitious rooting	No AR
79	No adventitious rooting	No AR
80	AR only on branches	No AR (+)
80	No adventitious rooting	No AR
83	Adventitious rooting only on branches	No AR (+)
83	Adventitious rooting only on branches	No AR (+)
87	Delayed branching	Others

Pool	Features	Group
87	Reduced height; No adventitious rooting; Leaf shape	No AR (+)
90	Long main stem; Greenish yellow plant; Delayed branching	Others
90	Long main stem; Greenish yellow plant; Delayed branching	Others
90	Long main stem; Greenish yellow plant; Delayed branching	Others
90	Long main stem; Greenish yellow plant; Delayed branching	Others
90	Delayed adventitious rooting; Long main stem; Reduced branching; Visible leaf vein	No AR (+)
96	Delayed branching	Others
96	Reduced height	Others
96	Reduced height	Others
97	No adventitious rooting	No AR
100	No adventitious rooting	No AR
111	Reduced growth; Leaf shape	Others
112	adventitious rooting only on branches	No AR (+)
113	Delayed adventitious rooting; Reduced branching	No AR (+)
114	Reduced height; Thick stem; Leaf curling; Leaf shape	Others
115	Reduced growth	Others
115	Adventitious rooting only on branches; Thick stem	No AR (+)
115	No adventitious rooting; Delayed branching	No AR (+)
115	No adventitious rooting; Delayed branching	No AR (+)
115	Reduced branching; Leaf shape; No adventitious rooting	No AR (+)
115	No adventitious rooting	No AR
115	No adventitious rooting	No AR
120	Whitish leaves; Reduced growth	Others
121	Dark green leaves, Reduced growth plant	Others
121	AR only on branches; Thick stem	No AR (+)
122	Reduced branching	Others
122	Reduced growth	Others
123	Branching pattern; Leaf shape; Structure	Others
123	No adventitious rooting	No AR
125	Delayed branching	Others
125	Delayed branching	Others
126	Reduced growth; Curled leaves	Others
126	No adventitious rooting	No AR
127	Reduced height	Others
130	No adventitious rooting; Reduced branching	No AR (+)
130	adventitious rooting only on branches	No AR (+)
130	adventitious rooting only on branches	No AR (+)
130	Aborted adventitious rooting	No AR (+)
130	No adventitious rooting	No AR
132	Reduced height	Others
132	Reduced height	Others
132	Reduced height	Others
132	Delayed branching; Reduced growth; Leaf shape	Others
133	Reduced height	Others
133	Reduced height	Others
133	Reduced height	Others
133	Reduced height	Others
134	Reduced branching	Others
134	Reduced branching	Others
134	adventitious rooting only on branches	No AR (+)

Pool	Features	Group
135	Long internodes; Yellowish plant	Others
135	Long internodes; Visible leaf veins	Others
137	Reduced growth (few leaves)	Others
138	Yellow leaves; Reduced growth	Others
138	Yellow leaves; Reduced growth	Others
140	Delayed branching	Others
141	Delayed branching	Others
141	Delayed branching	Others
141	Aborted adventitious rooting	No AR (+)
142	Delayed branching; Visible leaf vein	Others
144	No adventitious rooting; Delayed branching; Reduced growth	No AR (+)
145	Delayed branching; Curled leaves	Others
147	Reduced height	Others
148	Reduced branching	Others
148	Reduced growth; Curled leaves	Others
148	Reduced growth; Curled leaves	Others
149	Reduced height	Others
149	Reduced height	Others
149	Reduced height	Others
149	Reduced height	Others
149	Reduced height	Others
149	SAM absent	Others
149	SAM absent	Others
151	Reduced growth; Leaf shape	Others
151	Long internodes; Reduced growth	Others
151	No adventitious rooting	No AR
152	No adventitious rooting; 3 branches on one axil	No AR (+)
153	No adventitious rooting	No AR
153	No adventitious rooting	No AR
153	No adventitious rooting	No AR
153	No adventitious rooting	No AR
158	No adventitious rooting, Delayed branching	No AR (+)
160	No adventitious rooting	No AR
163	Reduced height, Bigger leaves	Others
163	Rosette, might not flower	Others
163	No adventitious rooting	No AR
164	No adventitious rooting	No AR
164(2)	No adventitious rooting, SAM Arrest	No AR (+)
167	No adventitious rooting	No AR
175	No adventitious rooting	No AR
176	No adventitious rooting	No AR
178	No adventitious rooting, Reduced branching	No AR (+)
184	No adventitious rooting, Reduced branching	No AR (+)
189	No adventitious rooting, Reduced branching	No AR (+)
212	Reduced growth	Others
217	No adventitious rooting	No AR
219	Small leaves, Accessory branching	Others
219(2)	Small leaves, Accessory branching	Others
220	No adventitious rooting, pointed serrations, SAM missing?	No AR (+)
221	No adventitious rooting	No AR

Pool	Features	Group
223	Reduced growth	Others
224	No branching	Others
225	No adventitious rooting, Reduced height, Cup-shaped leaves	No AR (+)
226	No adventitious rooting	No AR
233	No adventitious rooting	No AR
237	No adventitious rooting	No AR
243	Very small leaves	Others
245	Rosette, might not flower	Others
249	Rosette, accessory branches	Others
249	No adventitious rooting, Accessory branches	No AR (+)
250	No adventitious rooting	No AR
252	No adventitious rooting	No AR
252(2)	No adventitious rooting	No AR
253	Thin longer main stem, small serrated leaves	Others
256	No adventitious rooting	No AR
256(2)	No adventitious rooting	No AR
263	Reduced branching	Others
268	No adventitious rooting	No AR
274	Aborted AR, shorter internodes	No AR (+)
277	No adventitious rooting, thick stem, shorter internodes, broad leaves, late flowering	No AR (+)
277(2)	No adventitious rooting (could be delayed), narrow leaves, late flowering, delayed branching	No AR (+)
279	No adventitious rooting, thin stem, long internodes, delayed branching	No AR (+)
283	No adventitious rooting, late flowering	No AR (+)
292	No adventitious rooting, thin stem, long internodes, delayed branching	No AR (+)
296	No adventitious rooting	No AR
303	No adventitious rooting, shorter internodes, narrow leaves, late flowering	No AR (+)
307	No adventitious rooting	No AR
308	No adventitious rooting, thick stem, broad leaves, very delayed branching	No AR (+)
323	No adventitious rooting, narrow leaves, late flowering, delayed branching	No AR (+)
323(2)	No adventitious rooting, narrow leaves, late flowering, delayed branching	No AR (+)
336	No adventitious rooting, thin stem, late flowering, delayed branching	No AR (+)
339	No adventitious rooting, delayed branching	No AR (+)
343	No adventitious rooting, reduced growth, yellowish green	No AR (+)
350	No adventitious rooting, thin stem, fewer flowers per branch, narrow leaves	No AR (+)
354	No adventitious rooting, late flowering	No AR (+)
354(2)	No adventitious rooting, late flowering	No AR (+)

Supplementary Table 9. Table comparing mapped information for each sample in the experiment. Multiple alignment represents reads that were mapped to multiple regions in the genome. The name of the samples are in the form 'Time-Treatment-Zone-Replicate'. The time-points in this study include 'End of Vernalization' (EV), 6 hours (6h), 24 hours (24h), 72 hours (72h) and 120 hours (120h) after spray. Rooting and non-rooting zones are denoted as R and NR, respectively. The spray treatments are denoted as 0 (no spray), M (mock) and A (auxin, 1-NAA). Three biological replicates were used in this study.

Sample Name (Time-Treatment-Zone-Replicate)	Input	Mapped reads	Multiple alignment	% mapped reads	% multiple reads
EV-0-R-1	11754916	10560289	311019	89.84	2.65
EV-0-NR-1	11549989	10385994	285813	89.92	2.47
6h-M-R-1	13696923	12286487	347481	89.7	2.54
6h-M-NR-1	14388666	12946177	344675	89.97	2.4
24h-M-R-1	11691124	10374885	267410	88.74	2.29
24h-M-NR-1	13187951	12048256	324034	91.36	2.46
72h-M-R-1	13171642	11619453	303297	88.22	2.3
72h-M-NR-1	11604802	10462575	321078	90.16	2.77
120h-M-R-1	12323560	11219644	321475	91.04	2.61
120h-M-NR-1	11548574	10198040	277786	88.31	2.41
6h-A-R-1	13442716	12380022	312706	92.09	2.33
6h-A-NR-1	11735799	10695940	307189	91.14	2.62
24h-A-R-1	14779775	13354296	390986	90.36	2.65
24h-A-NR-1	12588738	11567883	306823	91.89	2.44
72h-A-R-1	12807607	11747286	269148	91.72	2.1
72h-A-NR-1	12811753	11613063	276905	90.64	2.16
120h-A-R-1	12172700	11144864	265312	91.56	2.18
120h-A-NR-1	13427646	12293161	304570	91.55	2.27
EV-0-R-2	13398468	12300977	327335	91.81	2.44
EV-0-NR-2	12868848	11829615	334596	91.92	2.6
6h-M-R-2	12161624	10946119	314715	90.01	2.59
6h-M-NR-2	13832270	12660206	368009	91.53	2.66
24h-M-R-2	11035043	10073486	291332	91.29	2.64
24h-M-NR-2	12810674	11596369	306632	90.52	2.39
72h-M-R-2	13250887	12162533	307428	91.79	2.32
72h-M-NR-2	12144415	9414671	610272	77.52	5.03
120h-M-R-2	13636415	12495640	350337	91.63	2.57
120h-M-NR-2	11402279	10190476	284226	89.37	2.49
6h-A-R-2	13721685	12374178	325935	90.18	2.38
6h-A-NR-2	13373281	12047329	319780	90.09	2.39
24h-A-R-2	10300015	9201674	213148	89.34	2.07
24h-A-NR-2	13448771	12065351	346780	89.71	2.58
72h-A-R-2	9926646	8935168	214073	90.01	2.16
72h-A-NR-2	10294342	9284842	230549	90.19	2.24
120h-A-R-2	9190653	8317304	199769	90.5	2.17
120h-A-NR-2	8808285	7893518	182819	89.61	2.08
EV-0-R-3	9723700	8752489	252584	90.01	2.6
EV-0-NR-3	13504931	12083520	325159	89.47	2.41
6h-M-R-3	9616880	8664892	234414	90.1	2.44
6h-M-NR-3	13596057	12050460	337824	88.63	2.48
24h-M-R-3	10590679	9369185	238207	88.47	2.25

Sample Name (Time-Treatment-Zone-Replicate)	Input	Mapped reads	Multiple alignment	% mapped reads	% multiple reads
24h-M-NR-3	13617524	12176810	318215	89.42	2.34
72h-M-R-3	10365706	9350962	234942	90.21	2.27
72h-M-NR-3	11425818	10112225	261916	88.5	2.29
120h-M-R-3	13166609	11913546	305655	90.48	2.32
120h-M-NR-3	10823432	9642743	248165	89.09	2.29
6h-A-R-3	21563640	16204706	469034	75.15	2.18
6h-A-NR-3	13590057	12308226	283278	90.57	2.08
24h-A-R-3	12963663	11523458	370177	88.89	2.86
24h-A-NR-3	13303171	11670590	302367	87.73	2.27
72h-A-R-3	13212306	11881942	272479	89.93	2.06
72h-A-NR-3	13527478	12015723	288044	88.82	2.13
120h-A-R-3	13445520	11988211	307615	89.16	2.29
120h-A-NR-3	14403727	12945975	302341	89.88	2.1
Average	12532044.07	11210063.59	304034.41	89.63	2.43

Supplementary Table 10. Table summarising KEGG pathways identified with all the *A. alpina* genes having homologs in *A. thaliana*. The highlighted KEGG pathways have more than 5% of the total genes associated to a KEGG pathway with the highest number of genes.

KEGG identifier	KEGG pathway	Number of genes	Percentage	
ath01100	Metabolic pathways	807	18.93	KEGG pathways with more than 1% of total genes identified during KEGG analysis
ath01110	Biosynthesis of secondary metabolites	485	11.38	
ath04075	Plant hormone signal transduction	158	3.71	
ath01200	Carbon metabolism	125	2.93	
ath01230	Biosynthesis of amino acids	111	2.6	
ath00940	Phenylpropanoid biosynthesis	77	1.81	
ath00230	Purine metabolism	70	1.64	
ath00500	Starch and sucrose metabolism	69	1.62	
ath03010	Ribosome	65	1.52	
ath00520	Amino sugar and nucleotide sugar metabolism	62	1.45	
ath00270	Cysteine and methionine metabolism	60	1.41	
ath04016	MAPK signaling pathway - plant	60	1.41	
ath04141	Protein processing in endoplasmic reticulum	59	1.38	
ath04626	Plant - pathogen inteaction	56	1.31	
ath00480	Glutathione metabolism	54	1.27	
ath00010	Glycolysis / Gluconeogenesis	52	1.22	
ath00240	Pyrimidine metabolism	46	1.08	
ath03008	Ribosome biogenesis in eukaryotes	45	1.06	
ath03018	RNA degradation	44	1.03	
ath00630	Glyoxylate and dicarboxylate metabolism	41	0.96	
ath00260	Glycine, serine and threonine metabolism	39	0.91	
ath00564	Glycerophospholipid metabolism	38	0.89	
ath00190	Oxidative phosphorylation	37	0.87	
ath04146	Peroxisome	36	0.84	
ath00040	Pentose and glucuronate interconversions	35	0.82	
ath00620	Pyruvate metabolism	35	0.82	
ath00195	Photosynthesis	35	0.82	
ath00710	Carbon fixation in photosynthetic organisms	34	0.8	
ath00030	Pentose phosphate pathway	34	0.8	
ath03013	RNA transport	32	0.75	
ath00561	Glycerolipid metabolism	31	0.73	
ath04144	Endocytosis	30	0.7	
ath00562	Inositol phosphate metabolism	30	0.7	
ath00051	Fructose and mannose metabolism	30	0.7	
ath00250	Alanine, aspartate and glutamate metabolism	29	0.68	
ath00460	Cyanoamino acid metabolism	28	0.66	
ath01212	Fatty acid metabolism	28	0.66	
ath00400	Phenylalanine, tyrosine and tryptophan biosynthesis	28	0.66	
ath00052	Galactose metabolism	27	0.63	
ath01210	2 - Oxocarboxylic acid metabolism	27	0.63	
ath00910	Nitrogen metabolism	26	0.61	
ath00053	Ascorbate and aldarate metabolism	26	0.61	
ath03040	Spliceosome	26	0.61	

KEGG identifier	KEGG pathway	Number of genes	Percentage	
ath00071	Fatty acid degradation	25	0.59	
ath00360	Phenylalanine metabolism	25	0.59	
ath03440	Homologous recombination	24	0.56	
ath00592	alpha	24	0.56	
ath04120	Ubiquitin mediated proteolysis	24	0.56	
ath00920	Sulfur metabolism	24	0.56	
ath00020	Citrate cycle	24	0.56	
ath04712	Circadian rhythm - plant	23	0.54	
ath00330	Arginine and proline metabolism	23	0.54	
ath00280	Valine, leucine and isoleucine degradation	23	0.54	
ath03030	DNA replication	22	0.52	
ath00380	Tryptophan metabolism	22	0.52	
ath00350	Tyrosine metabolism	22	0.52	
ath04145	Phagosome	21	0.49	
ath04070	Phosphatidylinositol signaling system	21	0.49	
ath03015	mRNA surveillance pathway	20	0.47	
ath00062	Fatty acid elongation	20	0.47	
ath00860	Porphyrin and chlorophyll metabolism	19	0.45	
ath00410	beta - Alanine metabolism	19	0.45	
ath00130	Ubiquinone and other terpenoid - quinone biosynthesis	18	0.42	
ath00960	Tropane, piperidine and pyridine alkaloid biosynthesis	17	0.4	
ath03015	mRNA surveillance pathway	20	0.47	
ath00062	Fatty acid elongation	20	0.47	
ath00860	Porphyrin and chlorophyll metabolism	19	0.45	
ath00410	beta - Alanine metabolism	19	0.45	
ath00130	Ubiquinone and other terpenoid - quinone biosynthesis	18	0.42	
ath00960	Tropane, piperidine and pyridine alkaloid biosynthesis	17	0.4	
ath00966	Glucosinolate biosynthesis	17	0.4	
ath00061	Fatty acid biosynthesis	16	0.38	
ath03060	Protein export	16	0.38	
ath00906	Carotenoid biosynthesis	16	0.38	
ath03410	Base excision repair	16	0.38	
ath00196	Photosynthesis - antenna proteins	16	0.38	
ath00900	Terpenoid backbone biosynthesis	15	0.35	
ath00950	Isoquinoline alkaloid biosynthesis	14	0.33	
ath00220	Arginine biosynthesis	14	0.33	
ath00073	Cutin, suberine and wax biosynthesis	14	0.33	
ath00908	Zeatin biosynthesis	14	0.33	
ath03420	Nucleotide excision repair	13	0.3	
ath00904	Diterpenoid biosynthesis	13	0.3	
ath03430	Mismatch repair	13	0.3	
ath00941	Flavonoid biosynthesis	12	0.28	
ath00640	Propanoate metabolism	12	0.28	
ath01040	Biosynthesis of unsaturated fatty acids	11	0.26	
ath03020	RNA polymerase	11	0.26	
ath00565	Ether lipid metabolism	11	0.26	
ath04136	Autophagy - other	11	0.26	
ath00450	Selenocompound metabolism	10	0.23	
ath00510	N-Glycan biosynthesis	10	0.23	
ath00310	Lysine degradation	10	0.23	
ath00670	One carbon pool by folate	10	0.23	
ath03022	Basal transcription factors	10	0.23	

KEGG identifier	KEGG pathway	Number of genes	Percentage	
ath02010	ABC transporters	9	0.21	
ath04933	AGE-RAGE signaling pathway in diabetic complications	9	0.21	
ath00261	Monobactam biosynthesis	9	0.21	
ath00970	Aminoacyl-tRNA biosynthesis	9	0.21	
ath00730	Thiamine metabolism	8	0.19	
ath00590	Arachidonic acid metabolism	8	0.19	
ath00100	Steroid biosynthesis	8	0.19	
ath00430	Taurine and hypotaurine metabolism	8	0.19	
ath00650	Butanoate metabolism	8	0.19	
ath00290	Valine, leucine and isoleucine biosynthesis	8	0.19	
ath00600	Sphingolipid metabolism	7	0.16	
ath00790	Folate biosynthesis	7	0.16	
ath00905	Brassinosteroid biosynthesis	7	0.16	
ath00760	Nicotinate and nicotinamide metabolism	7	0.16	
ath00740	Riboflavin metabolism	7	0.16	
ath00591	Linoleic acid metabolism	7	0.16	
ath00750	Vitamin B6 metabolism	6	0.14	
ath00340	Histidine metabolism	6	0.14	
ath00300	Lysine biosynthesis	6	0.14	
ath04130	SNARE interactions in vesicular transport	5	0.12	
ath00770	Pantothenate and CoA biosynthesis	5	0.12	
ath00511	Other glycan degradation	5	0.12	
ath00901	Indole alkaloid biosynthesis	5	0.12	
ath00780	Biotin metabolism	5	0.12	
ath00945	Stilbenoid, diarylheptanoid and gingerol biosynthesis	4	0.09	
ath00514	Other types of O-glycan biosynthesis	4	0.09	
ath00909	Sesquiterpenoid and triterpenoid biosynthesis	4	0.09	
ath00563	Glycosylphosphatidylinositol	4	0.09	
ath03050	Proteasome	3	0.07	
ath00603	Glycosphingolipid biosynthesis - globo and isoglobo series	3	0.07	
ath00531	Glycosaminoglycan degradation	2	0.05	
ath00944	Flavone and flavonol biosynthesis	2	0.05	
ath00604	Glycosphingolipid biosynthesis - ganglio series	2	0.05	
ath00660	C5-Branched dibasic acid metabolism	2	0.05	
ath00965	Betalain biosynthesis	2	0.05	
ath00785	Lipoic acid metabolism	2	0.05	
ath00072	Synthesis and degradation of ketone bodies	2	0.05	
ath04122	Sulfur relay system	2	0.05	
ath03450	Non-homologous end-joining	1	0.02	
ath00942	Anthocyanin biosynthesis	1	0.02	
ath00232	Caffeine metabolism	1	0.02	
ath00440	Phosphonate and phosphinate metabolism	1	0.02	

Supplementary Table 11. Table presenting the genes in each selected cluster with *A. thaliana* homologs. The information for each gene was taken from TAIR (http://arabidopsis.org/index.jsp). The highlighted represents the colour of the box in Figure 2-18B.

AALP name	At name	Common name
AALP_AA4G132200	AT2G36100	CASPARIAN STRIP MEMBRANE DOMAIN PROTEIN 1 (CASP1)
AALP_AA3G125100	AT3G11550	CASPARIAN STRIP MEMBRANE DOMAIN PROTEIN 2 (CASP2)
AALP_AA1G249400	AT1G22880	CELLULASE 5 (CEL5)
AALP_AA8G276600	AT5G50260	CYSTEINE ENDOPEPTIDASE 1 (CEP1)
AALP_AA1G060800	AT1G06350	DELTA 9 DESATURASE 4 (ADS4)
AALP_AA5G064900	AT3G30775	EARLY RESPONSIVE TO DEHYDRATION 5 (ERD5)
AALP_AA6G220900	AT2G28670	ENHANCED SUBERIN 1 (ESB1)
AALP_AA3G034400	AT3G03910	GLUTAMATE DEHYDROGENASE 3 (GDH3)
AALP_AA4G243200	AT2G45420	LOB DOMAIN-CONTAINING PROTEIN 18 (LBD18)
AALP_AA8G190700	AT5G17820	PEROXIDASE 57 (PER57)
AALP_AA8G500000	AT5G66390	PEROXIDASE 72 (PRX72)
AALP_AA4G202700	AT2G41850	POLYGALACTURONASE ABSCISSION ZONE A. THALIANA (PGAZAT)
AALP_AA4G090300	AT1G44800	SILIQUES ARE RED 1 (SIAR1)
AALP_AA2G213500	AT1G77210	SUGAR TRANSPORT PROTEIN 14 (STP14)
AALP_AA2G077000	AT1G66150	TRANSMEMBRANE KINASE 1 (TMK1)
AALP_AA6G072100	AT2G32300	UCLACYANIN 1 (UCC1)
AALP_AA1G239100	AT1G21890	USUALLY MULTIPLE ACIDS MOVE IN AND OUT TRANSPORTERS 19 (UMAMIT19)
AALP_AA8G054400	AT5G05810	ATL43
AALP_AA8G266100	AT5G49130	BIGE1B
AALP_AA4G206500	AT2G42380	BZIP34
AALP_AA5G225900	AT3G58120	BZIP61
AALP_AA5G119500	AT3G49720	CGR2
AALP_AA4G152300	AT2G37640	EXP3
AALP_AAs62283U000200	AT4G17460	HAT1
AALP_AA3G248500	AT4G15490	UGT84A3
AALP_AA3G162000	AT3G14370	WAG2
AALP_AA8G284400	AT4G08040	1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE 11 (ACS11)
AALP_AA6G288500	AT2G22810	1-AMINOCYCLOPROPANE-1-CARBOXYLATE SYNTHASE 4 (ACS4)
AALP_AA7G254400	AT4G37770	1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE 8 (ACS8)
AALP_AA1G001800	AT1G01120	3-KETOACYL-COA SYNTHASE 1 (KCS1)
AALP_AA7G138700	AT4G29140	ACTIVATED DISEASE SUSCEPTIBILITY 1 (ADS1)
AALP_AA8G419800	AT5G24330	ARABIDOPSIS TRITHORAX-RELATED PROTEIN 6 (ATXR6)
AALP_AA7G251700	AT4G37450	ARABINOGALACTAN PROTEIN 18 (AGP18)
AALP_AA3G148700	AT3G13520	ARABINOGALACTAN PROTEIN 12 (AGP12)
AALP_AA4G230200	AT2G44080	ARGOS-LIKE (ARL)
AALP_AA7G158600	AT5G49700	AT-HOOK MOTIF NUCLEAR LOCALIZED PROTEIN 17 (AHL17)
AALP_AA6G148000	AT5G43700	AUXIN INDUCIBLE 2-11 (ATAUX2-11)
AALP_AA6G062800	AT2G33310	AUXIN-INDUCED PROTEIN 13 (IAA13)
AALP_AA5G253500	AT3G59900	AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE (ARGOS)
AALP_AA8G155000	AT5G15160	BANQUO 2 (BNQ2)
AALP_AA7G028700	AT4G20270	BARELY ANY MERISTEM 3 (BAM3)
AALP_AA8G469000	AT5G63810	BETA-GALACTOSIDASE 10 (BGAL10)
AALP_AA1G201600	AT1G18400	BR ENHANCED EXPRESSION 1 (BEE1)
AALP_AA7G240400	AT4G36540	BR ENHANCED EXPRESSION 2 (BEE2)
AALP_AA2G175900	AT1G73830	BR ENHANCED EXPRESSION 3 (BEE3)
AALP_AA7G192200	AT4G32810	CAROTENOID CLEAVAGE DIOXYGENASE 8 (CCD8)
AALP_AA1G284800	AT1G47230	CYCLIN A3;4 (CYCA3;4)
AALP_AA1G215700	AT1G19630	CYTOCHROME P450, FAMILY 722, SUBFAMILY A, POLYPEPTIDE 1 (CYP722A1)
AALP_AA2G224300	AT5G56970	CYTOKININ OXIDASE 3 (CKX3)
AALP_AA8G432300	AT5G25460	DUF642 L-GALL RESPONSIVE GENE 2 (DGR2)
AALP_AA8G329100	AT5G54510	DWARF IN LIGHT 1 (DFL1)
AALP_AA2G242000	AT2G40550	E2F TARGET GENE 1 (ETG1)
AALP_AA8G160000	AT5G15350	EARLY NODULIN-LIKE PROTEIN 17 (ENODL17)
AALP_AA6G312000	AT1G64640	EARLY NODULIN-LIKE PROTEIN 8 (ENODL8)
AALP_AA7G015600	AT4G19120	EARLY-RESPONSIVE TO DEHYDRATION 3 (ERD3)
AALP_AA8G480500	AT5G64720	EGG CELL 1.5 (EC1.5)
AALP_AA4G090100	AT1G44830	ERF TRANSCRIPTION FACTOR 14 (ERF014)
AALP_AA8G429500	AT5G25190	ETHYLENE AND SALT INDUCIBLE 3 (ESE3)
AALP_AA4G267900	AT2G40940	ETHYLENE RESPONSE SENSOR 1 (ERS1)
AALP_AA6G241000	AT2G27050	ETHYLENE-INSENSITIVE3-LIKE 1 (EIL1)
AALP_AA1G220700	AT1G20190	EXPANSIN 11 (EXPA11)
AALP_AA3G374500	AT2G20750	EXPANSIN B1 (EXPB1)

AALP name	At name	Common name
AALP_AA6G157600	AT2G04780	FASCICLIN-LIKE ARABINOOGALACTAN 7 (FLA7)
AALP_AA1G082900	AT1G08010	GATA TRANSCRIPTION FACTOR 11 (GATA11)
AALP_AA8G294000	AT5G51810	GIBBERELLIN 20 OXIDASE 2 (GA20OX2)
AALP_AA3G163900	AT3G14570	GLUCAN SYNTHASE-LIKE 4 (GSL04)
AALP_AA1G288600	AT1G27950	GLYCOSYLPHOSPHATIDYLINOSITOL-ANCHORED LIPID PROTEIN TRANSFER 1 (LTPG1)
AALP_AA1G338300	AT1G33240	GT-2-LIKE 1 (GTL1)
AALP_AA3G113800	AT3G10520	HAEMOGLOBIN 2 (HB2)
AALP_AA4G228600	AT2G43910	HARMLESS TO OZONE LAYER 1 (HOL1)
AALP_AA8G154900	AT5G15150	HOMEOBOX 3 (HB-3)
AALP_AA5G131800	AT3G50890	HOMEOBOX PROTEIN 28 (HB28)
AALP_AA8G323100	AT5G53980	HOMEOBOX PROTEIN 52 (HB52)
AALP_AA7G157000	AT4G30410	IBH1-LIKE 1 (IBL1)
AALP_AA4G033200	AT1G52830	INDOLE-3-ACETIC ACID 6 (IAA6)
AALP_AA1G176600	AT1G15580	INDOLE-3-ACETIC ACID INDUCIBLE 5 (IAA5)
AALP_AA8G489800	AT5G65670	INDOLE-3-ACETIC ACID INDUCIBLE 9 (IAA9)
AALP_AA1G001700	AT1G01110	IQ-DOMAIN 18 (IQD18)
AALP_AA1G167200	AT4G14750	IQ-DOMAIN 19 (IQD19)
AALP_AA8G022100	AT5G03150	JACKDAW (JKD)
AALP_AA1G280300	AT1G26945	KIDARI (KDR)
AALP_AA8G123600	AT5G12330	LATERAL ROOT PRIMORDIUM 1 (LRP1)
AALP_AA2G016700	AT1G62440	LEUCINE-RICH REPEAT/EXTENSIN 2 (LRX2)
AALP_AA4G093700	AT4G13340	LEUCINE-RICH REPEAT/EXTENSIN 3 (LRX3)
AALP_AA8G117300	AT5G11950	LONELY GUY 8 (LOG8)
AALP_AA8G158400	AT5G15580	LONGIFOLIA1 (LNG1)
AALP_AA3G014100	AT3G02170	LONGIFOLIA2 (LNG2)
AALP_AA7G130400	AT4G28680	L-TYROSINE DECARBOXYLASE (TYRDC)
AALP_AA1G251700	AT1G23060	MICROTUBULE DESTABILIZING PROTEIN 40 (MDP40)
AALP_AA1G260200	AT1G24020	MLP-LIKE PROTEIN 423 (MLP423)
AALP_AA8G303700	AT5G52600	MYB DOMAIN PROTEIN 82 (MYB82)
AALP_AA7G001800	AT4G17980	NAC DOMAIN CONTAINING PROTEIN 71 (NAC071)
AALP_AA8G140100	AT5G14000	NAC DOMAIN CONTAINING PROTEIN 84 (NAC084)
AALP_AA6G114100	AT5G46590	NAC DOMAIN CONTAINING PROTEIN 96 (NAC096)
AALP_AA8G283300	AT5G50820	NAC DOMAIN CONTAINING PROTEIN 97 (NAC097)
AALP_AA6G286900	AT2G23050	NAKED PINS IN YUC MUTANTS 4 (NPY4)
AALP_AA4G038900	AT1G52190	NRT1/ PTR FAMILY 1.2 (NPF1.2)
AALP_AA2G050600	AT1G59740	NRT1/ PTR FAMILY 4.3 (NPF4.3)
AALP_AA6G341300	AT5G39860	PACLOBUTRAZOL RESISTANCE1 (PRE1)
AALP_AA5G056500	AT3G28857	PACLOBUTRAZOL RESISTANCE 5 (PRE5)
AALP_AA6G130500	AT5G45280	PECTIN ACETYLESTERASE 11 (PAE11)
AALP_AA5G236300	AT3G58850	PHY RAPIDLY REGULATED 2 (PAR2)
AALP_AA8G035100	AT5G04190	PHYTOCHROME KINASE SUBSTRATE 4 (PKS4)
AALP_AA1G039500	AT1G04520	PLASMODESMATA-LOCATED PROTEIN 2 (PDLP2)
AALP_AA4G202100	AT2G41820	PXY/TDR-CORRELATED 3 (PXC3)
AALP_AA6G124100	AT5G45750	RAB GTPASE HOMOLOG A1C (RABA1c)
AALP_AA8G437600	AT5G60860	RAB GTPASE HOMOLOG A1F (RABA1f)
AALP_AA8G186200	AT5G17490	RGA-LIKE PROTEIN 3 (RGL3)
AALP_AA1G345600	AT1G34110	RGF1 INSENSITIVE 5 (RGI5)
AALP_AA8G261200	AT4G16515	ROOT MERISTEM GROWTH FACTOR 6 (RGF6)
AALP_AA5G163300	AT3G53232	ROTUNDIFOLIA LIKE 1 (RTFL1)
AALP_AA8G272700	AT3G23635	ROTUNDIFOLIA LIKE 13 (RTFL13)
AALP_AA5G231700	AT3G25717	ROTUNDIFOLIA LIKE 16 (RTFL16)
AALP_AA5G292700	AT3G63470	SERINE CARBOXYPEPTIDASE-LIKE 40 (scpl40)
AALP_AA3G033100	AT3G03840	SMALL AUXIN UP RNA 27 (SAUR27)
AALP_AA3G033200	AT3G03830	SMALL AUXIN UP RNA 28 (SAUR28)
AALP_AA3G032900	AT3G03820	SMALL AUXIN UP RNA 29 (SAUR29)
AALP_AA7G266100	AT4G38850	SMALL AUXIN UPREGULATED 15 (SAUR15)
AALP_AA7G218400	AT4G34770	SMALL AUXIN UPREGULATED RNA 1 (SAUR1)
AALP_AA3G340300	AT2G18010	SMALL AUXIN UPREGULATED RNA 10 (SAUR10)
AALP_AA7G266000	AT4G38840	SMALL AUXIN UPREGULATED RNA 14 (SAUR14)
AALP_AA7G266200	AT4G38860	SMALL AUXIN UPREGULATED RNA 16 (SAUR16)
AALP_AA7G218700	AT4G34810	SMALL AUXIN UPREGULATED RNA 5 (SAUR5)
AALP_AA7G218300	AT4G34760	SMALL AUXIN UPREGULATED RNA 50 (SAUR50)
AALP_AA1G302500	AT1G29500	SMALL AUXIN UPREGULATED RNA 66 (SAUR66)
AALP_AA1G303000	AT1G29510	SMALL AUXIN UPREGULATED RNA 67 (SAUR67)

AALP name	At name	Common name
AALP_AA2G218300	AT5G20820	SMALL AUXIN UPREGULATED RNA 76 (SAUR76)
AALP_AA4G214100	AT2G42580	TETRATRICOPETIDE-REPEAT THIOREDOXIN-LIKE 3 (TTL3)
AALP_AA5G292200	AT3G63430	TON1 RECRUITING MOTIF 5 (TRM5)
AALP_AA6G150200	AT5G43380	TYPE ONE SERINE/THREONINE PROTEIN PHOSPHATASE 6 (TOPP6)
AALP_AA2G241800	AT2G40900	USUALLY MULTIPLE ACIDS MOVE IN AND OUT TRANSPORTERS 11 (UMAMIT11)
AALP_AA3G207600	AT3G18200	USUALLY MULTIPLE ACIDS MOVE IN AND OUT TRANSPORTERS 4 (UMAMIT4)
AALP_AAs45078U000600	AT4G14130	XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 15 (XTH15)
AALP_AA1G124700	AT1G11545	XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 8 (XTH8)
AALP_AA1G010500	AT1G01900	SBTI1.1
AALP_AA1G252200	AT1G23090	SULTR3;3
AALP_AA8G283000	AT5G50800	SWEET13
AALP_AAs60878U000100	AT4G14440	3-HYDROXYACYL-COA DEHYDRATASE 1 (HCD1)
AALP_AA6G253600	AT2G26250	3-KETOACYL-COA SYNTHASE 10 (KCS10)
AALP_AA1G078700	AT1G07720	3-KETOACYL-COA SYNTHASE 3 (KCS3)
AALP_AA1G267900	AT1G25450	3-KETOACYL-COA SYNTHASE 5 (KCS5)
AALP_AA4G005800	AT1G55870	ABA-HYPERSENSITIVE GERMINATION 2 (AHG2)
AALP_AA4G240700	AT2G45190	ABNORMAL FLORAL ORGANS (AFO)
AALP_AA7G221300	AT4G34970	ACTIN DEPOLYMERIZING FACTOR 9 (ADF9)
AALP_AAs50386U000100	AT5G28640	ANGUSTIFOLIA 3 (AN3)
AALP_AA4G270900	AT2G47930	ARABINOGALACTAN PROTEIN 26 (AGP26)
AALP_AA3G056400	AT3G05800	ATBS1(ACTIVATION-TAGGED BRI1 SUPPRESSOR 1)-INTERACTING FACTOR 1 (AIF1)
AALP_AA5G056700	AT3G28860	ATP-BINDING CASSETTE B19 (ABCB19)
AALP_AA7G090300	AT4G25960	ATP-BINDING CASSETTE B2 (ABCB2)
AALP_AA2G237200	AT1G17840	ATP-BINDING CASSETTE G11 (ABCG11)
AALP_AA4G045100	AT1G51500	ATP-BINDING CASSETTE G12 (ABCG12)
AALP_AA6G243800	AT2G26910	ATP-BINDING CASSETTE G32 (ABCG32)
AALP_AA3G312200	AT2G14580	BASIC PATHOGENESIS-RELATED PROTEIN 1 (PRB1)
AALP_AA4G247500	AT2G45760	BON ASSOCIATION PROTEIN 2 (BAP2)
AALP_AA1G190800	AT1G17200	CASP-LIKE PROTEIN 2A1 (CASPL2A1)
AALP_AA7G271000	AT4G39330	CINNAMYL ALCOHOL DEHYDROGENASE 9 (CAD9)
AALP_AA6G179900	AT1G11600	CYTOCHROME P450, FAMILY 77, SUBFAMILY B, POLYPEPTIDE 1 (CYP77B1)
AALP_AA4G255500	AT2G46660	CYTOCHROME P450, FAMILY 78, SUBFAMILY A, POLYPEPTIDE 6 (CYP78A6)
AALP_AA6G006200	AT4G00360	CYTOCHROME P450, FAMILY 86, SUBFAMILY A, POLYPEPTIDE 2 (CYP86A2)
AALP_AA5G109900	AT3G48720	DEFICIENT IN CUTIN FERULATE (DCF)
AALP_AA1G015600	AT1G02205	ECERIFERUM 1 (CER1)
AALP_AA7G205800	AT4G33790	ECERIFERUM 4 (CER4)
AALP_AA8G228400	AT5G20630	GERMIN 3 (GER3)
AALP_AA8G241600	AT5G25980	GLUCOSIDE GLUCOHYDROLASE 2 (TGG2)
AALP_AA1G005500	AT1G01610	GLYCEROL-3-PHOSPHATE SN-2-ACYLTRANSFERASE 4 (GPAT4)
AALP_AA6G069400	AT2G32690	GLYCINE-RICH PROTEIN 23 (GRP23)
AALP_AA3G147000	AT1G55260	GLYCOSYLPHOSPHATIDYLINOSITOL-ANCHORED LIPID PROTEIN TRANSFER 6 (LTPG6)
AALP_AA5G259600	AT3G60390	HOMEOBOX-LEUCINE ZIPPER PROTEIN 3 (HAT3)
AALP_AA3G251200	AT3G21760	HYPOSTATIN RESISTANCE 1 (HYR1)
AALP_AA8G140900	AT5G14090	LAZY 1 (LAZY1)
AALP_AA1G250800	AT1G23010	LOW PHOSPHATE ROOT1 (LPR1)
AALP_AA1G032000	AT1G03840	MAGPIE (MGP)
AALP_AA6G015600	AT4G01550	NAC DOMAIN CONTAINING PROTEIN 69 (NAC069)
AALP_AA3G183300	AT3G16180	NITRATE TRANSPORTER 1.12 (NRT1.12)
AALP_AA8G475700	AT5G64330	NON-PHOTOTROPIC HYPOCOTYL 3 (NPH3)
AALP_AA7G197000	AT4G33220	PECTIN METHYLESTERASE 44 (PME44)
AALP_AA3G312300	AT3G29670	PHENOLIC GLUCOSIDE MALONYLTRANSFERASE 2 (PMAT2)
AALP_AA4G216800	AT2G42870	PHY RAPIDLY REGULATED 1 (PAR1)
AALP_AA4G162600	AT2G38360	PRENYLATED RAB ACCEPTOR 1.B4 (PRA1.B4)
AALP_AA1G196100	AT1G17700	PRENYLATED RAB ACCEPTOR 1.F1 (PRA1.F1)
AALP_AA2G164600	AT2G21140	PROLINE-RICH PROTEIN 2 (PRP2)
AALP_AA8G433300	AT5G25610	RESPONSIVE TO DESICCATION 22 (RD22)
AALP_AA3G064800	AT3G03450	RGA-LIKE 2 (RGL2)
AALP_AA8G016400	AT5G02750	SHOOT GRAVITROPISM 9 (SGR9)
AALP_AA8G312000	AT4G12410	SMALL AUXIN UPREGULATED RNA 35 (SAUR35)
AALP_AAs68488U000700	AT1G56580	SMALLER WITH VARIABLE BRANCHES (SVB)
AALP_AA2G115100	AT1G68870	SOB FIVE-LIKE 2 (SOFL2)
AALP_AA2G006000	AT1G63260	TETRASPANIN10 (TET10)
AALP_AA4G255700	AT2G46640	TILLER ANGLE CONTROL 1 (TAC1)

AALP name	At name	Common name
AALP_AA8G485000	AT5G65140	TREHALOSE-6-PHOSPHATE PHOSPHATASE J (TPPJ)
AALP_AA2G134700	AT1G70560	TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS 1 (TAA1)
AALP_AA4G264700	AT2G47270	UPBEAT1 (UPB1)
AALP_AA8G480300	AT5G64700	USUALLY MULTIPLE ACIDS MOVE IN AND OUT TRANSPORTERS 21 (UMAMIT21)
AALP_AA1G293900	AT1G72290	WATER-SOLUBLE CHLOROPHYLL PROTEIN (ATWSCP)
AALP_AA1G086900	AT1G08465	YABBY2 (YAB2)
AALP_AA8G506400	AT5G66940	ATDOF5.8
AALP_AA7G251400	AT4G37390	BRU6
AALP_AA1G199000	AT1G18100	E12A11
AALP_AA3G316200	AT2G14960	GH3.1
AALP_AA6G286300	AT2G23170	GH3.3
AALP_AA6G104800	AT5G47370	HAT2
AALP_AA3G011200	AT3G02000	ROXY1
AALP_AA1G307000	AT1G29950	SACL3
AALP_AA6G173200	AT4G11280	1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID (ACC) SYNTHASE 6 (ACS6)
AALP_AA8G243200	AT5G19530	ACAULIS 5 (ACL5)
AALP_AA8G136400	AT5G13790	AGAMOUS-LIKE 15 (AGL15)
AALP_AA8G016500	AT5G02760	ARABIDOPSIS PP2C CLADE D 7 (APD7)
AALP_AA8G488500	AT5G65530	ARABIDOPSIS RECEPTOR-LIKE CYTOPLASMIC KINASE ATRLCK VI_A3 (ATRLCK VI_A3)
AALP_AA5G079100	AT3G45230	ARABINOXYLAN PECTIN ARABINOGALACTAN PROTEIN 1 (APAP1)
AALP_AA1G317700	AT1G31280	ARGONAUTE 2 (AGO2)
AALP_AA4G159200	AT2G38120	AUXIN RESISTANT 1 (AUX1)
AALP_AA2G223400	AT1G78100	AUXIN UP-REGULATED F-BOX PROTEIN 1 (AUF1)
AALP_AA5G230800	AT3G25710	BASIC HELIX-LOOP-HELIX 32 (BHLH32)
AALP_AA4G134500	AT5G38800	BASIC LEUCINE-ZIPPER 43 (bZIP43)
AALP_AA7G011700	AT4G18890	BES1/BZR1 HOMOLOG 3 (BEH3)
AALP_AA8G383000	AT5G59010	BRASSINOSTEROID-SIGNALING KINASE 5 (BSK5)
AALP_AA8G227600	AT5G20540	BREVIS RADIX-LIKE 4 (BRXL4)
AALP_AA5G090500	AT5G18930	BUSHY AND DWARF 2 (BUD2)
AALP_AAs66918U000100	AT4G17615	CALCINEURIN B-LIKE PROTEIN 1 (CBL1)
AALP_AA4G238700	AT2G44990	CAROTENOID CLEAVAGE DIOXYGENASE 7 (CCD7)
AALP_AAs49657U000100	AT4G14723	CHALLAH-LIKE 2 (CLL2)
AALP_AA2G051400	AT1G59720	CHLORORESPIRATORY REDUCTION28 (CRR28)
AALP_AA4G083100	AT1G47485	C-TERMINALLY ENCODED PEPTIDE 1 (CEP1)
AALP_AA8G145200	AT5G14400	CYTOCHROME P450, FAMILY 724, SUBFAMILY A, POLYPEPTIDE 1 (CYP724A1)
AALP_AA8G314600	AT5G53290	CYTOKININ RESPONSE FACTOR 3 (CRF3)
AALP_AA2G248900	AT1G79760	DOWNSTREAM TARGET OF AGL15-4 (DTA4)
AALP_AA1G040900	AT1G04635	EMBRYO DEFECTIVE 1687 (EMB1687)
AALP_AA2G157100	AT1G72470	EXOCYST SUBUNIT EXO70 FAMILY PROTEIN D1 (EXO70D1)
AALP_AA3G059300	AT3G06020	FANTASTIC FOUR 4 (FAF4)
AALP_AA4G024100	AT1G53920	GDSL-MOTIF LIPASE 5 (GLIP5)
AALP_AA6G266800	AT2G24762	GLUTAMINE DUMPER 4 (GDU4)
AALP_AA5G004700	AT2G01430	HOMEOBOX-LEUCINE ZIPPER PROTEIN 17 (HB17)
AALP_AAs59668U000200	AT4G14560	INDOLE-3-ACETIC ACID INDUCIBLE 1 (IAA1)
AALP_AA1G039800	AT1G04550	INDOLE-3-ACETIC ACID INDUCIBLE 12 (IAA12)
AALP_AAs58011U000100	AT4G14550	INDOLE-3-ACETIC ACID INDUCIBLE 14 (IAA14)
AALP_AA3G175100	AT3G15540	INDOLE-3-ACETIC ACID INDUCIBLE 19 (IAA19)
AALP_AA3G264100	AT3G23030	INDOLE-3-ACETIC ACID INDUCIBLE 2 (IAA2)
AALP_AA7G185100	AT4G32280	INDOLE-3-ACETIC ACID INDUCIBLE 29 (IAA29)
AALP_AA5G279400	AT3G62100	INDOLE-3-ACETIC ACID INDUCIBLE 30 (IAA30)
AALP_AA3G199400	AT3G17600	INDOLE-3-ACETIC ACID INDUCIBLE 31 (IAA31)
AALP_AA5G001500	AT2G01200	INDOLE-3-ACETIC ACID INDUCIBLE 32 (IAA32)
AALP_AA4G208000	AT2G21050	LIKE AUXIN RESISTANT 2 (LAX2)
AALP_AA7G165100	AT4G30980	LJRHL1-LIKE 2 (LRL2)
AALP_AA5G166100	AT3G53450	LONELY GUY 4 (LOG4)
AALP_AA3G150900	AT3G13682	LSD1-LIKE2 (LDL2)
AALP_AA3G009800	AT3G01840	LYSM-CONTAINING RECEPTOR-LIKE KINASE 2 (LYK2)
AALP_AA8G305300	AT5G52870	MEMBRANE-ASSOCIATED KINASE REGULATOR 5 (MAKR5)
AALP_AA8G306900	AT5G52900	MEMBRANE-ASSOCIATED KINASE REGULATOR 6 (MAKR6)
AALP_AAs67696U000200	AT2G30040	MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 14 (MAPKKK14)
AALP_AA4G200300	AT2G41660	MIZU-KUSSEI 1 (MIZ1)
AALP_AA5G196700	AT3G55730	MYB DOMAIN PROTEIN 109 (MYB109)
AALP_AA8G498600	AT5G66300	NAC DOMAIN CONTAINING PROTEIN 105 (NAC105)
AALP_AA1G308500	AT1G30100	NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 5 (NCED5)

AALP name	At name	Common name
AALP_AA5G162200	AT3G53180	NODULIN/GLUTAMINE SYNTHASE-LIKE PROTEIN (NodGS)
AALP_AA8G167900	AT5G16000	NSP-INTERACTING KINASE 1 (NIK1)
AALP_AA5G126000	AT3G50410	OBF BINDING PROTEIN 1 (OBP1)
AALP_AA5G192500	AT3G55370	OBF-BINDING PROTEIN 3 (OBP3)
AALP_AA8G297500	AT2G30400	OVATE FAMILY PROTEIN 2 (OFP2)
AALP_AA2G253100	AT1G80110	PHLOEM PROTEIN 2-B11 (PP2-B11)
AALP_AA1G240400	AT1G21980	PHOSPHATIDYLINOSITOL-4-PHOSPHATE 5-KINASE 1 (PIP5K1)
AALP_AA6G337900	AT2G26710	PHYB ACTIVATION TAGGED SUPPRESSOR 1 (BAS1)
AALP_AA2G140400	AT1G70940	PIN-FORMED 3 (PIN3)
AALP_AA5G004800	AT2G01420	PIN-FORMED 4 (PIN4)
AALP_AA2G212500	AT1G77110	PIN-FORMED 6 (PIN6)
AALP_AA1G252100	AT1G23080	PIN-FORMED 7 (PIN7)
AALP_AA8G011600	AT5G01890	PXY/TDR-CORRELATED 2 (PXC2)
AALP_AA3G205500	AT3G18130	RECEPTOR FOR ACTIVATED C KINASE 1C (RACK1C_AT)
AALP_AA1G142600	AT1G12950	ROOT HAIR SPECIFIC 2 (RSH2)
AALP_AA1G145700	AT1G13245	ROTUNDIFOLIA LIKE 17 (RTFL17)
AALP_AA1G104000	AT1G09840	SHAGGY-LIKE PROTEIN KINASE 41 (SK41)
AALP_AA7G253400	AT4G37650	SHORT ROOT (SHR)
AALP_AA5G237300	AT1G20140	SKP1-LIKE 4 (SK4)
AALP_AA3G066600	AT3G06370	SODIUM HYDROGEN EXCHANGER 4 (NHX4)
AALP_AA6G043900	AT4G03330	SYNTAXIN OF PLANTS 123 (SYP123)
AALP_AA8G397200	AT5G60200	TARGET OF MONOPTEROS 6 (TMO6)
AALP_AA2G228800	AT1G78580	TREHALOSE-6-PHOSPHATE SYNTHASE (TPS1)
AALP_AA1G228100	AT1G21070	UDP-RHA/UDP-GAL TRANSPORTER 2 (URGT2)
AALP_AA6G000800	AT4G00050	UNFERTILIZED EMBRYO SAC 10 (UNE10)
AALP_AA3G240000	AT3G20830	UNICORN-LIKE (UCNL)
AALP_AA2G197400	AT1G75500	WALLS ARE THIN 1 (WAT1)
AALP_AA7G073500	AT4G24240	WRKY DNA-BINDING PROTEIN 7 (WRKY7)
AALP_AA3G030400	AT3G03660	WUSCHEL RELATED HOMEOBOX 11 (WOX11)

Supplementary Table 12. Significance test for endogenous IAA levels in *A. alpina*. Summary data for an ANOVA for the levels of free endogenous IAA levels in the main stem of *A. alpina* plants before vernalization, and 4, 8, 12, 16 and 21 weeks after vernalization.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	5	355.76	71,152	19,215	0.1642
Residuals	12	444.35	37,029	-	-

***p<0.001, **p<0.01, *p<0.05

Supplementary Table 13. Significance test for endogenous IAA levels in *A. alpina*. Summary data for an ANOVA for the levels of free endogenous IAA levels in the main stem of *A. alpina* plants at the end and 5 days after vernalization of 12, 16 and 21 weeks after vernalization.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Treatment	2	345	172.5	6.602	0.00956 **
Day	1	131.1	131.1	5.018	0.04184 *
Residuals	14	365.8	26.13	-	-

***p<0.001, **p<0.01, *p<0.05

Supplementary Table 14. Primers and their sequences used in this study.

Gene	Direction	Sequence (5' -> 3')	Comment
AaGH3.3	Forward	ACAATTCCGCTCCACAGTTC	Transcript abundance
AaGH3.3	Reverse	CAAGTAACACCGATGCGTTC	Transcript abundance
AaGH3.6	Forward	CACCTTGTTCCGTTCGATG	Transcript abundance
AaGH3.6	Reverse	TACCATTCATGCAAAGCTCC	Transcript abundance
AaPP2A	Forward	AGTATCGCTTCTCGCTCCAG	Transcript abundance
AaPP2A	Reverse	AACCGGTTGGTCGACTATTG	Transcript abundance
AaARF6	Forward	AAATGGGAAAAAGAGGCCTC	Transcript abundance
AaARF6	Reverse	GATGCGATACCGTTACCGAG	Transcript abundance
AaARF8	Forward	GATCCTTGGGAGTCATTTGTG	Transcript abundance
AaARF8	Reverse	TAGAAATGGGTCAGGTTCTGTG	Transcript abundance
AaARF17	Forward	GAAATGAACACGTTGGAAACC	Transcript abundance
AaARF17	Reverse	CTGGATGATTTACTTTCTTCTTCG	Transcript abundance
AaLRP1	Forward	GTTTTGATACAAGCTCTAGTCGCC	Transcript abundance
AaLRP1	Reverse	ACTCATCGTCCCCATCCTC	Transcript abundance
GH3.3	Forward	TTCTATATCCTCAATTCATCAAACC	cDNA Sequencing
GH3.3	Forward	AAAGGGAAGGCTCTATACTTCCTG	cDNA Sequencing
GH3.3	Forward	CCGGAGCAATGGCTCAGTATATC	cDNA Sequencing
GH3.3	Forward	ACTTCCATTTCTTCGGAATTACGG	cDNA Sequencing
GH3.3	Reverse	GATTCATCACAGGCATGAGAAG	cDNA Sequencing
GH3.3	Reverse	TGATGCCTTCCCAATTATTGTC	cDNA Sequencing
GH3.3	Reverse	GGGCATAGTCTTCGTCTCTG	cDNA Sequencing
GH3.3	Reverse	GGTTTGATCCTTCATTAGAAGCTC	cDNA Sequencing
GH3.6	Forward	TTTTACTCTTCTTCTCTAATCTCTCTC	cDNA Sequencing
GH3.6	Forward	ACAAAGGCAAAGGCATGTACTTTC	cDNA Sequencing
GH3.6	Forward	TGCACAATGTATGCTTCTTCCG	cDNA Sequencing
GH3.6	Forward	TCGAGGATTGCTGTTTAACC	cDNA Sequencing
GH3.6	Reverse	CCAGGAACAAACTGGTCCATC	cDNA Sequencing
GH3.6	Reverse	CAAACCATTGCTGTAATAGTCCAG	cDNA Sequencing
GH3.6	Reverse	GAGGGAGGAATTGCCGTGTTAC	cDNA Sequencing
GH3.6	Reverse	AAATTAGACACACAGACACAGAC	cDNA Sequencing
LRP1	Forward	TAGAGAGAGAAAGTGTGAATAGGG	cDNA Sequencing
LRP1	Forward	AGATGGGCATGGTCGGTTTAAGAG	cDNA Sequencing
LRP1	Forward	ACCAAGAAGCCACGGATCGTTG	cDNA Sequencing
LRP1	Reverse	ATCCCGGAAGCCATGTAGGAAC	cDNA Sequencing
LRP1	Reverse	AGAGGAAGTCGAAAGCGACGAG	cDNA Sequencing
LRP1	Reverse	TTGCAGCATGAGTTAGTGAAC	cDNA Sequencing

7.3. List of abbreviations

°C	degree Celsius
µg	microgram
µL	microLitre
1-NAA	1-Naphthaleneacetic acid
2-NAA	2-Naphthaleneacetic acid
3', 5'	3-prime, 5-prime
A	Auxin-treated
A. alpina	*Arabis alpina*
A. thaliana	*Arabidopsis thaliana*
A. tumefaciens	*Agrobacterium tumefaciens*
Aa	*Arabis alpina*
ANOVA	Analysis of variance
AR	Adventitious roots
At	*Arabidopsis thaliana*
BACA	bubble chArt to compare annotations
bp	basepair
cDNA	Complementary DNA
CDS	Coding sequence
DNA	deoxyribonucleic acid
DNase	Deoxy-ribonuclease
dNTP	deoxyribonucleic triphosphate
Dor	Dorfertal
EMS	Ethyl methyl sulfonate
FPKM	Fragments Per Kilobase of transcript per Million mapped reads
g	gram
GO	Gene Ontology
GUS	Beta-glucuronidase
h	Hour
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
L	Litre
LD	Long day
M	Mock-treated
M	Mol
miR	micro RNA
mRNA	Messenger RNA
nm	nanometer
NPA	Naphthylphthalamic acid
NR	Non-rooting zone
PCR	Polymerase Chain Reaction
PEP1	*PERPETUALLY FLOWERING 1*

R	Rooting zone
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcription polymerase chain reaction
SD	Short day
Tot	Totes Gebirge
w	Week
Wca	West Carpathians
wLD	Weeks in LD
wV	Weeks vernalized

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