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WOX-CLAVATA system and its targets in the regulation of storage root and spontaneous tumour development in radish (*Raphanus sativus* L.)

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LIST OF ABBREVIATIONS AND SYMBOLS

ABA – abscisic acid

CLE – CLAVATA3/EMBRYO SURROUNDING REGION-related

CK - cytokinin

HD-ZIPIII – HOMEODOMAIN-LEUCINE ZIPPER класса III

IAA - indole-3-acetic acid

InDel – **in**sertion or **del**etion of bases

LM - lateral meristem

PCR - polymerase chain reaction

qPCR - real-time PCR

RAM – root apical meristem

SAM – shoot apical meristem

SNP – single nucleotide polymorphism

SNV - single nucleotide variants

TDIF –Tracheary Element Differentiation Inhibitory Factor

TDR/PXY – TDIF RECEPTOR/PHLOEM INTERCALATED WITH XYLEM

TF - transcription factor

WOX – WUSCHEL-RELATED HOMEOBOX

3-AT – 3-Amino-1,2,4-triazole

INTRODUCTION

The relevance of the topic. This research aims to bring to light the mechanisms of storage root development in radish. This phenomenon is based on several processes, including the activity of genes that control the lateral meristem cambium, as well as various external signals and metabolic changes (see review by Kuznetsova et al., 2020). The study of cell division and differentiation processes in higher plants is one of the most promising tasks of modern plant developmental genetics. The mechanisms of genetic control of lateral meristems, including secondary meristems, to which cambium belongs, are currently under active investigation (Turley and Etchells, 2022, Fernández-Piñán et al., 2021, Ikeuchi et al., 2022). One of the most important regulators of cambium activity is the WOX-CLAVATA system. It is a highly conserved regulatory module comprising CLAVATA3/EMBRYO SURROUNDING REGION-related (CLE) family peptides, their receptors, and target genes encoding homeodomain-containing TFs of the WUSCHEL-RELATED HOMEOBOX (WOX) family. This module controls the maintenance of stem cells and various meristems (Stahl and Simon, 2009; Lee and Torii, 2012).

At the same time, research on the functions of the WOX-CLAVATA module in storage root development is currently at the initial stage (Hoang et al., 2020; Kuznetsova et al., 2020; Kuznetsova et al., 2022). Radish (*Raphanus sativus* L.) as an annual crop related to *Arabidopsis thaliana*, the model object of genetics, is a promising model object for studying the genetics of storage root development. According to the data of comparative transcriptome analysis (Hoang et al., 2020), different species of plants with a storage root are characterised by changes in the expression of the same regulatory genes during root growth, and, therefore, the mechanisms of storage root development may be similar in different species. Thus, the data obtained in radish can be further extrapolated to other crops with storage roots. The study of the genetic control of storage root formation, particularly in radish, is an important task for modern biology and agriculture. Our work contributes to the study of genes which can be the targets for genome editing when creating improved varieties of root crops.

The extent of development of the topic. Since root crops are among the most massively cultivated agricultural plants (Fernie and Yan, 2019), the study of the mechanisms of storage root development is currently important for plant genetics problem solving. The storage root is formed as a result of intensive secondary root growth

due to the high activity of the cambium lateral meristem. Genetic mechanisms of cambium development regulation, including the role of conserved meristem regulators in controlling its activity, have also been investigated in the last decade. Due to that, a number of regulators of cambium activity and storage root development have been identified, including phytohormones (cytokinins, CK, indole-3-acetic acid, IAA, and peptide phytohormones); transcription factors, TFs, with WOX family proteins among others; and epigenetic regulators. The CLE family peptide hormones belong to a group of mobile regulators of meristem gene expression, particularly *WOX* genes, which are conserved regulators of meristem development (Hirakawa and Bowman, 2015; Yamaguchi et al., 2016). The CLE peptides and WOX TF are involved in cambium development (Hirakawa et al., 2010) and the formation of storage roots, including those in radish (Gancheva et al., 2016; Gancheva et al., 2018). Therefore, the aim of this work is to study the mechanisms for the WOX4 and WOX14 TFs and the CLE41 peptide functioning in root development of radish.

In our work we used inbred radish lines from the genetic collection created and maintained at the Department of Genetics and Biotechnology of St. Petersburg State University (Narbut, 1966; Buzovkina and Lutova, 2007). The radish genetic collection is a source of material for investigating the genetics of storage root development. The collection contains lines that differ in storage root characteristics, including those with developmental abnormalities, such as spontaneous tumours formed on the roots of certain lines.

The study of mechanisms of spontaneous tumour development in radish inbred lines is also the objective of the work. Tumours arise as a result of disruption to the processes of systemic control of cell division at the level of individual organs or the whole organism. Most of the studies of tumour formation in plants have been carried out on the example of tumours induced by phytopathogens. There are few studies on spontaneous plant tumours because they are rare due to the regulation of a plant body system by a flexible system with multiple feedback loops (Dodueva et al., 2020). In this regard, the study of the mechanisms of spontaneous tumour development contributes to the identification of systemic regulators of cell proliferation in higher plants. For example, studies of spontaneous tumours in *Arabidopsis* mutants and interspecific tobacco hybrids have identified novel participants in the systemic control of plant cell division, including regulators of cell adhesion (Frank et al., 2002; Krupkova et al., 2007).

Spontaneous tumours in radish inbred lines are developed on the root and are derived from another meristem, the cambium (Lebedeva et al., 2015), which contributes significantly to the development of the storage root. Consequently, their study will provide new data on the functions of cambial regulators, which play a role in storage root formation, in more general processes, such as the systemic control of plant cell division.

Purpose of the work: to study the role of meristem regulators belonging to the WOX-CLAVATA system in the process of root growth and development in radish (*Raphanus sativus* L.).

The following **objectives** were formulated to achieve the goal:

1. To analyse the genomes of two lines of radish genetic collection contrasting in spontaneous tumour formation ability:

1.1 To assess the quality of genome assembly of radish lines;

1.2 To identify genes with single nucleotide substitutions (SNPs), insertions and deletions (InDel) in the tumour line;

1.3 To verify the presence of SNPs and InDel in the tumour line using amplicon sequencing.

2. To identify the *RsWOX* and *RsCLE* genes, perform their sequence analysis and predict the domain structure of the encoded proteins:

2.1. To identify the *RsWOX* genes and localise them on chromosomes; to analyse the RsWOX proteins structure;

2.2. To identify the *RsCLE* genes and localise them on chromosomes; to analyse the RsCLE proteins structure.

3. To investigate the role of *RsWOX4*, *RsWOX14* and *RsCLE41* genes in root development and gene expression in radish:

3.1. To provide a transcriptome analysis of roots with *RsCLE41* overexpression;

3.2. To study the effect of *RsWOX4* and *RsWOX14* overexpression on radish root development;

4. To search for targets of RsWOX4 and RsWOX14 transcription factors:

4.1. To provide in silico search for possible targets of RsWOX4 and RsWOX14 TFs;

4.2. To conduct a quantitative analysis of candidate gene expression in control plants and plants with overexpression and silencing of *RsWOX4-2* gene;

4.3. To analyse the interaction of the RsWOX4 TF homeodomain with the promoters of candidate genes using a yeast one-hybrid assay.

Scientific novelty of the work. This work is devoted to the study of the meristem regulators role in root development in radish. The study of genetic mechanisms of storage root development in different plant species is currently at the initial stage of research, and a number of data were obtained in this work for the first time. In addition, due to the rare occurrence of developmental anomalies such as spontaneous tumours, there is scarce data on their developmental mechanisms, which also increases the novelty of this work. In our work, we have obtained genome assemblies of related tumour-forming and non-tumour lines of radish for the first time. Besides that, we have first identified single nucleotide polymorphism (SNPs) and **in**sertions or **de**letions (InDels) in radish lines contrasting in the ability to form spontaneous tumours. In the genome assemblies obtained we have identified *WOX* and *CLE* family genes regulating meristem development and then have analysed sequences of the corresponding proteins. Also for the first time we have performed a transcriptome analysis of plants with *RsCLE41-1* overexpression. We have identified the *RsLOG3* gene, which encodes the cytokinin biosynthesis enzyme, as a probable direct target of the RsWOX4 transcription factor. Thus, the work is characterised by a high degree of scientific novelty.

Theoretical and practical significance of the work. In our work, we searched for new candidate genes for the role of regulators of storage root development based on the *Raphanus sativus* model plant. We analysed root anatomy and gene expression in plants with altered expression levels of the cambium development regulatory genes *RsCLE41, RsWOX4* and *RsWOX14*, which allowed us to obtain data on their role in the storage root development. An important result of our work is the identification of the target of the main regulator of cambium activity, the RsWOX4 transcription factor: this is the *RsLOG3* gene encoding an enzyme of cytokinin biosynthesis. In the future, *RsWOX4* and *RsLOG3* genes may become targets for genomic editing of radish aimed at improving the traits important for agriculture.

The theoretical significance of the results of this work is the identification and analysis of the sequences of the *RsCLE* and *RsWOX* gene families of radish and the study of their functions, as well as their probable target genes, which contributes to the field of genetics of plant development. The study of spontaneous tumour formation in radish inbred lines is also an important objective of the work. Sequencing and comparative

analysis of the genomes of tumour and non-tumour lines of radish provides an opportunity to identify candidate genes for the role of regulators of spontaneous tumour formation and systemic control of cell division.

Methodology and methods of research. We have used a wide range of molecular genetics and bioinformatic methods in this work. Bioinformatics methods including sequencing, genome assembly and annotation, as well as search for single-nucleotide substitutions and insertions/deletions were used *to analyse genome sequencing data of radish lines.* In silico sequence analysis methods, phylogenetic analysis methods, DNA isolation methods, polymerase chain reaction (PCR), Gateway gene cloning system, bacterial and plant transformation methods, and light microscopy methods were used *to investigate the role of genes encoding components of WOX-CLAVATA systems.* RNA sequencing of transcriptomes and methods for analysing transcriptome data and qPCR were used f*or quantitative analysis of gene expression.* The yeast one-hybrid assay was used t*o analyse the interactions of* the WOX4 TF *with the promoters of putative target genes*. Statistical methods were used to validate the results of the study.

The degree of validity and approbation of the results. The main results of the thesis work were presented and discussed at 6 international conferences and published in 6 articles in peer-reviewed scientific journals:

1. **Kuznetsova, X**.; Dodueva, I.; Afonin, A.; Gribchenko, E.; Danilov, L.; Gancheva, M.; Tvorogova, V.; Galynin, N.; Lutova, L. Whole-Genome Sequencing and Analysis of Tumour-Forming Radish (*Raphanus sativus* L.) Line. Int. J. Mol. Sci. 2024. Vol. 25. P. 6236.
2. **Kuznetsova KA**, Dodueva IE, Lutova LA. The homeodomain of the *Raphanus sativus* WOX4 binds to the promoter of the *LOG3* cytokinin biosynthesis gene. Ecological genetics. 2024a. Vol. 22, №. 1. P. 33–46.
3. **Kuznetsova K.,** Efremova E., Dodueva I., Lebedeva M., Lutova L. Functional Modules in the Meristems: “Tinkering” in Action. Plants (Basel). 2023. Vol. 12, №. 20. P. 3661;
4. **Kuznetsova K**., Dodueva I., Gancheva M., Lutova L. Transcriptomic Analysis of Radish (*Raphanus sativus* L.) Roots with *CLE41* Overexpression. Plants (Basel). 2022. Vol. 11, №. 16. P. 2163;

5. Tkachenko A., Dodueva I., Tvorogova V., Predeus A., Pravdina O., **Kuznetsova K.**, Lutova L. Transcriptomic Analysis of Radish (*Raphanus sativus* L.) Spontaneous Tumor. Plants (Basel). 2021. Vol. 10, №. 5. P. 919;
6. **Kuznetsova K.A.**, Dodueva I.E., Pautov A.A., Krylova E.G., Lutova L.A. Genetic control of storage root development. Russian Journal of plant physiology. 2020. Vol. 67, №. 4. P. 589-605 (In Russian).

Other publications on the topic of the thesis:

1. Kuznetsova KA, Efremova EP, Buzovkina IS, Dodueva IE, Lutova LA. Stanislava I. Narbut, the author of the first radish genetic collection in Russia (In Russian). Ecological genetics. 2023. Vol. 21, №. 2. P.167–1822.

2. Dodueva I.E., Lebedeva M.A., **Kuznetsova K.A.**, Gancheva M.S., Paponova S.S., Lutova L.A. Plant tumors: a hundred years of study. Planta. 2020. Vol. 251, №. 4. P. 82.

The scope and structure of the work. The thesis consists of an introduction and four chapters. The full volume of the thesis is 199 pages with 40 figures and 7 tables. The list of references contains 585 titles.

Main scientific results.

1) Genome assemblies of two inbred lines of the radish genetic collection, which contrastingly differ in their ability to spontaneously form tumours, were obtained. 108 genes with single nucleotide substitutions affecting the sequence of the encoded proteins were identified in the tumour-forming line (Kuznetsova et al., 2024a; the main results are presented on pages 4-10; the degree of personal involvement is 80%: obtaining plant material, bioinformatics analysis, preparation of figures, writing 18 of 21 pages of the article, preparation of the article for publication).

2) Genes encoding components of the WOX-CLAVATA system were identified in the genomes of radish inbred lines and their chromosomal localisation was determined (Kuznetsova et al., 2024a; the main results are presented on pages 6-9; the degree of personal participation is 80%: obtaining plant material, bioinformatics analysis,

chromosomal location of genes, preparation of figures, writing 18 of 21 pages of the article text, preparation of the article for publication).

3) New putative targets for the signal peptide RsCLE41 were identified (Kuznetsova et al., 2022; main results are presented on pages 12-14; 80% of personal involvement: experimental methodology, RNA isolation, transcriptome analysis, data visualisation, data validation, writing 14 of 19 pages of the article, preparation of the article for publication).

4) The interaction of the RsWOX4 TF with the TAATCC site in the promoter of the *RsLOG3* gene regulating cytokinin biosynthesis has been demonstrated (Kuznetsova et al., 2024b; the main results are presented on pages 5-8; the degree of personal participation is 80%: obtaining plant material, obtaining vectors for plant transformation, *in silico* analysis, setting up the experiment with yeast one-hybrid assay, histological analysis, statistical analysis, writing 12 of 14 pages of text, preparing the article for publication).

5) An extensive analysis of data on targets and mechanisms of the conserved WOX-CLAVATA systems in different species of higher plants, including on their role in storage root development (Kuznetsova et al., 2020; the main results are presented on page 14; the degree of personal participation is 80%, analysis of data on the conserved role of the WOX-CLAVATA systems, TF and environmental factors in the control of cambium activity and development of storage root in model objects and root crops, writing 15 of 18 pages of text). The concept of conserved regulatory modules (so called gene modules) participating in different aspects of plant development was formulated; a connection between the tinkering concept put forward by F. Jacob in 1977 and their role in modules formation was established (Kuznetsova et al., 2023; the main results are presented on page 29; the degree of personal participation is 80%: formulation of the regulatory module concept at the molecular level during plant development; clarification of the significance of the modular principle in the control of plant organ systems development (on the example of meristems) and in the implementation of molecular mechanisms of development control; analysis of data on the role of the WOX-CLAVATA systems as the most conservative gene module in plant development; writing 29 out of 40 pages of text).

6) Using data on transcriptome analysis of radish tumours, it was shown that genes with identified InDel and SNP are differentially expressed in tumour line 19. Expression levels of the *PCNA1* and the *LOC108817684* genes were shown to be up-

regulated in roots of radish, while expression levels of *SAUR32*, *ERF019*, *LRR-RK*, and *ERF018* were shown to be down-regulated (Tkachenko et al, 2021a; main results are presented on pages 12-14; 50% of personal involvement: growing plants, RNA isolation, analysing the transcriptome for individual genes, validating data with real-time PCR, writing 6 of 20 pages of text).

Defense provisions.

1. For the first time, the genomes of inbred lines of radish of the SPbSU genetic collection, which contrast in their ability to spontaneous tumour formation, were sequenced and analysed. 36 genes with single nucleotide substitutions (SNP) and 72 genes with insertions/deletions (InDel), presumably causing loss of function of encoded proteins, were identified in the tumour-forming line of the SPbSU radish genetic collection compared to the non-tumour line.

2. Genes of the WOX-CLAVATA system (24 genes of the WOX family and 52 genes of the CLE family) were identified in the lines of radish genetics collection and their chromosomal localisation was determined. The genes *RsWOX4-1* and *2*, *RsWOX14*, *RsCLE41-1*, *2*, and *3*, *RsCLE42-1* and *2* were shown to be presumably involved in the control of cambium activity in accordance with the functions of their homologues in other plant species.

3. Overexpression of the *RsWOX14* and *RsWOX4* genes has been shown to cause changes in root stele structure and an increase in the number of secondary xylem cells.

4. New putative targets of the signal peptide RsCLE41 and the transcription factor RsWOX4 were identified. The interaction of the RsWOX4 TF with the TAATCC site in the promoter of the *RsLOG3* gene regulating cytokinin biosynthesis was demonstrated.

CHAPTER 1. LITERATURE REVIEW

This work is aimed at the study of the mechanisms of storage root development, which is among the topical tasks of modern developmental biology and agriculture. One of the most promising objects for storage root genetics studies is radish (*Raphanus sativus* var. *radicula* Pers.), a root crop related to *Arabidopsis thaliana*, the model object of genetics research.

Radish belongs to the *Brassicaceae* family (Cabbage family) and is a variety of sown radish (*Raphanus sativus* L.). The radish genome has been sequenced and annotated (Kitashiba et al., 2014; Xu et al., 2023). The wild ancestor of radish, as well as of daikon, is the wild radish *Raphanus raphanistrum* L. (Lewis-Jones et al., 1982). Radish is an annual plant with a short vegetation period and is characterised by very rapid (within 30 days or less) growth of the taproot and the lower part of the hypocotyl, which makes radish the earliest root crop and a promising object for studying the genetics of storage root development. It is known that the development of the storage root depends on the increased activity of the cambium lateral meristem, which gives rise to the conductive tissues in the plant (Kuznetsova et al., 2020). According to the anatomical classification, radish develops a monocambial storage root of xylem type, with a single cambial ring and a parenchyma consisting of multiple differentiating xylem cells (Sazanova, 1985; Zaki et al., 2012).

A genetic collection of inbred radish lines created by self-pollination of individual plants of different cultivars is maintained at SPbSU (Narbut, 1966; Buzovkina and Lutova, 2007). The collection contains lines originating from three varieties and differing in a number of marker traits, including the degree of storage root development. In addition, the radish genetic collection also contains lines with various morphogenetic anomalies, including lines with spontaneous tumour formation. These tumours which are morphologically and anatomically similar to crown gall induced by *Agrobacterium tumefaciens* are formed on the storage root of certain radish lines during the transition to flowering (Narbut, 1967; Buzovkina and Lutova, 2007; Lebedeva et al., 2015). Due to the complexly regulated multilevel organisation of the systemic control of cell division, as well as due to the modular organisation of the plant organism, plant varieties with spontaneous tumours development are very rare (Dodueva et al., 2020), and therefore such varieties and such tumours could be the objects of interest. According to the data

obtained, spontaneous tumours on radish roots are of cambial origin (Ilina et al., 2006; Lebedeva et al., 2015), and their formation is associated with an increase in the free CKs level (Matveeva et al., 2004) and activation of meristem regulator gene expression (Lutova and Dodueva, 2007; Lebedeva et al., 2015; Tkachenko et al., 2021a). Thus, studies of the processes underlying spontaneous tumour formation are important for understanding the mechanisms of meristematic competence control of cambium cells.

Although the genetic control of cambium activity has now been investigated in detail (Fischer et al., 2019; Wang et al., 2021a), the role for individual cambial regulators in the storage roots development is now at the initial stage of study (Gancheva et al., 2016; Gancheva et al., 2018; Kuznetsova et al., 2020). In this regard, at the SPbSU Department of Genetics and Biotechnology we are studying the genetic mechanisms underlying the control of cambium activity and development of the storage root on the model of radish. In particular, the functions of some components of the WOX-CLAVATA regulatory module (see below), which controls cambium activity and development of the vascular system, in the formation of the storage root in radish and its wild ancestor *Raphanus raphanistrum* have been extensively studied at this Department (Gancheva et al., 2016; Gancheva et al., 2018). A number of unique results were also obtained: studying the anatomy of spontaneous tumours in radish lines revealed their meristematic nature (Lebedeva et al., 2015); the localisation of cell proliferation zones was associated with the distribution of IAA and CK in tissues (Lebedeva et al., 2015; Matveeva et al, 2004); transcriptome analysis of spontaneous tumours revealed increased expression of a large number of genes involved in all stages of cell cycle control in plants (Tkachenko et al., 2021a). However, now we could only speculate about genetics control of spontaneous tumour development in certain radish inbred lines of SPbSU collection. Identifying genes whose sequences differ between tumour and non-tumour lines was an important step in this direction and one of the aims of sequencing the genomes of two inbred radish lines contrasting in tumour formation.

Our work continues the earlier studies on the development of the storage root and spontaneous tumours in radish. This work may contribute to the understanding of the genetic control of cambium lateral meristem activity, as well as the development of structures of cambial origin - the storage root and spontaneous tumours.

1.1. Lateral meristems of higher plants

Meristems are complexly organised aggregates of plant cells that intensively divide and remain physiologically active throughout ontogenesis. Meristems ensure the formation of new organs and tissues and the continuous increase in plant growth. The general properties of meristems include:

• the ability to maintain an undifferentiated state of cells and to produce cells capable of differentiation;

• the presence of stem cells reservoir;

• the presence of organising centres in which conservative regulators, which are TFs, function to maintain the nondeterministic state of meristems.

According to localisation, meristems could be classified as apical: shoot apical meristem (SAM), which can be transformed into inflorescence meristem and then into floral meristem, and root apical meristem (RAM), lateral meristems (procambium; pericycle; cambium which produce xylem and phloem cells; cork cambium, or phellogen), intercalary meristems (meristems of internodes), marginal meristems (meristems of leaf plate margins) and irregular, or facultative meristems (nodule meristem; wound callus; galls and tumours, which may develop *de novo* by cell dedifferentiation or arise from other meristems).

This literature review will focus on lateral meristems (LM), as the object of our study is the storage root of inbred radish lines. The development of the storage root is associated with secondary thickening processes that depend on the cambium LM activity (Kuznetsova et al., 2020). Spontaneous tumours developing on the roots of some radish lines are also LM derived processes and are of cambial or pericyclic origin (Lebedeva et al., 2015).

LMs are located parallel to the lateral surface of the organ in which they are located, and seem like tracts or cylindrical layers of stem cells which give rise to the new tissues. LMs ensure the realisation of such important processes as growth of axial organs by thickening (secondary growth); vascular system and protective tissues development; formation of secondary AMs and LMs; regeneration; interactions with pathogens and symbionts by several ways; nutrient storage (especially important for the development of the storage root), etc. (Dodueva et al., 2014; Serra et al., 2022). The location of LMs in the plant axial organs and their alterations during ontogenesis are presented in Figure 1.

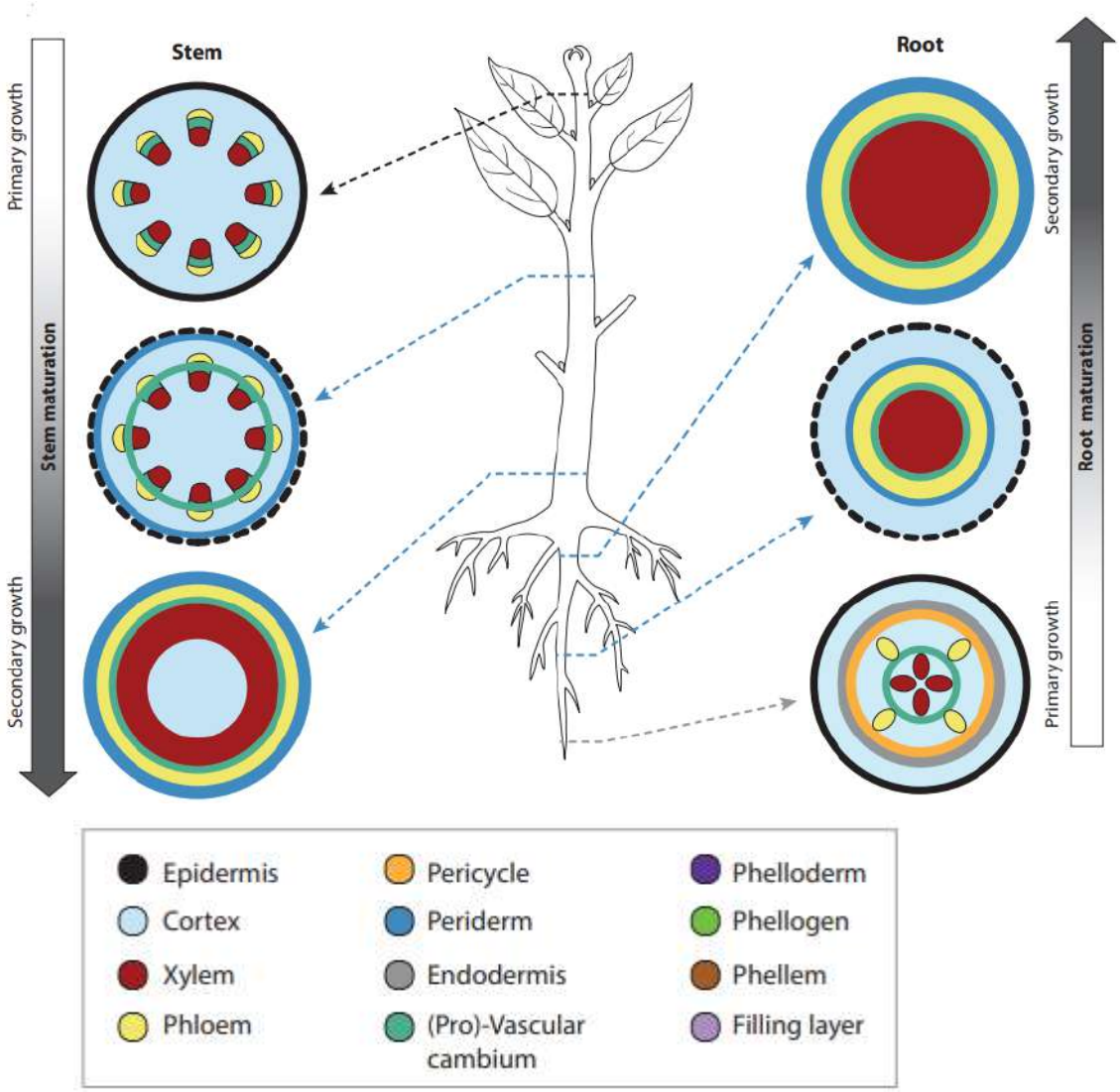

Figure 1. Localisation of lateral meristems and tissues during stem and root development (from Serra et al., 2022).

All LMs are classified into primary LMs, which are formed during embryonic development, and secondary LMs, which arise in postembryonic development from primary LMs. Primary LMs in turn are classified into procambium and pericycle, while secondary LMs are classified into cambium and cork cambium, or phellogen (Pautov et al., 2012). During plant development, procambium forms vascular tissues of stem and root, ensuring their growth by thickening. Pericycle is the most pluripotent meristem that gives rise to LMs (cambium, phellogen), AMs of lateral roots, as well as callus and AM of shoots during *in vitro* regeneration (see the review by Dodueva et al., 2014).

LM development is regulated by a number of factors, including phytohormones and TFs. Antagonistic interaction between CK and IAA are central to the process of LM development (Bishopp et al., 2011). Phytohormones, in particular IAA and CK, linked by

multiple feedback loops with TFs of the WOX and KNOX (KNOTTED1-related homeobox) families, which control stem cell maintenance and proliferation in meristems. Such interactions were initially described in AMs (Jasinski et al., 2005, Leibfried et al., 2005; Zhang et al., 2017; Tian et al., 2014), but subsequently similar connections have been identified in LM, particularly in cambium and phellogen (Suer et al., 2011; Smetana et al., 2019; Fu et al., 2021). Besides WOX and KNOX, other families of TFs, such as several classes of homeodomain and leucine zip (HD-ZIP) TFs (Smetana et al., 2019) and AINTEGUMENTA (ANT) TFs from the APETALA2 family, are also involved in the regulation of LM development (Randall et al., 2015). Overall, WOX TFs in LMs, as in other meristems, act as TFs of organising centre and stem cell pool maintenance signals. At the same time, other groups of TFs fulfil diverse functions, from meristem establishment and proliferation of its cells to differentiation of specific tissues on its periphery (Dodueva et al., 2014; Kuznetsova et al., 2020; Serra et al., 2022).

Also, CLE peptide phytohormones and their receptors play an important role in the control of LM activity (at least in the cambium) and regulate the expression levels of *WOX* genes functioning there (Hirakawa et al., 2010). CLE peptides, alongwith their receptors of the serine-threonine-rich repeat receptor protein kinases (leucine-rich repeat receptor kinases, LRR-RK) family and their targets, *WOX* genes, constitute the so-called WOX-CLAVATA system, a conserved regulatory module that controls stem cell pool size in various meristems (Dodueva et al., 2016; Kuznetsova et al., 2023). An essential part of this regulatory module is also the GRAS HAIRY-MERISTEM (HAM) family TFs, which interact with WOX TFs in different meristems and, at least in AMs, influence WOXes movement from the organising centre to the stem cell proliferation zone (Zhou et al., 2015; Geng, Zhou, 2021).

The regulatory pathways controlling the functioning of LMs will be discussed in detail further this literature review.

1.1.1. Pericycle as a pluripotent primary meristem

The pericycle consists of a layer of undifferentiated pluripotent cells that surrounds the vascular bundle and can form other types of tissue, including meristematic tissues, under different conditions. This primary LM plays a central role in the formation of various secondary meristems such as lateral root (De Smet et al., 2006), symbiotic nodule formed under rhizobial inoculation of legumes (Ferguson et al, 2010), cambium ring formation and secondary thickening in dicotyledons (Baum et al., 2002), phellogen differentiation with the formation of periderm, which is a stress-protecting plant suberised barrier, as well as during the callus formation and plant regeneration *in vitro* (Atta et al., 2009; Figure 2). Pericycle is laid down in embryogenesis simultaneously with procambium (Scheres et al., 1994).

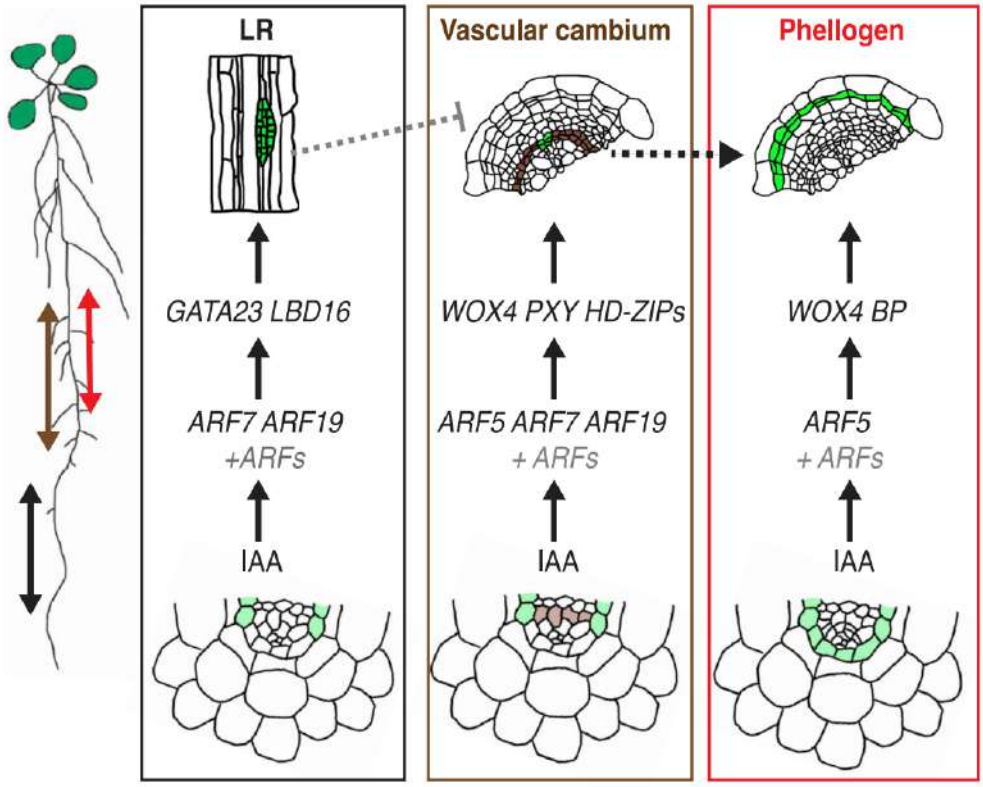

Figure 2. Regulation of lateral root, phellogen and vascular cambium formation from pericycle cells (by Xiao et al., 2020). Light green, pericycle cells; green, pericycle-derived cells; light brown, procambium cells; brown, procambium-derived cells. Bidirectional arrows indicate root zones where initiation occurs: lateral root (black); periderm (red); vascular cambium (brown). Genes and arrows highlighted in grey and all dashed lines indicate putative interactions. The development of the vascular cambium is initiated after the repression of the lateral root (LR) developmental programme and probably depends on the repression of the unstudied ARF activity. Phellogen formation depends on vascular cambium activity.

Among the two types of pericycle cells (xylem and phloem), only the cells of the first type are able to form other meristem types because of their undifferentiated state (Baum et al., 2002, Parizot et al., 2008). In xylem pericycle cells, genes of the primary response to IAA are mainly expressed; in phloem pericycle cells, as well as in cambium, genes of the primary response to CK are expressed. These dependencies illustrate the connection between the character of vascular tissues and pericycle development and the distribution of these antagonist hormones (Bishopp et al., 2011). Among TFs, the SHORTROOT TF, which forms a heterodimer with the SCARECROW TF that controls the vascular system development, is involved in the regulation of the xylem pericycle (Helariutta et al., 2000, Cui et al., 2011). The targets of such dimers are genes encoding TFs of the HD-ZIPIII (Homeodomain/Leucin rich Zipper III) family, which regulate vascular development (Emery et al., 2003).

The lateral root development is the main function of the pericycle. The lateral root is formed from pericycle cells located opposite the xylem pole (Dubrovsky et al., 2000), which retain mitotic activity for a long time and are called lateral root founder cells (De Smet et al., 2006). Auxins play a key role in lateral root formation (Dubrovsky et al., 2008) by acting on the cyclin-dependent kinases CDKA and CDKB1;1 and stimulating plant cell division. Auxins can also diminish the expression of genes encoding KRP cell cycle inhibitor proteins by stimulating pericycle cell division (Beeckman et al., 2001). The concentration of auxins in xylem pericycle cells fluctuates, which controls the periodicity of cell divisions and lateral root establishment (De Smet et al., 2006). Auxin targets in lateral root founder cells are GATA23 (De Rybel et al., 2010) and LBD16 (Xiao et al., 2020) TFs. CKs repress lateral root formation (Laplaze et al., 2007) by affecting the expression of *PIN-FORMED (PIN)* genes, which encode proteins for IAA polar transport, and by disrupting auxin gradient formation (Ruzicka et al., 2009). In the pericycle, a high level of CK response is observed in the zone between the lateral root primordia. The genes of the WOX-CLAVATA system regulating RAM activity, the *WOX5*, encoding TF, and the *Arabidopsis Crinkly 4 (ACR4)*, encoding the receptor for CLE40 peptide, are also expressed in the lateral root primordia at early stages of root development. The *WOX5* gene expression in the pericycle in lateral root primordia cells is regulated by auxins (Stahl, Simon, 2009; Gonzali et al., 2005). The activity of *ACR4* reppresses cell divisions in pericycle cells neighboring the lateral root primordium (Stahl, Simon, 2009).

In the *Arabidopsis* root, cells of the xylem pericycle participate in the formation of the secondary LM **cambium** along with the procambium; as a result, the cambium acquires the form of a continuous cell layer. In particular, it is the pericycle that plays a central role in cambium formation in monocotyledons (Jura-Morawiec et al., 2021). The WOX4, PXY, and HD-ZIPIII TFs are involved in the control of cambium activity (see *Cambium* section).

At the same time, pericycle cells of phloem type give rise to the **phellogen**, or cork cambium, which, like the cambium, subsequently form an enclosed ring. The cork formed from phellogen cells, after the deadening and banishment of cortex and endoderm cells, fulfils the function of the covering tissue of the root. Among the TFs that stimulate phellogen formation are WOX4 (WOX family) and KNAT1/BREVIPEDICELLUS (BP) (KNOX family) (Xiao et al., 2020), whose gene expression is regulated by auxin. These TFs and their auxin-dependent regulation are important for cambium specification (Liebsch et al., 2014, Hirakawa et al., 2010, Suer et al., 2011, see *Phellogen* section).

Pericycle cells located opposite the poles of the protoxylem participate in **callus formation and regeneration processes** (Atta et al., 2009). Due to the division of xylem pericycle cells, outgrowths similar to the primordia of lateral roots are formed on plant explants. *PLETHORA1*, *SCR*, *SHR*, and *GLABRA2* genes characteristic of lateral roots are expressed in these outgrowths (Atta et al., 2009, Sugimoto et al., 2010). The *WOX5* gene is expressed in subepidermal cell layers (Sugimoto et al., 2010). Transfer of explants to medium with auxin induces the transformation of callus primordia into lateral roots; when transferring to medium with CK, callus primordia were transformed into shoots (Atta et al., 2009).

The pericycle is involved in some types of **plant-microbial interactions**, namely the formation of nitrogen-fixing nodules, as well as root outgrowths induced by *Agrobacterium* and parasitic nematodes. The development of nodules and lateral roots are controlled by common components (Searle et al., 2003); the formation of their primordia initiates cell divisions in the xylem pericycle. The nodule has its own meristem, the development of which is controlled by the *WOX5* gene, which is the target of the autoregulation of nodulation system including CLE peptides and their receptors (Osipova et al., 2012). Pericycle is involved in the formation of undetermined-type nodules, which, in contrast to determinate-type nodules, are able to maintain their meristematic activity for a long time (Hirsch et al., 1989). Pericycle is also important for the interaction of

plants with pathogens that cause the formation of **galls and tumours** on roots (see *Tumours in higher plants* section). The T-DNA transfer and integration during infection of root explants with *Agrobacterium tumefaciens* occurs predominantly in pericycle cells (De Buck et al., 2000). When the plant is infected by nematodes, nematode feeding sites are formed where pericycle and xylem parenchyma cell divisions are activated. The formation of such sites is associated with changes in the expression pattern of cell cycle genes (De Almeida, Gheysen, 2013).

Thus, the pericycle gives rise to a variety of secondary meristem types, including such LMs as cambium and phellogen, as well as pathogen-induced plant tumours.

1.1.2. Procambium and cambium as meristems of vascular tissues

The procambium primary meristem is forming during embryogenesis at the heart stage, simultaneously with another primary LM, the pericycle (Scheres et al., 1994). During postembryonic development, the tissues that form the plant's vascular system, the primary phloem and xylem, are developed from the procambium (Baum et al., 2002). The cambium is a secondary LM that gives rise to the secondary vascular tissues of plants and is almost identical to the procambium in structure, function, and regulatory pathways. In the root, the cambium is formed with the participation of the primary LMs, the procambium and pericycle (Smetana et al., 2019; Shi et al., 2019).

During the evolution of seed plants, two types of secondary LMs that give rise to vascular tissues have emerged: the vascular cambium, characteristic of the gymnosperms and dicotyledons, and the monocot cambium. The cambium of dicotyledons is formed from pluripotent procambium cells in vascular bundles (bundle cambium) and pericycle (interfascicular cambium) and has a shape of a continuous layer of cells. Cells of the bundle cambium, dividing asymmetrically with subsequent differentiation, give rise to conductive elements of xylem and phloem, and derivatives of the interfascicular cambium give rise to parenchyma ray cells (Fisher et al., 2019).

The monocot cambium forms outside the primary vascular bundles from the pericycle. Its cells then divide periclinally and deposits secondary cortex on the outside and secondary tissue with secondary xylem and phloem located in the vascular bundles on the inside (Jura-Morawiec et al., 2021). Both types of cambium are similar in their concentric structure and contain initial cells that undergo periclinal divisions and

differentiate to form secondary vascular tissues, and play a similar role in the radial growth of axial plant organs.

The cambium zone of dicotyledonous plants consists of one layer of asymmetrically dividing undifferentiated cambial initiates (stem cells) and mother cells of phloem and xylem (progenitor cells). Accordingly, there are three domains in the cambium: proximal (consisting of xylem mother cells), distal (including phloem mother cells) and central (consisting of stem cells). The cell layer of the proximal domain, adjacent to the xylem, acts as an organising centre in the cambium (Smetana et al., 2019).

Cambium stem cells undergo asymmetric periclinal divisions, each resulting in one xylem and one phloem progenitor cell (Smetana et al., 2019; Shi et al., 2019). These cells divide symmetrically on either side of the cambial zone, forming differentiating secondary xylem cells towards the centre and secondary phloem cells towards the periphery. The secondary xylem and phloem include conductive and mechanical elements of different types, cells of axial parenchyma and radial rays. The phloem and xylem also serve as sources of signals that determine cambium development: IAA, which regulating the maintenance of the organising centre of the cambium, are delivered via the xylem and its progenitor cells, and CLE peptides of the TDIF family, that maintain the proliferative activity of the cambium, are delivered via the phloem (Smetana et al., 2019; Hirakawa et al., 2010).

The cambium activity depends on the balance of cell proliferation and differentiation. Numerous studies have identified various cambium regulators including TFs of different families and phytohormones interacting with them (reviewed by Kuznetsova et al., 2020).

1.1.2.1. WOX-CLAVATA system in the regulation of cambium activity

The main role in the regulation of cambium stem cell maintenance belongs to the WOX4 TF (Ji et al., 2010), and its interacting cofactor, HAM4 TF (Zhou et al., 2015), as well as to its partner WOX14 TF (Etchells et al., 2013). The *WOX4* and *WOX14* genes are targets of a signalling pathway induced by the CLE peptide group (Hirakawa et al., 2010; Etchells et al., 2013), and thus are part of the WOX-CLATATA cambial module. The involvement of the WOX-CLAVATA system in cambium development is summarised in Figure 3.

The WOX-CLAVATA system controls the homeostasis of cambium LM through a positive regulatory cascade that targets the expression of the *WOX4* and *WOX14* genes. The balance of the cambium stem cell pool is under the control of a small specific group of CLE peptides called TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF), also known as a group B CLE peptides. In *Arabidopsis*, the TDIF group includes the CLE41/CLE44 peptides (products of the *CLE41* and *CLE44* genes, yielding identical mature peptides) and CLE42 (Ito et al., 2005). The concentration gradient of TDIF peptides formed when TDIF enters from the phloem is important for the proper orientation of cambium cell division. A shift of this gradient leads to a change in the orientation of cell divisions and disruption of the ordered structure of the stele. TDIF peptides are produced by phloem cells and then move to the cambium where they bind to their receptor called TDIF RECEPTOR/ PHLOEM INTERCALATED WITH XYLEM (TDR/PXY), a member of the LRR-RLK family, and its homologues called PXY-like (PXL1 and PXL2) (Hirakawa et al., 2010; Jung et al., 2015). In contrast to apical meristems, where CLE peptides act as negative regulators of stem cell maintenance and expression of *WUS* in SAM and *WOX5* in RAM (Schoof et al., 2000; Stahl et al., 2009), the interaction of TDIF peptides with the PXY receptor represses cambium stem cell differentiation into secondary xylem and promotes the maintenance of this LM identity (Hirakawa et al., 2010).

Another pathway for positive regulation of the *WOX4* expression is auxin-dependent control through Auxin-Response Factor (ARF) and HD-ZIPIII TF families. These TFs are involved in xylem formation; in addition, this pathway is important for the establishment of the cambium organising centre (Smetana et al., 2019; Shi et al., 2019).

In contrast, TDIF-dependent control of cambium cell division requires a functionally active PXY receptor and is independent of WOX4 TF activity (Etchells and Turner, 2010). The *WOX4* has been implicated in the regulation of cell proliferation but not xylem differentiation, suggesting different targets for TDIF-PXY action (Hirakawa et al., 2010). There are several evidence that *WOX4* acts downstream of *PXY* in the pathway, promoting vascular cell division together with *WOX14*, whose expression is increased in response to prolonged TDIF treatment, as *wox14* enhances the cell division defect in *wox4* mutants (Etchells et al., 2013). TDIF peptides are thought to convey positional information through the PXY receptor, supporting activity and asymmetric cambial stem cell divisions through three distinct pathways:

Firstly, activation of the PXY receptor represses xylem cell differentiation by associating with BRASSINOSTEROID INSENSITIVE2 (BIN2), BIN2-LIKE1 (BIL1) and BIL2 glycogen synthase kinase 3 (GSK3) family proteins at the plasma membrane (Kondo et al., 2014). This interaction, in turn, leads to phosphorylation and subsequent destabilisation of brassinosteroid-dependent BRASSINAZOLE-RESISTANT1/BRI1-EMS-SUPPRESSOR1 (BES1/BZR1) TF, which promote xylem differentiation (Saito et al., 2018). Thus, TDIF signalling through the PXY receptor represses differentiation of cambium stem cells without the regulation of *WOX4* and *WOX14* expression (Etchells et al., 2013).

Secondly, TDIF-PXY signalling promotes cambial cell proliferation through activation of *WOX4* and *WOX14* gene expression, and it has been suggested that the TDIF-PXY-WOX4/14 regulatory module controlling cambial cell division acts in parallel with the TDIF-PXY-BIN2-BES1 pathway regulating differentiation of cambial stem cells into xylem (Etchells et al., 2013).

Thirdly, the TDIF-PXY interaction affects the recruitment of cambium stem cells to the phloem lineage. This pathway is mediated by PXY-dependent regulation of WOX14 TF, a probable target of which is the gene encoding TARGET OF MONOPTEROS6 (TMO6) TF, which is also regulated by auxin and MONOPTEROS TFs of the ARF family (Schlereth et al., 2010). The WOX14 and TMO6 TFs regulate the expression of the *LATERAL ORGAN BOUNDARIES DOMAIN 4 (LBD4)* gene, which encodes a TF active in cells localised at the procambium-phloem interface and involved in cambium cell division and phloem differentiation (Smit et al., 2020).

Other components of the WOX-CLAVATA cambial system in *Arabidopsis* include two close homologues of TDR/PXY receptors, PXY-LIKE (PXL1 and PXL2) (Jung et al., 2015) and SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) group co-receptors that interact with PXY to bind TDIF peptide ligands (Zhang et al., 2016c). In addition, two other LRR-RK receptors, MORE LATERAL GROWTH 1 (MOL1) and REDUCED LATERAL GROWTH 1 (RUL1), are also involved in cambium development: the phenotype of *mol1* and *rul1* mutants allow to conclude that the MOL1 receptor is a negative regulator represses cambial cell proliferation independently of TDR/ PXY, whereas RUL is a positive regulator (Gursanscky et al., 2016).

Differentiation of cambium cells along the phloem or xylem pathway is also controlled by CLAVATA-like (possibly WOX-CLAVATA) systems. In particular,

development of phloem, a tissue that produces TDIF peptides, is controlled by CLE25 and CLE45 peptides, their receptors CLV2/CRN and BAM3, and co-receptors CIK and CLERK (Ren et al., 2019). The CLE10 peptide restricts vascular development by inhibiting the expression of *A-type ARR*, *ARR5* and *ARR6* genes encoding repressors of CK signal transduction (Kondo et al., 2011). In addition, the identification of a novel gene, *AtCLE33*, which has a high percentage of similarity to *AtCLE45* and controls protophloem formation, was recently reported in *Arabidopsis*. The *AtCLE33* has been shown to be expressed in the developing protophloem and perceived by BAM3. The *CLE33 CLE45* double mutants exhibited ectopic differentiation of the protophloem in neighbouring cells, consistent with the model that phloem-specific CLE peptides act as paracrine signals to maintain a single functional protophloem cell. The orthologues of *CLE33* have been found in angiosperms, monocotyledons, and eudicots, and duplication of the gene that gave rise to *CLE45* in *Arabidopsis* and other *Brassicaceae* appears to be recent (Carbonnel et al., 2023). In contrast, CLE19 peptide activates xylem development: the *CLE19* overexpression leads to the formation of xylem 'islands', unrelated to the plant vascular system, in *Arabidopsis* flower and cabbage organs (Fiers et al., 2004), and increases the number of xylem elements - vessels and woody xylem parenchyma - in the radish storage root (Gancheva et al., 2016).

Possible targets of WOX TFs in cambium

Thus, the WOX-CLAVATA signalling module plays a central role in the control of cambium activity and differentiation of its derivatives. Numerous data on the genetic control of xylem and phloem differentiation have been obtained (see below). At the same time, little is currently known about direct targets of WOX4 and WOX14 TFs (except for the data on the participation of WOX14 TFs in the regulation of *TMO6* and *LBD4* expression, mentioned above), and the search for such targets is an important task of plant development genetics.

At the same time, data were obtained on the targets of other WOX family TFs that control apical meristem development, WUS and WOX5 (Table 1). Since data on the sequences of WOX TF binding sites in the promoters of target genes have been obtained (Lohmann et al., 2001; Busch et al., 2010) and a number of WUS and WOX5 TF target genes have been found, the initial search for putative candidates for the role of WOX4 and WOX14 targets can be based on the presence of certain binding sites in the

promoters, as well as on the belonging to certain gene families that are regulated by other WOX TFs.

The domain structure of WOX TFs suggests that they can be both positive and negative regulators of transcription (Rodriguez et al., 2016). According to the literature (Table 1), identified targets of WUS TFs include *CLE* family genes, *CLV3* and *CLE40*, which in turn regulate *WUS* gene expression as part of the WOX-CLAVATA systems. Interestingly, expression of the *CLV3* gene (a negative regulator of *WUS* gene expression) is positively regulated by WUS TFs (Schoof et al., 2000), while expression of the *CLE40* gene (a positive regulator of *WUS* gene expression) is negatively regulated (Schlegel et al., 2021). Thus, both positive and negative feedback loops are operative in the regulation of the SAM stem cell pool. Also, WUS TF negatively controls the expression of genes encoding A-type ARR repressors of CK signal transduction (Leibfried et al., 2005) and one of the CK biosynthesis enzymes, LOG4 (Chickarmane et al., 2012). The targets of negative regulation of WUS TFs also include genes that control leaf primordium formation: *ASYMMETRIC LEAF 2 (AS2), KANADY1 (KAN1),* and *YABBY3 (YAB3)* (Yadav et al., 2013). At the same time, WOX5 TF as a central regulator of RAM stem cells negatively regulates the expression of the cell cycle regulator gene *CYCD3;1*, ensuring slow division of organising centre cells (Forzani et al., 2014), and the gene encoding CYCLING DOF FACTOR 4 (CDF4) TF, a regulator of columella initialis differentiation (Pi et al., 2015). Targets of direct positive regulation of WOX5 TF include genes of the IAA biosynthesis pathway via indole pyruvic acid (Savina et al., 2020) and the PLETHORA3 (PLT3) TF coding gene, a regulator of RAM stem cell division and IAA transport (Burkart et al., 2022).

Genes expressed in cambium and/or vascular tissues of radish root (according to transcriptome analysis performed by Tkachenko et al., 2021) and belonging to the same families as the abovementioned WUS and WOX5 TF targets were screened in our work as possible WOX4 TF targets (see *Results* section).

Table 1. Known targets of WOX family TFs controlling meristem development. Genes with decreased levels of expression are highlighted in red; genes with increased levels of expression are highlighted in green.

WOX TF	Target gene (what it encodes)	Function of the target gene	Reference
WUS (regulator of the SAM)	*CLV3* (signal peptide)	Repression of the *WUS* (negative feedback loop), restriction of the SAM stem cell pool	Schoof et al., 2000; Schlegel et al., 2021
	CLE40 (signal peptide)	Activation of *WUS* (positive connection) and maintenance of the stem cell pool in the SAM; repression of *WOX5* and limiting the size of the RAM stem cell pool	Schlegel et al., 2021
	ARR5, ARR6, ARR7, ARR15 (signal transduction repressors)	Repressing of the CKs response	Leibfried et al., 2005
	AS2 (TF)	Laying of leaf primordia, development of the upper side of the leaf	Yadav et al., 2013
	KAN1 (TF)	Development of the lower side of the leaf, inhibition of IAA transport	Yadav et al., 2013
	YAB3 (TF)	Development of leaf underside, proliferation of leaf cells	Yadav et al., 2013
	LOG4	CK biosynthesis in the SAM epidermis; negatively regulated by *WUS*	Chickarmane et al., 2012
WOX5 (regulator of the RAM)	*CDF4* (TF)	Columella cell differentiation	Pi et al., 2015
	PLT3 (TF)	Root meristem stem cell division, maintenance of IAA transport	Burkart et al., 2022
	TAA1, YUC1-7 (enzymes)	Biosynthesis of IAA via indole pyruvic acid	Savina et al., 2020
	CYCD3;3	Columella cell proliferation	Forzani et al., 2014

1.1.2.2. Other groups of TFs in the regulation of cambium activity

The development of the cambium and vascular system of plants is controlled by several families of TFs. The participation of TFs of different groups in cambium regulation is shown in Figure 3.

KNOX TFs were initially identified as central regulators of the SAM development. They also play a role in the development of the cambium and the vascular system. The STM and KNAT1 TFs exert vascular and xylem fibre differentiation by repressing transcription of genes encoding BLADE-ON-PETIOLE1 and BLADE-ON-PETIOLE2 TFs, which define leaf primordia boundaries (Woerlen et al., 2017).

PHABULOSA, CORONA, REVOLUTA, and ARABIDOPSIS THALIANA HOMEOBOX8 of **HD-ZIPIII** TFs control polar transport of IAA, the early stages of xylem differentiation (Baima et al., 2001; Smetana et al., 2019), and the maintenance of the central domain of the cambial zone and the expression of its controlling genes, the *WOX4* and the *ANT* (Smetana et al., 2019).

In contrast, mobile TFs of the **PHLOEM EARLY DOF 1 and 2 (PEAR1, 2)** groups transported from the phloem stimulate periclinal cambial cell divisions by activating the expression of genes encoding HD-ZIPIII TFs (Miyashima et al., 2019).

The **ANT TF**, which belongs to the APETALA2 family, is required for cambium stem cell proliferation: its direct targets include cyclin class D genes. The *ANT* gene is active in the cambium and also determines the size of plant organs (Randall et al., 2015).

The IAA-regulated **AUXIN RESPONSE FACTOR5/MONOPTEROS (ARF5/MR) TF**, which controls the expression of *PIN* genes, also regulates cambium development (Krogan et al., 2016). The MR limits the number of cambium stem cells through *WOX4* gene repression; the ARF3 and ARF4 regulate WOX4 in the cambium positively (Brackmann et al., 2018). The gene encoding the **TARGET OF MONOPTEROS5 (TMO5) TF** is also a target of MR TF: TMO5 interacts with the **LONESOME HIGHWAY (LHW) TF** and enables specification of proto- and metaxylem cells (Ohashi-Ito et al., 2013).

The **MADS-boxed TFs** also regulate cambium development. They are involved in root development in *Arabidopsis* (Alvarez-Buylla et al., 2019), potato (Gao et al., 2018), yam (Noh et al., 2010, Ku et al., 2008). Overexpression of the *SRD1* gene of yam has

been shown to cause enhanced proliferation of cambium and metaxylem cells in this plant (Ku et al., 2008).

Two group of TFs with **NAC domain**, **VASCULAR-RELATED NAC DOMAIN PROTEIN (VND)** and **SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN (SND),** are responsible for the differentiation of xylem elements, controlling cell wall lignification and protoplast die-off. Putative targets of SND TFs are cellulose and lignin biosynthesis genes, and targets of VND TFs are genes controlling vascular protoplast destruction (Kubo et al., 2005, Ohashi-Ito et al., 2010).

The **ALTERED FLOEM DEVELOPMENT TFs** of the MYB family control protophloem differentiation. It is likely that phloem and xylem differentiation programmes competitively inhibit each other (Bonke et al., 2003). **The LATERAL ORGAN BOUNDARIES1 TF** regulates the early stages of phloem differentiation. In *LBD1* mutants, the number of phloem cells is reduced, and the expression level of *KNOX* genes that stimulate xylem differentiation is increased (Yordanov et al., 2010).

1.1.2.3. Phytohormones in the regulation of cambium activity

Since all stages of plant development are regulated by phytohormones, different groups of these signal molecules are also involved in controlling the development of cambium and other LMs. Signal cascades regulate the cambium activity and differentiation of vascular tissues with the participation of phytohormones and TFs are schematically shown in Figure 3.

As mentioned above, CLE peptide hormones are central regulators of meristem activity, including cambium, and of plant vascular system development.

In addition to CLE peptides, another group of peptide phytohormones, **EPFL (EPIDERMAL PATERNING FACTOR LIKE) peptides**, are also involved in the regulation of cambium activity and also play a role in controlling the development of epidermal derivatives, such as stomata and trichomes (Torii, 2021). Subsequently, it was shown that some EPFL family peptides also act as positive regulators of cambium activity. The EPFL6 and EPFL4 peptides are synthesised in the endoderm and move to the phloem, where they bind to receptors of the ERECTA (ER) family, also belonging to LRR-RK (Tameshige et al., 2017). The targets of ER receptor protein kinases are the components of MAP kinase cascade (Jewaria et al., 2013). In the regulation of stomatal

differentiation, TFs of the basic helix-loop-helix (bHLH) SPECHLESS, MUTE and FAMA families act as targets of MAP kinases acting downstream of ER receptors, each regulating a different stage of stomatal closure cell differentiation (Chowdhury et al., 2021). These TFs do not participate in the control of cambium development, and the targets of the EPFL-activated MAP kinase cascade in this process are unknown.

The antagonistic interaction of **IAA** and **CK** is important for the development of the procambium during embryogenesis, cambium formation and the process of secondary growth. These hormones are asymmetrically distributed in the stele of *Arabidopsis* roots: IAA is localised in the primary xylem and pericycle cells, whereas CK is localised in the procambium and primary phloem (Bishopp et al., 2011).

IAA is a major contributor to xylem development: the IAA-regulated MONOPTEROS/AUXIN RESPONSE FACTOR 5 (MP/ARF5) TF regulated the expression of genes encoding the TARGET OF MONOPTEROS 5 (TMO5) and the HD-ZIPIII TFs, which control xylem-type differentiation of cambium cells (Smetana et al., 2019, Brackmann et al., 2018). In addition, IAA also controls cambium development: the interaction between TMO5 and LHW TFs regulated by IAA determines periclinal divisions of the cambium (Ohashi-Ito et al., 2010), and HD-ZIPIII TFs are essential for maintaining the cambium organising centre (Smetana et al., 2019). High levels of IAA in the proximal domain of the cambium are maintained through a IAA polar transport system (Bennett et al., 2016).

CKs control cambium specification and proliferation of its cells (Bishopp et al., 2011, Matsumoto-Kitano et al., 2008) and are concentrated in the cambium and phloem (Bishopp et al., 2011), where they activate the expression of genes responsible for cell divisions: the *ANT* and *CYCD3;1* genes in the central domain of the cambium (Randall et al., 2015), and the *PEAR* genes in the phloem and distal domain of the cambium (Miyashima et al., 2019). The antagonism of CKs and IAA in the cambium is manifested as follows: CKs negatively regulate the expression of the IAA polar transport regulators, the *PIN1* and the *PIN7*; while IAA induces the expression of the *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6 (AHP6)* gene, whose product is a negative regulator of CK signal transduction (Bishopp et al., 2011).

In addition, other phytohormones like gibberellins, ethylene, brassinosteroids, and strigolactones are also important for cambium development.

Gibberellins were shown to stimulate the differentiation of xylem lignified elements (Mauriat and Moritz, 2009, Ragni et al., 2011). The gibberellin biosynthesis genes are targets of KNOX TFs negative regulation (Jasinski et al., 2005). It is likely that gibberellin biosynthesis or transport is controlled by the WOX14 TF, as overexpression of the *WOX14* increases the level of active gibberellins in the stele, and developmental defects in the *wox14* mutant are compensated for by the application of gibberellins (Denis et al., 2017).

Ethylene stimulates cambium cell division, increasing cambium and xylem zones. The *ASO (ACC OXIDASE)* gene, which controls the limiting step of ethylene biosynthesis, is expressed only in differentiating secondary xylem (Love et al., 2009). The gene encoding the ERF1 (Ethylene Response Factor) TF also controls cambium and xylem cell development. In *tdr/pxy* mutants, increased expression of *ERF1* indicates a possible antagonistic interaction between TDR/PXY-dependent and ethylene-dependent pathways regulating cambium activity (Etchells et al., 2012).

Strigolactones stimulate cambium activity and secondary stem and root growth: these processes are controlled by the *MAX (MORE AXILLARY BRANCHES)* family genes and their antagonists, the *SUPPRESSOR OF MAX 2 (SMAX2)-LIKE* family genes *(SMXL3, SMXL4* and *SMXL5),* which encode strigolactone signal transduction repressors and control phloem development (Agusti et al., 2011, Wallner et al., 2017).

Brassinosteroids positively regulate xylem vascular differentiation (Yamamoto et al., 2001) and are controlled by the *BZR1* and *BES1* brassinosteroid-dependent genes (Kondo et al., 2014). The *GSK3* family genes, *BIN2, BIL1* and *BIL2*, repress brassinosteroid signalling through inhibitory phosphorylation of the BZR1 and BES1 TFs. As mentioned above, BIN2, BIL1, and BIL2 proteins also interact with the TDR/PXY protein, a component of the WOX-CLAVATA system that regulates the cambium/xylem balance (Kondo et al., 2014). In addition, GSK3 proteins also regulate the response to IAA by phosphorylating IAA-dependent ARF TFs to release them from complexes with transcriptional repressors. Thus, the target of BIL1 kinase is the MR TF, a key regulator of xylem differentiation (Sehr et al., 2010).

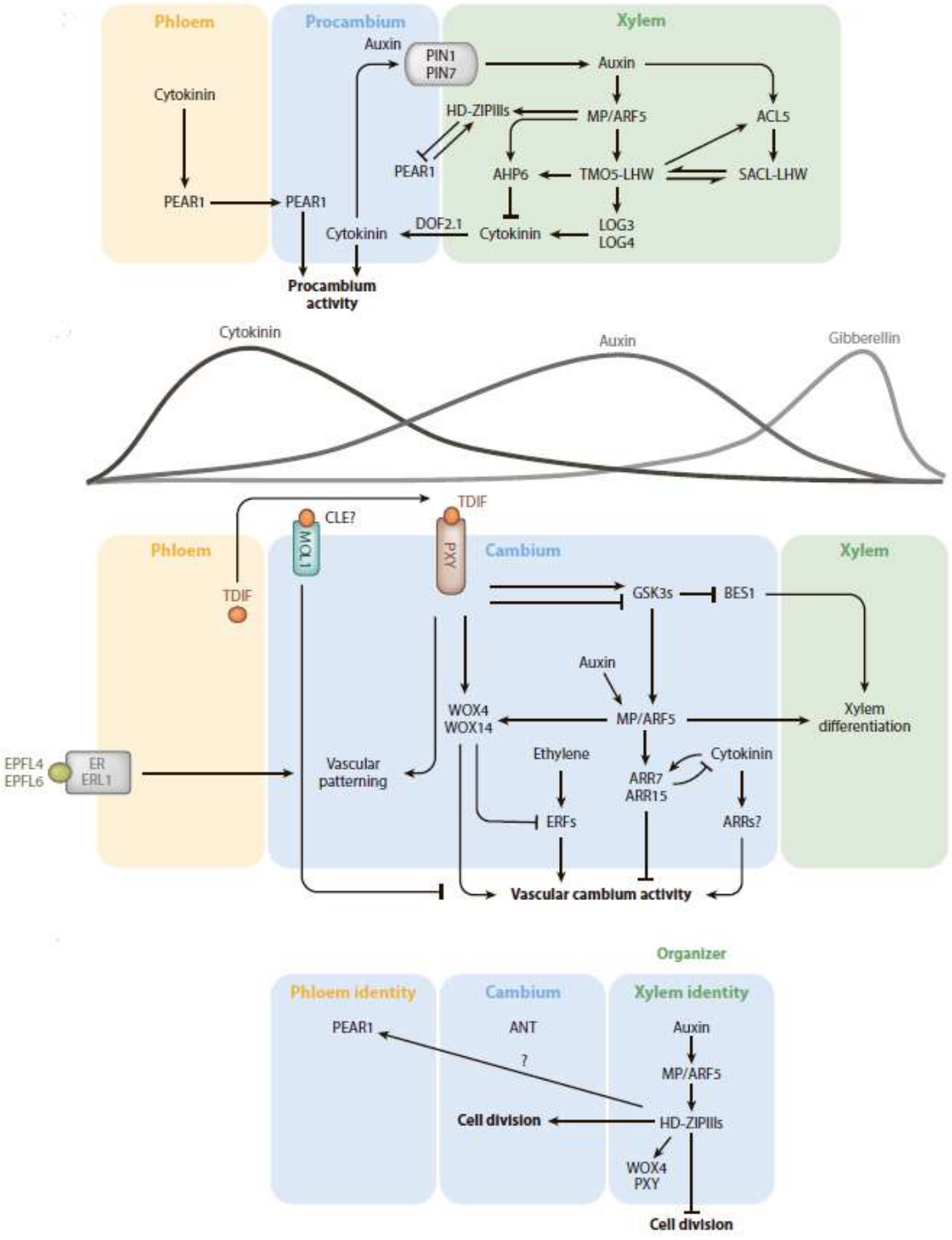

Figure 3. Involvement of TFs and phytohormones in the control of cambium activity and vascular tissue differentiation (from Fisher et al., 2019, details in text).

1.1.3. Phellogen as a cork cambium

The secondary LM phellogen is a part of the periderm, the covering tissue of the axial organs of perennial plants, which also includes the cork (phellem) and phelloderm. The phellogen (also called cork cambium) is the middle layer of the periderm, which consists of living dividing cells. Cell division of the phellogen results in the differentiation of the protective covering tissue cork to the outside and one or more layers of cells of the phelloderm, which is composed of parenchyma cells, to the inside.

The formation of the periderm begins after the laying of the vascular cambium. In stems of most perennial plants, the phellogen is formed of the subepidermal layer cells or, less frequently, of epidermis, phloem or cortex cells (Serra et al., 2022). In roots, the site of phellogen embedding is the pericycle (Wunderling et al., 2018). In potato tubers, the periderm is formed of hypodermis cells during radial growth (Kumar and Ginzberg, 2022).

The processes leading to the differentiation of the phellem from phellogen cells have been studied in detail and include the formation of a secondary cell wall and the biosynthesis and deposition of suberin, lignin, triterpenes and soluble aromatic compounds (Fernández-Piñán et al., 2021).

The gene expression patterns during phellogen development has been studied on the woody stems of poplar, in which the development of cork cambium depends on the season (Fernández-Piñán et al., 2021), as well as on the *Arabidopsis* root (Wunderling et al., 2018). A number of similarities in organisation and gene expression between phellogen and vascular cambium were observed, as schematically depicted in Figure 4.

Thus, in the poplar stem, the expression of cyclin genes (e.g. *CYCD3;1*) responsible for cell division as well as genes controlling maintenance of meristem activity increases during phellogen development. The latter category includes genes known to be central regulators of SAM activity, *WUSCHEL (WUS,* of *WOX* family) and *SHOOTMERISTEMLEESS* (*STM,* of *KNOX* family), as well as genes regulating the development of vascular cambium and conducting tissues, such as *WOX4, PXY, BIN2, HAM3, SCARECROW-LIKE28, KNAT1/BP, ANT, ANT-LIKE,* and *PHB* (Fernández-Piñán, et al, 2021; Zhang et al., 2019). The *Ultrapetala 1 (ULT1)* is another important regulator of seasonal phellogen activity which acts as a factor limiting the activity of this LM, and the *STM* gene is its putative target (Fernández-Piñán, et al., 2021).

It has been shown in *Arabidopsis* that vascular cambium activity is required for phellogen formation from the pericycle. During phellogen development, the IAA level is increased and the expression of the *ARF5* and *ARF8* genes encoding auxin-dependent TFs activates (Wunderling et al., 2018). Their influence is thought to activate *WOX4* expression and initiate phellogen formation in certain pericycle and/or subepidermal layer cells (Serra et al., 2021).

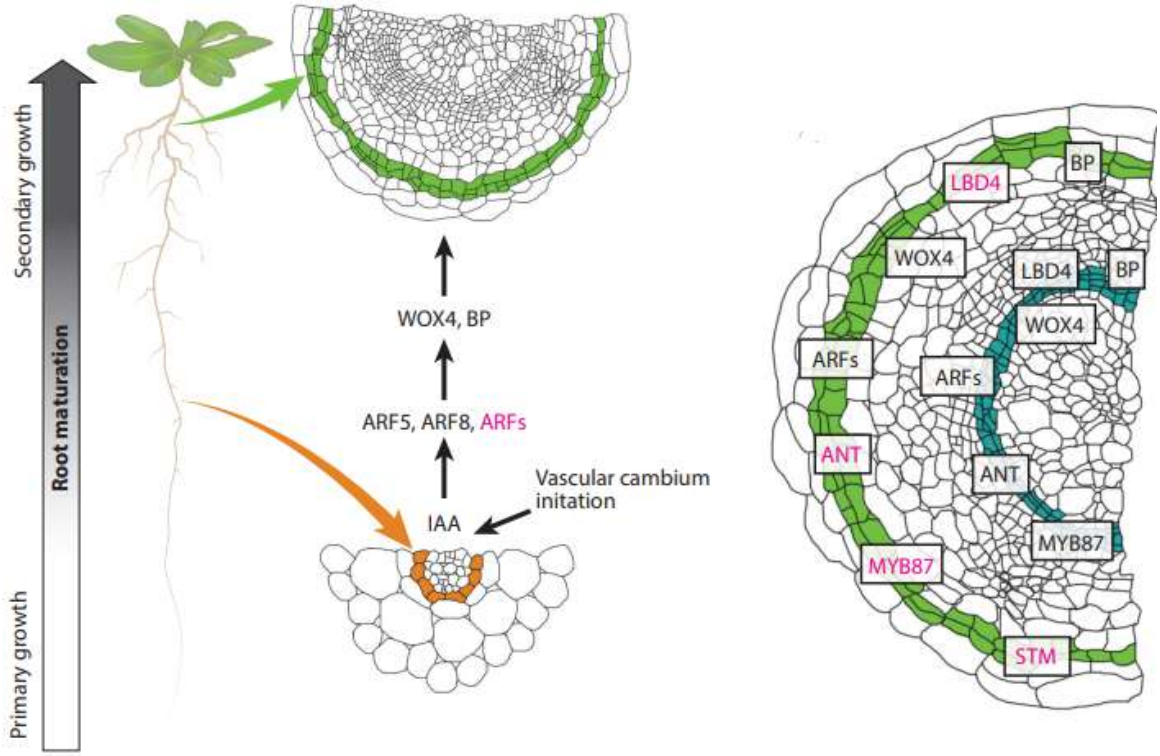

Figure 4. Similarities in the genetic regulation of vascular cambium and phellogen development (from Serra et al., 2022). Putative regulator genes are highlighted in pink, known regulator genes are highlighted in black. Cells of different tissues are marked in light green (phellogen), orange (pericycle), and dark green (cambium).

Metabolic processes such as the biosynthesis of suberin, lignin, triterpenes and soluble aromatic compounds are enhanced during growth of cork. Suberin synthesis increases the expression of genes related to fatty acid elongation (Teixeira et al., 2014, Lopes et al., 2020). Among the suberin biosynthesis genes *GPAT5, CYP86A1, CYP86B1, AtHHT/ASFT, CYP86A33* and *FHT* are active in the phellogen (Boher et al., 2018). In contrast to suberin, the molecular processes of lignin and cell wall polysaccharide formation in the phellem are currently unexplored. The group of these genes includes the secondary cell wall regulators cellulose synthase, hemicellulose and laccase, which may be involved in late cork formation, providing higher strength, stiffness and hydrophobicity (Fernández-Piñán et al., 2021).

Phytohormones such as auxins and brassinosteroids also play an important role in phellogen regulation. Increased auxin levels lead to the production of ethylene, a major activator of genes involved in the initial stages of phellogen formation (Lev-Yadun et al., 1990). Orthologues of *PIN3*, an auxin transporter, and *DWF1*, encoding the brassinosteroid biosynthesis enzyme, are expressed at high levels in phellogen cells (Boher et al., 2018). The AREB/ABF and MYC/MYB TFs are more upregulated in trees with a more pronounced cork layer (Teixeira et al., 2014). In addition, genes encoding EIN3 (Ethylene insensitive 3), ETR1/ETR2 and EIN4 ethylene-sensitive TFs were expressed at high levels in these cork samples (Teixeira et al., 2018). Increased expression of jasmonate genes was also observed in cork layers (Lopes et al., 2020). Genes associated with programmed cell death (PCD), encoding caspases and proteasome subunits, were also expressed during cork development (Rantong et al., 2015). PCD begins simultaneously with rapid cell wall suberisation and differentiation of phellem cells and is ontogenetic in its nature (Inácio et al., 2021).

The LM activity is thus under the control of a complex gene network involving TFs of different families and phytohormones. Different LMs are related by their origin and genetic control. Their activity, among other things, has resulted in the vascular system of plants, the growth of whose parenchyma can lead to the formation of storage axial organs, including the storage root.

1.2. Factors influencing storage root development

Development of any plant storage organ is a complex trait that is controlled by a number of factors. Firstly, the parenchyma of vascular tissues, which forms the major part of the storage root, originates from the cambium. In addition, prolonged cambial activity is often a characteristic of the vascular tissue parenchyma in the storage root, as evidenced by parenchyma proliferation and sometimes the forming of tertiary meristem foci and additional vascular bundles in its layers (Hearn et al., 2018; Kuznetsova et al., 2020). The activity of the cambium and the differentiation of xylem and phloem, in turn, are under the control of external factors and the internal environment (phytohormones). Secondly, the formation of the storage root (an organ acts as a receptor for nutrients) depends on the coordination of the development of other organs of the plant, its above-ground and underground parts. Thirdly, the formation of the storage parenchyma and deposition of nutrients itself is regulated by a complex system of genetic mechanisms.

Forming of xylem and phloem storage parenchyma from cambium is important for the development of storage roots in crop root plants. In addition, the parenchyma in storage roots retains the ability to proliferate and even develop the tertiary cambial foci for a long time (Tonn and Greb, 2017). The genetic control of cambium, the LM underlying the formation of the storage root, was covered in detail in the previous part of the review. In this section, we briefly review the mechanisms by which external cues influence the development of the storage root, as well as the mechanisms of nutrient storage in roots.

1.2.1. Environmental and internal signals

The storage root development depends largely on its communication with the above-ground part of the plant, from where metabolites and growth regulators are supplied to the root. Carbohydrates from the aboveground part are utilised for the formation of cell walls and root tissues and for starch storage in the storage parenchyma. Sucrose, in addition, controls the expression level of *CYCD* cell cycle regulator genes (Riou-Khamlichi et al., 1999), as well as root elongation and secondary growth (Kircher and Schopfer, 2012).

Hormones (in particular IAA), proteins and RNAs also move to the root, modifying its developmental programme towards storage (Figure 5). The example of such mobile signals are regulatory peptides, products of the *FLOWERING LOCUS T (FT)* gene family, which in *Arabidopsis* regulate the photoperiodic control of flowering by acting as florigen (Corbesier et al., 2007). The role of FT protein homologues during the formation of storage organs has been unravelled in potato (Navarro et al., 2011) and lily (Lee et al., 2013; Noy-Porat et al., 2013); FT proteins have also been identified in sugar beet (Pin et al., 2010), radish (Jung et al., 2016) and cassava (Adeyemo et al., 2019). Since root thickening has been shown to be dependent on day length in some plants, it is hypothesised that FT-like proteins may also control root formation and storage root formation (Guoa et al., 2019).

Small RNAs and mRNAs also regulate processes of secondary growth that are the basis for the formation of the storage root. Transcripts of genes encoding homeodomain-containing TFs of the BEL family play a central role in this process, as they are able to form heterodimers with KNOX TFs, which is important for organogenesis (Di Giacomo et al., 2013). In potato, this group of regulators includes the StBEL5 TF, which moves from leaves to stolons, and the POTATO HOMEOBOX 1 (POTH1) TF of the KNOX family, which is a StBEL5 interaction partner in the stolon (Ghate et al., 2017). Overexpression of *StBEL5* activates the expression of the genes for IAA *(YUCCA)* and CK *(IPT, LOG)* metabolism, suggesting a possible role of this TF in tuber development (Lin et al., 2013). The *StBEL5* and *POTH1* orthologues have been identified in many root and tuberous plants. They may control root formation through regulation of phytohormone biosynthesis genes because conserved binding motifs for BEL5-POTH1 dimers were found in the promoter regions of the *YUCCA*, *IPT*, *ARF8*, and *GA20Ox* genes in beet (Natarajan et al., 2019; Kondhare et al., 2018). Chaperone proteins of the Polypyrimidine Tract Binding Protein family are also involved in stabilising mRNAs during their transport (Kondhare et al., 2018). Among the small RNAs regulating the process of storage root formation, the miR156 and the miR172, which fulfil opposite functions in the transition from juvenile to adult phase of plant development, play a key role (Natarajan et al., 2019).

Thus, the regulation of storage root development by signals from the aboveground part of the plant has been mainly studied in potato tubers; their role in the control of storage roots is still under investigation.

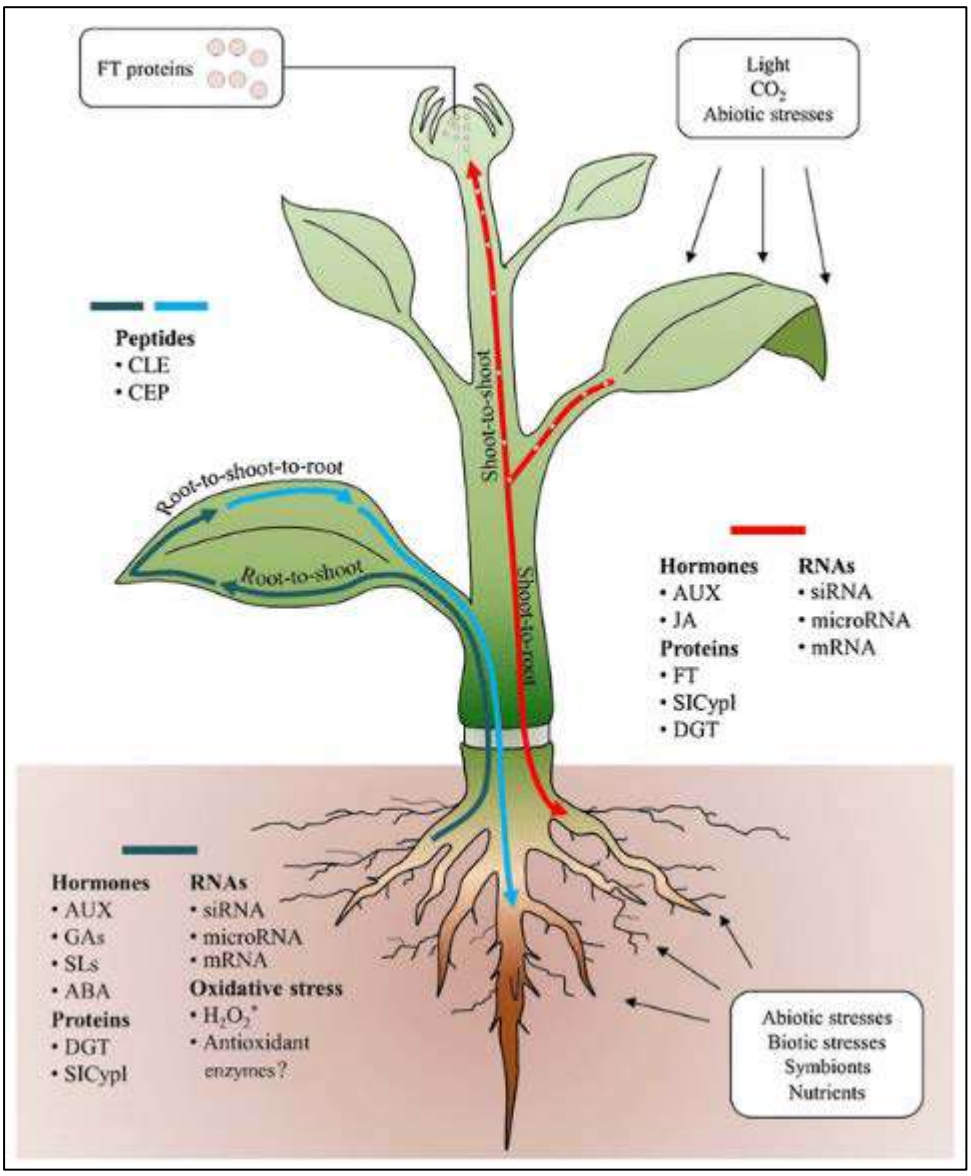

Figure 5: Main factors mediating the interaction between aboveground and belowground parts of a plant (from Gaion et al., 2018).

1.2.2. Metabolic changes

Plants use storage roots to survive unfavourable conditions and to vegetative reproduction, and also as sources of nutrition for humans and animals through the accumulation of carbohydrates (usually in the form of starch). It has been shown that the main metabolic factors of storage root formation in plants are an increased starch and sugar levels and a decreased lignin biosynthesis level (Eserman et al., 2018; Wang et al., 2016a; Firon et al., 2013). Starch, a mixture of amylose and amylopectin which are alpha-

glucose polymers, is a predominant carbohydrate deposited in storage roots. Starch is mainly synthesised and stored in amyloplasts (plastids of underground organs) of plant storage root. The material for starch synthesis is transported into the root from the aboveground part of the plant via the phloem (in the form of sucrose), then distributed by membrane transporters to both intercellular channels (plasmodesmata) and root tissues (Hennion et al., 2019). Starch synthesis is catalysed by several highly conserved groups of enzymes (Pfister and Zeeman, 2016). Their involvement in the process of storage root formation has been shown for various agricultural plants (Firon et al., 2013; Wang et al., 2016a; Eserman et al., 2018). Anatomical and biochemical studies have demonstrated that storage roots of wild ancestors of agricultural plants, as well as roots of rootless plants, produce a low number of parenchyma cells and accumulate little amounts of starch (Eserman et al., 2018), and there the starch biosynthesis genes are expressed at low levels (Wang et al., 2016a).

In addition to starch and sugars, fructose polymers fructans, mainly of the inulin type, consisting of many β-D-fructose molecules and one α-D-glucose molecule, can accumulate in the storage roots of *Asteraceae* plants. Sugar beet (and its wild ancestor) accumulates sucrose instead of polysaccharides, which is probably related to its strategy of adaptation to saline conditions. Genes encoding enzymes of the starch biosynthesis pathway have been identified in the beet genome, and their expression levels increase during root formation. To date, there is no explanation for this paradox (Turesson et al., 2014).

Lignin, a mixture of aromatic polymers, which are phenylpropane derivatives, is also accumulated in storage roots. It is formed during the processes of biosynthesis and polymerisation of monomers and is a part of the lignified cell walls of plants, including the walls of vascular elements. Enzymes of lignin mono- and polymerisation biosynthesis regulate the content of this substance in plant cells (Liu et al., 2018). In storage roots, lignin content is reduced (Wang et al., 2016a; Yang et al., 2011) due to the formation of a large amount of storage parenchyma with poorly lignified cell walls. In addition, there is a relationship between phenylpropanoid and starch metabolism in plants with developing storage roots. Thus, in transgenic yam with an increased level of lignin biosynthesis, the amount of sucrose and hexoses in leaves and the level of starch in the root decreased. At the same time, the thickening of storage roots was also reduced. The high level of starch

degradation in these plants is determined by the redistribution of photoassimilates towards lignin accumulation (Wang et al., 2016a).

Thus, the key trend in the process of nutrient storage in plants is, firstly, an increase in starch and sugars biosynthesis, and secondly, a decrease in lignin biosynthesis. The general scheme of metabolic control of storage root formation is presented in Figure 6.

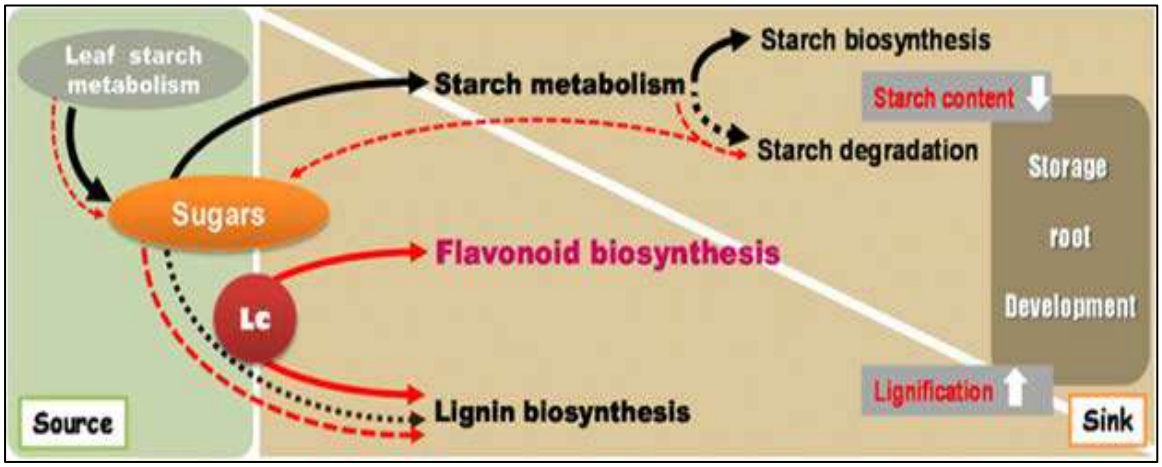

Figure 6. Major metabolic changes occurring during storage root development (from Wang et al., 2016a).

Thus, such a complex trait as a storing root is developed due to: 1) processes of cambium activity and secondary root growth control; 2) signals coming from the aboveground part of the plant and 3) accumulation of such organic compounds as starch, sucrose, and fructans). Due to the development of "omics" technologies (genomics, transcriptomics, proteomics, metabolomics), data on the genetic regulation of these processes in different plant species that form storage roots have been obtained (see reviews by Kuznetsova et al., 2020; Hoang et al., 2020). However, the list of root crops for which such data have been obtained is limited, and in the case of many plant species, additional studies are required.

Currently, numerous investigations have identified regulators of storage root development in diverse plant species, including the most important root crops (see review by Kuznetsova et al., 2020). Our work is devoted to the study of the different cambial regulators in radish storage root development.

1.3. Radish as an object of storage root genetics

One of the most promising objects for the study of storage root development is the radish (*Raphanus sativus* var. *radicula* Pers.), a root crop belonging to the *Brassicaceae* family and related to the model object of genetics, *Arabidopsis thaliana*. Radish forms a monocambial storage root of the xylem type, with a single cambial ring and differentiation of a storage parenchyma, consisting of multiple differentiating xylem cells (Sazanova, 1985; Zaki et al., 2012). It should be noted that the radish storage root is a composite structure, the upper part of which is a derivative of the hypocotyl (Sazanova, 1985); however, in the following we will use the term ‘storage root’ for describing the object of our studies.

The growth of radish storage root is positively regulated by nitrate (Geem et al., 2023), photoperiod (Guoa et al., 2019), and CK (Jang et al., 2015).

Transcriptome analyses of different stages of radish storage root development identified several groups of transcripts whose levels increase most significantly during root growth and development. Among them, the most widely represented are genes regulating cell division (some genes of cyclins and cyclin-dependent kinases) and cell stretching (genes encoding cell wall destabilisation enzymes, which are expansins and xyloglucan endotransglycosylases, Wang et al, 2013b), regulators of various metabolic pathways and signalling (e.g. genes encoding TFs of different families and components of the MAP kinase cascade), microRNA genes that target some genes encoding TFs (Yu et al., 2015), and genes regulating starch and sucrose biosynthesis and metabolism and biosynthesis of secondary metabolites (Wang et al., 2013b; Xie et al., 2018b). Also, an increase in the expression levels of some genes regulating the metabolism and/or signalling of phytohormones, such as IAA, CK, ethylene and ABA, was also detected during radish root development (Xie et al., 2018b).

Among the numerous radish *CLE* genes, two candidates were identified as regulators of root development: the *RsCLE41*, a regulator of cambium cell division and a repressor of xylem differentiation, and the *RsCLE19*, a probable stimulator of vascular differentiation. For these genes, a multiple increase in expression levels during radish root growth and a localisation of expression in strictly defined tissues (the *CLE41* in phloem and cambium, the *CLE19* in xylem) were shown. Experiments on the overexpression of the *CLE41* and the *CLE19* in radish *R. sativus* and its wild ancestor *R. raphanistrum*

shown that the *CLE41* causes the outgrowth of vascular bundles, an increase in the number of cambium cells and thin-walled xylem parenchyma, whereas the *CLE19* increases the number of lignified xylem elements (Gancheva et al., 2016; Gancheva et al., 2018).

There is a genetic collection of inbred radish lines created by self-pollination of single plants of different cultivars maintained at SPbSU. Currently, the collection consists of 33 lines of the 50th inbreeding generation, with lines differ in a number of marker traits, including the degree of storage root development (Narbut, 1966; Buzovkina and Lutova, 2007). In addition, the radish genetic collection also contains lines with root development abnormalities such as spontaneous tumour formation (see below). Spontaneous tumours in radish are of cambial origin (Lebedeva et al., 2015), therefore tumour formation regulatory genes may be unknown components of the cambium development and meristematic competence control system of root cells.

Therefore, the objectives of this work included the search for genes regulating spontaneous tumour formation by genome analysis of related tumour and non-tumour radish lines. Some of the identified regulators of storage root and tumour formation in radish are shown in Figure 7.

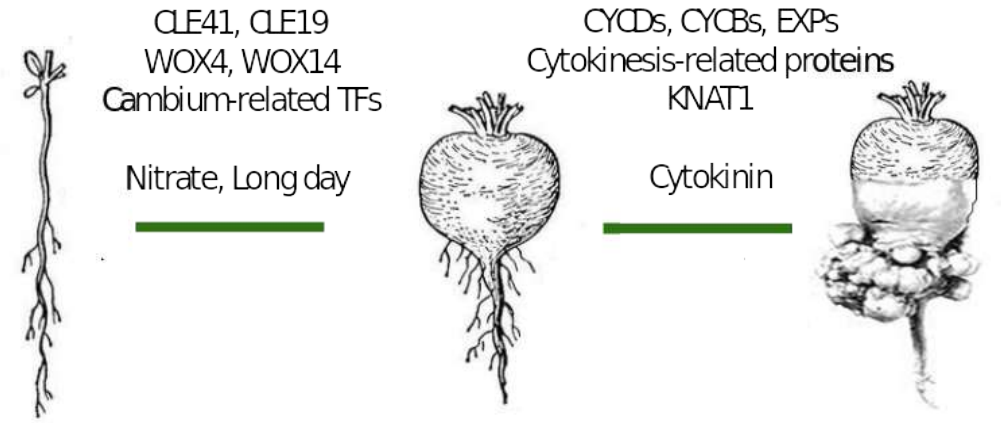

Figure 7. Several identified regulators of storage root and tumour development in radish (based on Guo et al., 2019, Jang et al., 2015, Gancheva et al., 2016, Xie et al., 2018).

1.4. Tumours in higher plants

Tumours, or neoplasia, are pathological entities resulting from uncontrolled proliferation of a group of cells, which may be caused by disruption of stem cell maintenance processes or dysregulation of mitogenic signalling (Doonan and Sablowski, 2010; Ullrich et al., 2019). Since multicellularity evolved independently in plants and animals (Meyerowitz, 2002), the systemic regulation of cell proliferation and differentiation in these two groups of higher eukaryotes is organised differently, and there are significant differences in tumour formation in plants and animals. Firstly, tumours in plants are much less common than in animals. Most plant tumours are formed under the influence of pathogens, whereas spontaneous tumours, which depend on the plant genotype, are rare (Dodueva et al., 2020; Doonan and Sablowski, 2010). In contrast to animals, in higher plants, disruption of individual components of cell cycle control does not lead to tumour formation due to the fact that 'extra' plant cells produced by enhanced cell proliferation can be incorporated into an already existing body plan (Dewitte et al., 2007).

One of the reasons for this may be the fact that in higher plants the processes of cell proliferation and differentiation are mainly concentrated in meristems, which are structures containing pools of stem cells and are the main zones of organogenesis and histogenesis (Ivanov, 2003; Heidstra and Sabatini, 2014). In addition to cell cycle regulators, cell proliferation in meristems is regulated by meristem-specific TFs as well as upstream signalling systems, in particular mobile peptides and phytohormones (Hazak and Hardke, 2016; Schaller et al., 2015; Lee et al., 2019). Indeed, in most examples of spontaneous and pathogen-induced tumourigenesis in plants, an increase in the level of phytohormones (mainly CKs) in tissues as a trigger of tumour development, as well as an increase in the expression level of several genes encoding meristem regulators, in particular TFs, have been reported (for reviews, see Dodueva et al., 2020; Doonan and Sablowski, 2010).

Plant tumours can be developed on organs of any type, including leaves, stems, roots, and flowers (Ahuja, 1998) and characterised by different degree of cell differentiation. Tumours (structures with undetermined growth and absence of secondary cell differentiation) as well as galls (structures with determinate growth and features of differentiation) can be formed in plants. The former divides for a long time and resembles

a callus, sometimes can partially undergo secondary differentiation and even form new tissues and organs. For example, tumours induced by *Agrobacterium tumefaciens*, which can evolve a limited number of differentiated tissue types such as vessels (Ullrich and Aloni, 2000) or epidermis (Veselov et al., 2003). Other non-deterministic tumours are able to develop abnormal organs, such as leaf tumours caused by the bacterium *Rhodococcus fascians* (Depuydt et al., 2008). Galls, the latter type, are more ordered structures composed of differentiated cells and tissues, and a typical example of them could be galls induced by parasitic arthropods (Paponova et al., 2017; Giron et al., 2016; Shorthouse et al., 2005).

Long-term studies of spontaneous and pathogen-induced plant tumours have revealed various mechanisms underlying their development, including changes in the levels of major phytohormones, dysregulation of the cell cycle and cell adhesion, and others. Since plant tumours are very diverse, multiple studies have focused on the different types of them.

Most examples of tumour formation in plants are related to the development of neoplasia by a variety of pathogens: bacteria, fungi, protozoa, nematodes, and arthropods (Figure 8). Numerous studies have identified both parasitic genes necessary for tumour formation and plant genes whose expression promotes the development of neoplasia (see below).

At the same time, spontaneous tumours in plants are relatively rare and are formed in plants with a specific genotype (mutants, interspecific hybrids or inbred lines, Doonan and Sablowski, 2010; Dodueva et al., 2020). Basically, data on the mechanisms of spontaneous tumour formation were obtained for the group of monogenic tumour-forming mutants of *Arabidopsis* (Figure 8). Among this group, tumour formation may result from disruption of cell wall structure and reduced cell adhesion (Krupkova et al., 2007), hypersensitivity to certain groups of growth regulators (Pelagio-Flores et al., 2013), or operational irregularities of plant cell signal transduction components with unknown functions (Smyczynski et al., 2006; Da Costa et al., 2006). Spontaneous tumours can also be formed in plants of interspecific hybrids (between several *Nicotiana* species) as well as in inbred lines of white melilot *Melilotus albus* (Littau and Black, 1952) and radish *Raphanus sativus* (Narbut, 1967). The genetic control of such tumours remains poorly understood.

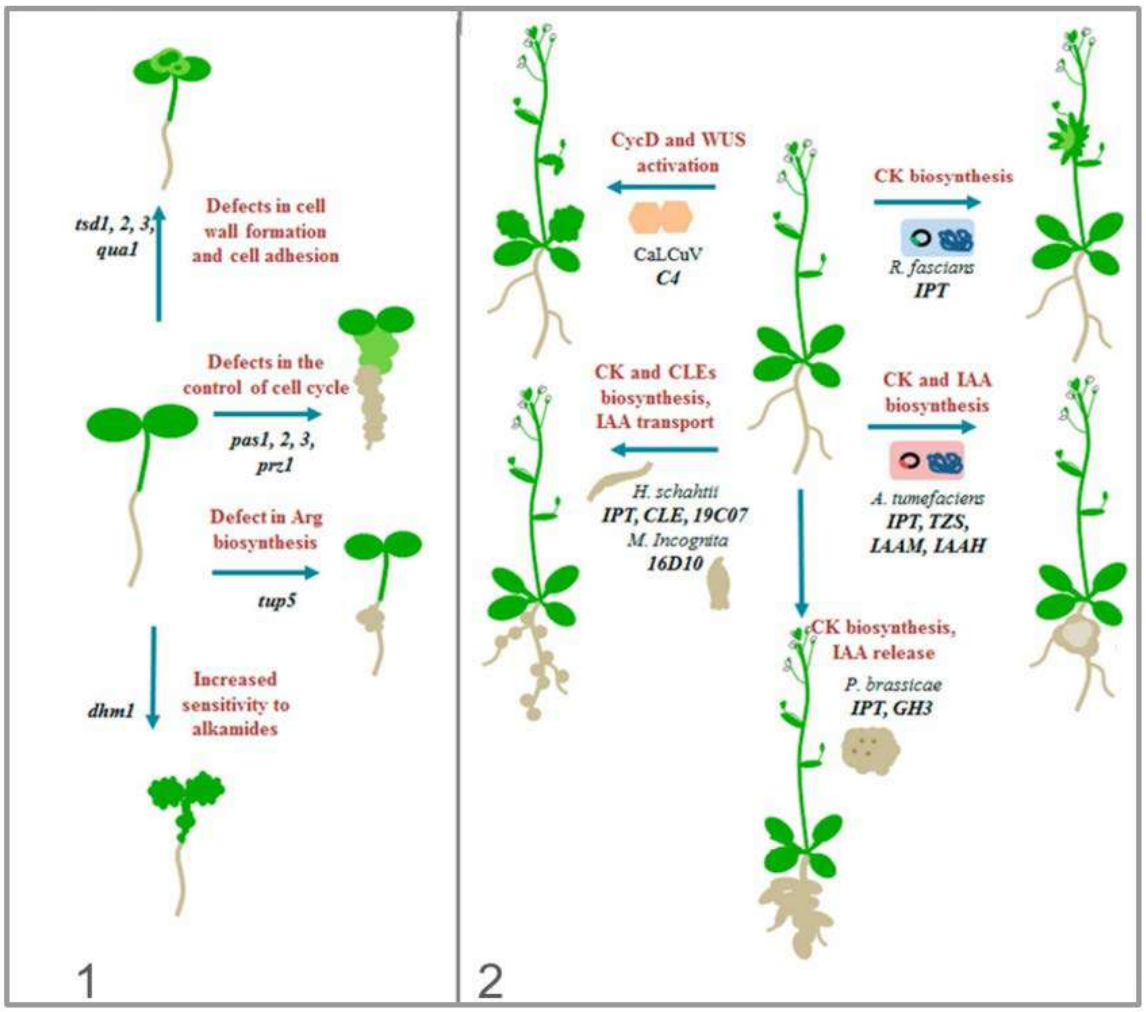

Figure 8. Spontaneous (1) and pathogen-induced (2) tumours in *Arabidopsis* (from Dodueva et al., 2020). The genes shown in Figure 8(2) belong to pathogens and are homologs of the corresponding plant genes. When plants are infected with different types of pathogens, different structures are formed on the plant bodies: leaf curling (when infected with CaLCuV); increased SAM activity and development of specific structures on the shoot (when infected with the bacterium *R. fascians*); formation of galls on plant roots (when infected with the nematodes *Heterodera schahtii* and *Meloidogyne incognita*); formation of clubroot when infected with the protist *Plasmodiophora brassica*; formation of crown gall on plant roots (when infected with the bacterium *Agrobacterium tumefaciens*). The parasites are shown schematically under their species names.

1.4.1. Pathogen-induced tumours

Phytohormones, especially IAA and CK, play a central role in basic plant developmental processes and are also involved in pathogen-induced tumour growth processes (Boivin et al., 2016). A range of biotrophic plant pathogens manipulate levels of IAA and/or CKs to induce host tissue overgrowth for their own benefit, to create a habitat or nutrient source for themselves.

To date, different groups of phytopathogens have been found to have genes for the biosynthesis of IAA and/or CK, the expression of which is important for the infection of plants and the induction of plant tissue overgrowth. Such genes have also been found in a variety of bacteria (including both tumour-inducing pathogens and non-tumour-inducing pathogens, as well as symbiotic and free-living species). Interestingly, enzymes for the biosynthesis of IAA and CKs in other organisms have been identified earlier than in plants. For example, isopentenyl transferases (IPTs) regulating the first step of CK biosynthesis were first identified in the amoeba *Dictyostelium discoideum* (Taya et al., 1978) and the bacterium *A. tumefaciens* (Akiyoshi et al., 1983; Barry et al., 1984) and much later in plants (Takei et al., 2001). Some phytopathogens are also capable of modulating the homeostasis of host phytohormones by disrupting their transport. For example, the tumour-inducing bacteria *Pantoea agglomerans* and parasitic nematodes that cause galls on roots can manipulate the flux of IAA through their effector proteins, which in plant cells interact with proteins that form IAA influx and outflow channels (Lee et al., 2011; Chalupowicz et al., 2013). Different phytopathogens also have multiple effectors that interact with the signal transduction pathways of different phytohormone groups to induce tissue outgrowth and suppress host defences (for review, see Dodueva et al., 2022). In addition, some phytopathogens, including those capable of inducing hyperplasias, are able to synthesise and secrete peptide phytohormones of different groups (Dodueva et al., 2021).

Bacteria of the *Agrobacterium* genus, in particular *A. tumefaciens*, which causes the development of undetermined tumours with low capacity for secondary differentiation (so-called crown gall), and *A. rhizogenes*, which causes the development of numerous adventitious roots on the affected organ, are two best known phytopathogens causing hyperplasia in plants (Tepfer, 1984). All pathogenic species of the *Agrobacterium* genus contain virulent plasmids called Ti (Tumor inducing) in *A. tumefaciens* and Ri (Root

inducing) in *A. rhizogenes*. The reason for the development of hyperplasias of *Agrobacterium* origin is gene expression of a specific section of DNA, T-DNA, which is a part of virulent plasmids of *Agrobacterium*. The T-DNA is a 10-30 kbp fragment bounded by direct repeats (Huffman et al., 1984). The excision of the T-DNA and its transfer to the plant cell nucleus are controlled by the gene products of the *Vir* region of the *Agrobacterium* plasmid *(VirA-VirF),* as well as some chromosomal genes (Gelvin, 2003; Permyakova et al., 2009; Pitzschke and Hirt, 2010). In nature, infection of plants by *Agrobacterium* occurs through wounding, and some compounds such as phenols and sugars released during this process and serve as inducers of *Vir* gene expression (Gelvin, 2003).

Expression of T-DNA genes in plant cells causes uncontrolled proliferation and abnormal growth of tissues, as well as synthesis of opines which are derivatives of amino acids used by *Agrobacterium* as a source of nutrition (Dessaux et al., 1986). Thus, most of the T-DNA genes studied belong to one of two functional groups: genes controlling the biosynthesis and secretion of opines and genes stimulating the proliferation of cells of the infected plant (the latter are called *Agrobacterium* oncogenes). The diverse pathogenesis strategies of *A. tumefaciens* and *A. rhizogenes* are determined by the differences in their T-DNA oncogene composition. In general, the most studied oncogenes of *A. tumefaciens* cause synthesis of a large amount of IAA and CKs, whereas the oncogenes of *A. rhizogenes* cause IAA synthesis and increased sensitivity of plant tissues to phytohormones (Capone et al., 1989).

For example, the *tms (tumour morphology shoot)* locus of *A. tumefaciens* contains genes controlling the steps of IAA biosynthesis from indole-3-acetamide: *tms1 (iaaM)* and *tms2 (iaaH)* (Kemper et al., 1985), and the *tmr (tumour morphology root)* locus contains the *ipt* gene encoding a key enzyme of CK biosynthesis (Barry et al., 1984). Another gene associated with CK biosynthesis, *tzs (trans-zeatin synthase),* is present only on certain types of Ti plasmids (Akiyoshi et al., 1983).

At the same time, the functions of a number of *A. tumefaciens* T-DNA genes are not directly related to hormone biosynthesis. For example, the *tml (tumor morphology large)* locus includes the *6b* oncogene, the expression of which in transgenic plants stimulates tumour formation, callus cell division and changes in leaf morphology (Helfer et al., 2003). The *6b* gene is not associated with phytohormone metabolism and belongs to the *plast* (phenotypic plasticity genes) gene family (Otten, 2018), and the effector

protein encoded by it is transported into plant cells where it interacts with a small group of nuclear proteins with unknown function (Kitakura et al., 2008). Protein 6b also affects the chromatin status of plant DNA by activating the expression of cell cycle genes *(CYCB, CYCD)* and meristem regulators *(KNOX class I)* (Terakura et al., 2007).

Infection with *A. tumefaciens* also causes changes in the expression levels of certain host plant genes. For example, transcriptome analysis of crown gall on radish seedlings revealed activation of expression of a large number of genes encoding TFs and negative regulation of genes involved in stress response (Tkachenko et al., 2021b).

Most of the T-DNA genes of *A. rhizogenes* also belong to the *plast* family (Otten, 2018). Infections of plants with this bacterium lead to the development of so-called hairy-root syndrome when stimulation of pericycle cell division causes formation of many adventitious roots on the affected organ.

The best known among the T-DNA genes of *A. rhizogenes* are the *rol (root locus)* genes *A, B, C, D,* which determine hairy roots development (Capone et al., 1989). The exact functions of the *rol* genes are unknown. The products of some of them were shown to be capable of interacting with plant proteins involved in signalling cascades (Moriuchi et al., 2004; Dubrovina et al., 2009). The products of the *aux1* and *aux2* T-DNA genes of *A. rhizogenes*, as well as the products of the *tms* locus genes of *A. tumefaciens* (Gaudin and Jouanin, 1995), are auxin biosynthesis enzymes.

Neoplastic tissue growth in plants can also be induced by a heterogeneous group of bacteria that affect the formation of tumour anatomically similar to the crown gall induced by *A. tumefaciens* (Clark et al., 1989; Chalupowicz et al., 2006), but incapable of transforming the plant cells. Such groups of bacteria include *Pseudomonas savastanoi* causing tumours in olive and oleander, *Rhizobacter dauci* causing bacterial galls in carrot, and *Pantoea agglomerans* and *Rhodococcus fascians* causing tumours in various species. All of the abovementioned bacterial species are capable of producing IAA and CKs, and IAA and CK biosynthesis genes located on chromosomes or plasmids have been identified in their genomes (Inze et al., 1987; Clark et al., 1993). Phytohormone biosynthesis genes determine the efficiency of plant infection and the development of bacterial neoplasia (Bardaji et al., 2011). Active cell proliferation in leaf galls produced by these bacteria is associated with activation of the cell cycle gene *CYCD3* (Depuydt et al., 2009) and the meristem regulator genes *STM* and *KNAT1* (De O-Manes et al., 2001;

Depuydt et al., 2008). Thus, the life strategy of all tumour-inducing phytopathogenic bacteria is related to the use of those phytohormone biosynthesis genes that they possess.

The ability to synthesise and secrete IAA and CKs is also widespread in the **fungal kingdom**. Fungus are found in both tumour-inducing phytopathogens and those that do not cause hyperplasias in plants (Shen et al., 2018; Chanclud and Morrel, 2016, Tanaka et al., 2011, Hinsch et al., 2015, Miransari et al., 2014, Hilbert et al., 2012).

Moreover, life cycles of several fungi, for instance *Ustilago* basidiomycetes, can include non-pathogenic haploid and pathogenic diploid stages; the fungi infect plants through aboveground meristematic tissues. The cell walls of infected plants are destroyed at the sites of contact between hyphae and plant cell membranes, which lead to the formation of biotrophic surfaces with enhanced vesicular transport. Hypertrophy and proliferation of host plant cells evolve soon after infection (Doehlemann et al., 2008). IAA biosynthesis genes have been identified in *U. maydis*, but the ability of *Ustilago* to synthesise IAA is not a key factor in tumour induction (Reineke et al., 2008). In contrast, CK biosynthesis genes have not been identified in *Ustilago* (Morrison et al., 2015). Tumour formation induced by *U. maydis* is also dependent on gibberellins (Walbot and Skibbe, 2010).

Taphrina ascomycetes are also capable of causing hyperplasia of plant tissues (Tsai et al., 2014). Infection of *T. deformans* is associated with increased levels of CK (Johnston and Trione, 1974; Testone et al., 2008) and IAA (Yamada et al., 1990) in host leaves. In species that induce hyperplasia, genes for the synthesis of IAA, CKs, gibberellins, and ABA, including orthologues of *Ustilago* IAA biosynthesis genes, have been identified in species that induce hyperplasia, assuming that ascomycetes could use a similar strategy for IAA synthesis (Cissé et al., 2013; Tsai et al., 2014). Other enzymes for IAA biosynthesis, the YUCCA orthologues, have also been found in ascomycetes (Tsai et al. 2014). Increased proliferation of palisade mesophyll cells, an effect similar to that of *KNOX* class I gene overexpression, was observed in the leaves of plants affected by ascomycetes of the *Taphrina* genus (Testone et al., 2008).

Fungi of the *Fusarium* genus cause the formation of structures resembling leaf galls caused by *R. fascians* in affected plants. Genes for IAA *(iaaM, iaaH, YUCCA),* CK *(IPT, LOG)* and gibberellins synthesis have been identified in *Fusarium* (Vanstaden and Nicolson, 1989; Tsavkelova et al., 2012; Niehaus et al., 2016, Sørensen et al., 2018). In addition, *Fusarium* fungi are able to synthesise the peptide phytohormone RALF (RAPID

ALKALINIZATION FACTOR), which is responsible for the development of pathogenesis symptom (Masachis et al., 2016).

Protozoa that cause plant tumours include *Plasmodiophora brassicae*, an obligate protist that causes clubroot disease, which manifests as formation of scales on the hypocotyls and roots of infected plants of the *Brassicaceae* family (Kageyama and Asano, 2009). Infection of *Arabidopsis* hypocotyls with *P. brassicae* enhances cambium and phloem parenchyma cell division (Kobelt et al., 2000), as evidenced by increased expression levels of *CYCB1*, *ANT*, and *EXP* genes; in addition, the infection suppressed xylem differentiation, as evidenced by decreased levels of the xylogenesis regulators *VND6, VND7*, and *MYB4* (Malinowski et al., 2012; Irani et al., 2018). Transgenic plants with overexpression of the cell cycle inhibitor *KRP1* as well as *cle41* mutants with reduced numbers of cambium stem cells had a reduced ability to form such outgrowths (Malinowski et al., 2012). The formation of clubroot results from a change in the balance of host plant phytohormones that occurs during *P. brassicae* infection, with a several-fold increase in the content of free and conjugated forms of IAA and CK (Dekhuijzen and Overeem, 1971; Devos et al., 2005). Explants of roots and stems infected with *P. brassica* grew independently of CK *in vitro* (Dekhuijzen and Overeem, 1971). The early stage of cotyledon formation is characterised by cell division processes due to the synthesis of CK by bacteria (Dekhuijzen and Overeem, 1971; Devos et al., 2005), while in the further stages the growth processes were predominant (Kobelt et al., 2000), due to the increased levels of IAA (Grsic-Rausch et al., 2000). One of the genes that maintains the balance of IAA in *P. brassicae* is *PbGH3*, whose product conjugates IAA and jasmonates to amino acids *in vitro*. This gene is absent in the closest relatives of *P. brassicae* (Schwelm et al., 2015).

Phytoparasitic **nematodes**, in addition to the standard strategy of tumour-inducing pathogens (production of CKs and influence on the distribution of IAA in plant tissues), can use a unique strategy of biosynthesis and secretion of effector peptides that mimic plant peptides of CLE, CEP (C-TERMINALLY ENCODED PEPTIDEs) and IDA (INFLORESCENCE DEFICIENT IN ABSCISSION) families. Among the nematodes that induce tumours in plants, there are gall nematodes (*Meloidogyne* spp.) and cyst nematodes (*Globodera* spp. and *Heterodera* spp.). They both penetrate root cells and incline host plant tissues to form galls (Palomares-Rius et al., 2017). A cell cycle is triggered in the plant cell selected by the nematode for feeding, after which neighbouring

cells begin to expand. Nematode feeding sites are then formed, either by cell fusion into syncytia (in cyst-forming nematodes) or by the formation of giant cells in the absence of cytokinesis (in gall nematodes). Early stages of nematode parasitism activate cell cycle genes, which is important for the formation of such feeding sites (de Almeida Engler and Gheysen, 2013; Siddique et al., 2015).

Nematodes inject their pharyngeal gland secretion, which contains phytohormones and TFs, into plant cells (Mitchum et al., 2012; Ali et al., 2017). When plants are infected by cyst nematodes, expression of CK-dependent genes is activated in syncytia and neighbouring cells (Siddique et al., 2015; Dowd et al., 2017).

Neither cyst nor gall nematodes are capable of producing IAA (Goverse et al., 2000), but they can manipulate the flux of IAA in host plants via the AUX/LAX and PIN transporters (Kyndt et al., 2016). Cyst and gall nematodes differ in manipulating the action of IAA: one of the targets of IAA in gall induced by gall nematodes is the IAA-regulated gene *LBD16*, which controls cell divisions in the xylem pericycle during lateral root formation (Cabrera et al., 2015).

Nematodes are also capable of producing CLE peptides, which is their unique feature (Wang et al., 2001; Gao et al., 2003). CLE peptides of cyst-forming nematodes belong to the same group as CLE1-7 peptides of *Arabidopsis* (Ali et al., 2017). A group of CLE peptides similar to B-type CLE peptides has also been identified in *H. schachtii*, as well as a TDR protein that binds B-type CLE peptides. Both types of CLE peptides, A and B, can promote cell proliferation during the formation of feeding sites (Guo et al., 2017). In *M. incognita*, the *16D10* gene, which encodes a protein similar to CLE peptides and stimulates root growth, was also identified. In *Arabidopsis* roots, 16D10 interacts with SCL6 and SCL21 TFs (Huang et al., 2006).

The syncytium-forming nematode *Rotylenchulus reniformis* secretes peptides similar to plant CEP peptides. The *RrCEP* family genes are activated during the infection phase of the nematode life cycle and are expressed in pharyngeal gland cells. The synthetic peptide RrCEP1 enhanced the expression of the nitrate transporter gene *AtNRT2.1* (Eves-Van Den Akker et al., 2016). *Meloidogyne* nematodes also secrete IDA peptides, the function of which is to weaken the connections between plant cells, making it easier for nematodes to penetrate the root (Kim et al., 2018).

Such **Arthropods**, as insects and mites, in various kinds in plants induce hyperplasias, ranging from small swellings on leaves or stems formed by overgrown

epidermal cells to complex structures with differentiated tissues (Giron et al., 2016; Shorthouse et al., 2005). Insect- and tick-induced galls share common characteristics and are regulated by similar mechanisms. Insects that induce gall formation can inject secretions, likely containing IAA and CKs, into host plant tissues, causing abnormal growth (Hough, 1953). Gall-inducing insects also affect the expression of cambium regulator genes *(CLE44, TDR/PXY, WOX4* and *ERECTA)* as well as flowering and fruit formation (Schultz et al., 2019).

Gall-inducing mites pierce the epidermal tissue of host plants and inject there saliva possessing a weak IAA- and CK-like activity (De Lillo and Monfreda, 2004), inducing abnormal growth. Mite-induced galls are characterised by enhanced mesophyll proliferation leading to the formation of homogeneous parenchyma and changes in the adaxial-abaxial polarity of the leaf lamina (Kane et al., 1997; Paponova et al., 2017). During mite-induced gall development on strawberry, *KNOX* and *WOX* genes, genes controlling leaf development *(YAB2, REV, AS2)*, cell cycle genes *(CYCD3, CYCB1)*, and primary response genes to IAA and CK were activated (Paponova et al., 2017).

Viruses of two groups, *Reoviridae* (reoviruses) and *Geminiviridae* (geminiviruses), cause tumour formation in plants, while the mechanisms of hyperplasia induction differ from those of the abovementioned groups of phytopathogens and are not related to changes in the balance of phytohormones (Hibino, 1996; Roumagnac et al., 2015; Wei and Li., 2016). Reoviruses infecting plants spread through the phloem, causing hyperplasia of sieve elements and increased proliferation of phloem parenchyma cells (Lv et al., 2017). Possible mechanisms underlying reovirus-induced tumour formation are associated with the effects on the phytohormonal balance of the host. For example, the P2 protein of rice dwarf virus binds to the key enzyme of gibberellin biosynthesis ent-kaurene oxidase, negatively regulating gibberellin levels and thus plant growth (Zhu et al., 2005). Geminiviruses are able to stimulate endoreduplication in host plant cells and increase mitosis frequency (Latham et al., 1997; Hanley-Bowdoin et al., 2004) through interaction with cell cycle proteins. Some geminivirus proteins contain conserved sequences for binding to plant RETINOBLASTOMA-RELATED protein (Kong et al., 2000; Arguello-Astorga et al., 2004). Indeed, infection of *Arabidopsis* with cabbage leaf curl virus decreases the level of genes regulating the G1-S transition (*CYCD3*) and increases the level of genes regulating the G2 and M phases. Overexpression of *CYCD3;1* and *E2FB*, whose activity normally stimulates cell proliferation and reduces the number

of endocycles, blocks the development of virus infection, while overexpression of *E2FA* and *E2FC*, which stimulate endomitosis, promotes tumour development in infected plants (Ascencio-Ibáñez et al., 2008). To trigger plant cell proliferation, geminiviruses also use a strategy involving the interaction of viral proteins with plant cell cycle proteins and regulators of meristem activity. The C4 protein of beet curl virus is key to tumour development (Latham et al., 1997; Mills-Lujan and Deom, 2010). Expression of the *CYCA1;1* and *CYCB1;4* genes was enhanced in plant seedlings transformed with *C4* (Mills-Lujan and Deom, 2010). In plant cells, C4 proteins bind to SHAGGY kinases, which are homologues of key regulators of signalling pathways in animals (Piroux et al., 2007; Deom and Mills-Lujan, 2015) and are involved in brassinosteroid signalling in *Arabidopsis* (Yan et al., 2009).

1.4.2. Spontaneous tumours

Spontaneous (genetic, or genetically determined) tumours develop in plants with a specific genotype without infection by a pathogen. In higher plants, spontaneous tumours are much less common than pathogen-induced tumours; they can occur in different species on different organs: leaves, seedlings, lateral shoots, ovaries (Ahuja, 1998). Spontaneous tumours also form in inbred lines and interspecific hybrids. The genetic control of spontaneous plant tumour development (with the exception of tumour monogenic mutants of *Arabidopsis*) remains poorly understood.

In interspecific hybrids

Spontaneous tumour development in interspecific hybrids has been reported in the *Datura, Briofillum, Lilium, Gossipium, Lycopersicon, Brassica* and *Nicotiana* genera (Ahuja, 1998). The first studies on spontaneous tumour formation were devoted to the tumours in interspecific hybrids of the *Nicotiana* genus (Smith, 1988). Tumours in tobacco develop during flowering in the lower part of the stem, are histologically similar to crown galls induced by *A. tumefaciens*, but are capable of secondary differentiation and the formation of rudimentary shoots and leaf-like structures (Hagen, 1962; Aoki and Syono, 1999). Two groups ('+' and '-') are distinguished within the *Nicotiana* genus, and tumour formation has been observed in hybrids of species from different groups, as

opposed to hybrids of species from the same group (Naf, 1958). This is probably due to the fact that the genomes of "+" species have an initiator gene that controls the onset of tumour formation, while the genomes of "-" species have multiple loci of expression enhancers (Ahuja, 1968). Plants of the "-" group showed a greater ability to root explants, while plants of the "+" group showed an increased ability to regenerate shoots (Bogani et al., 1997).

In interspecific tobacco hybrids, there was a connection between tumour formation and hormonal balance, expression of different groups of genes, and T-DNA gene expression. Elevated levels of free IAA and CK were observed in tumour tobacco hybrids (Ahuja, 1971). When hybrids (Qu et al., 2006) or isolated RAMs (Ames, 1972; Ames and Mistretta, 1975) were treated with phytohormones, CK stimulated tumour formation, while IAA inhibited it. Also, the expression of *CYCD3* (Wang et al., 1999; Jin et al., 2008) and *KNOX I* (Lee et al., 2004) genes were increased in developing tumours of tobacco hybrids.

One of the possible causes of tumour formation in interspecific tobacco hybrids was considered to be the expression of homologues of *Agrobacterium* oncogenes, which are present in the genomes of many species of the *Nicotiana* "-" group and have been termed as cellular T-DNA (cT-DNA). The *N. glauca* cT-DNA is an imperfect inverted repeat containing homologues of several *A. rhizogenes* T-DNA oncogenes *(NgrolB, NgrolC, Ngorf13, Ngorf14)* and an opine synthase gene (Aoki and Syono, 1999). Subsequently, cT-DNA sequences have been identified in many *Nicotiana* species of *Tomentosae* and *Noctiflorae* sections (Intrieri and Buiatti, 2001; Chen and Otten, 2017). Most of the cT-DNA sequences are expressed, which suggest their role in plant life; moreover, their expression is dramatically increased during tumour induction by wounding and decreased during plant regeneration from tumours (Aoki and Syono, 1999). Subsequently, cT-DNA, the presence of which may be the result of horizontal gene transfer, has been found in the genomes of various plant species out of the *Nicotiana* genus. None of these species form tumours, and it has been suggested that cT-DNA may play a role in plant adaptation to stresses and the synthesis of certain secondary metabolites (Chen et al., 2018; Matveeva and Otten, 2021).

In addition, spontaneous tumour formation was observed in transgenic *Nicotiana tabacum* plants with suppression of the *CHRK1* gene encoding a receptor kinase with a chitinase-like sequence in the extracellular domain (Kim et al., 2000), which also has

anti-oncogenic activity (Lee et al., 2003). The *CHRK1* gene is expressed in SAM, young leaves, and in the vascular system (Lee et al., 2003). The callus with the *CHRK1* suppressed is capable of rapid hormone-independent cell proliferation and formation of shoot-like structures, sharing a phenotypic similarity with tumours of interspecific *Nicotiana hybrids*. The *CHRK1* is probably involved in the control of CK levels in plant tissues (Lee et al., 2003). The level of *CycD3* gene expression increased dramatically in cells of transgenic seedlings as well as in calluses cultured on medium with CK (Lee et al., 2004). There is also data on the participation of CHRK1 kinase in the regulation of the response to stress. Increased level of expression of genes involved in stress response was observed in tumours of plants with *CHRK1-s* (Lee et al., 2004); increased expression of stress response genes was also observed in tumours of interspecific tobacco hybrids (Lee et al., 2004). Thus, repression of the CHRK1 pathway caused by deletion of the extracellular domain of the CHRK1 kinase, combined with hormonal imbalance caused by cT-DNA gene expression, may be responsible for tumour formation in *Nicotiana* interspecific hybrids (Lee et al., 2003). The *Hl-1* and *Hl-2* oncogenes, *habituated leaves* (Meins and Tomas, 2003), which induce CK-independent tissue growth *in vitro*, may also be involved in tumour formation in tobacco. The *Hl-1* and *Hl-2* transgenic plants had increased levels of CK in tissues compared with the wild type (Hewelt et al., 2000).

Thus, the mechanisms underlying spontaneous tumour formation in interspecific hybrids are not fully understood, although there are a number of candidate genes for the role of regulators of tumour formation in *Nicotiana* hybrids.

In *Arabidopsis thaliana* mutants

The most well-studied example of spontaneous tumour formation in plants are tumours in *A. thaliana* mutants. Tumour development in these mutants begins soon after germination and affects SAM, leaf buds and hypocotyl. The mutants show increased expression of CK- and IAA-dependent genes, as well as cell cycle and meristem control genes (Frank et al., 2002; Sieberer et al., 2003; Krupkova et al., 2007), but the levels of IAA and CK in the mutants are similar to those in wild type plants. The causes of oncogenesis in *Arabidopsis* mutants are diverse. Various monogenic mutations resulting in tumour growth can lead to disruption of cell wall structure and reduced cell adhesion (in *tsd* mutants), impaired arginine biosynthesis (in the *tup5* mutant), hypersensitivity to a

growth regulator (in the *dhm1* mutant), and impaired functioning of cell signalling components and cell cycle activation (in *pas* mutants).

Tumorous shoot development mutants *(tsd1, 2, 3)* develop callus-like structures with aberrant leaf-like organs instead of SAM (Frank et al., 2002). The developmental defects of *tsd* mutants are caused by impaired cell wall formation due to changes in the orientation of cellulose microfibres and pectin composition, as well as impaired orientation of cytoskeleton microtubules (Krupkova et al., 2007; Paredez et al., 2008). The product of *TSD1* is endo-1,4-β-d-glucanase, which is involved in cellulose biosynthesis (Krupkova and Schmülling, 2009), and *TSD2* encodes a pectin methyltransferase (Krupkova et al., 2007). Another gene in this group, *QUA1*, encodes a glycosyltransferase that is also involved in pectin biosynthesis. Loss-of-function *qua1* mutants reveal impaired cell adhesion, resulting in spontaneous callus formation on the surface of the cotyledons, roots and hypocotyl (Bouton et al., 2002). Callus formation from shoots and seedlings, which results from disruption of the *NpGUT1* gene encoding a glucuronyltransferase involved in pectin synthesis, is also characteristic of the *nolac-H18 (non-organogenic callus with loosely attached cells)* mutant of tobacco (Iwai et al., 2002).

The *pasticcino* mutants *(pas1, 2, 3)* form zones of ectopic cell proliferation on the aboveground parts of plants. In *pas1* seedlings, an additional layer of chaotically dividing cells is formed in the hypocotyl, and there is also a zone of ectopic cell division in the epidermis and primary cortex. As in *tsd* mutants, cell proliferation in *pas* mutants is enhanced by CKs (Faure et al., 1998). The *PAS1* gene product is an immunophilin protein (Smyczynski et al., 2006). The *PAS2* encodes a tyrosine phosphatase-like PTPL protein characterised by the absence of phosphatase activity (Bellec et al., 2002). The PAS2 protein interacts with the phosphorylated form of CDKA and competes with active phosphatases for interaction with substrates. It is possible that the *pas2* phenotype is formed by stimulation of cell division resulting from increased catalytic activity of CDKA (Da Costa et al., 2006).

The phenotype of the *proporz (prz1)* mutant seedlings responds to the introduction of exogenous CKs or IAA that induce tumour callus formation on the surface of all organs (Sieberer et al., 2003). The *PRZ1* gene encodes the chromatin remodelling factor AtADA2b (Mao et al., 2006), which has a number of targets including *KRP* genes (Sieberer et al., 2003; Anzola et al., 2010). IAA and CK are antagonists of KRP proteins:

IAA treatment represses *KRP* genes, especially in *prz1* mutants, causing pericycle deregulation and uncontrolled cell proliferation (Anzola et al., 2010). Other targets of PRZ1 protein include *PLT* genes, key regulators of the RAM stem cell niche (Kornet and Scheres, 2009).

The *tumor prone 5 (tup5)* mutant is characterised by the formation of stunted root rudiments that form callus-like structures. The *TUP5* gene encodes an acetylornithinaminotransferase involved in arginine biosynthesis (Frémont et al., 2013). The *N-isobutyl decanamide-hypersensitive1 (dhm1)* mutant shows increased sensitivity to alkamides and forms tumours on petioles, leaves and stems (Pelagio-Flores et al., 2013). High concentrations of N-isobutyl decanamide, an active natural alkamide, induce callus formation on leaves and enhance lateral root formation, indicating that the compound has biological activity (Lopez-Bucio et al., 2007).

Thus, the regulators of spontaneous tumour formation in *Arabidopsis* mutants are highly diverse, although the mechanisms of tumour formation are only understood for mutants with impaired cell adhesion (*tsd, qua, nolac-H18*).

In inbred lines

Spontaneous tumour formation has been observed in inbred lines of *Melilotus albus* (Littau, Black, 1952) and *Raphanus sativus* (Narbut, 1967), the latter being the subject of the most important studies. The genetic collection of radish (*Raphanus sativus* var. *radicula* Pers.) lines was created at SPbSU by inbreeding individual plants of different radish varieties (Narbut, 1966, 1967); at present it consists of 33 highly inbred lines (Figure 9, Table 2, Buzovkina, Lutova, 2007). The formation of spontaneous tumours on roots was observed in some lines of the collection already after 2-3 generations of inbreeding (Narbut, 1967), and currently ten inbred lines of radish spontaneously form tumours on the main root and hypocotyl during flowering (Buzovkina, Lutova, 2007). It has been shown that in some hybrid combinations of Saxa lines, including crosses of lines 18x19, tumour formation is inherited as a monogenic recessive trait (Matveeva et al., 2004). In this regard, the objects of our study were related lines 18 and 19 of the genetic collection of radish lines of SPbSU.

Studies of the anatomical structure of tumours have shown that tumour formation in plants is associated with strengthening of cell division and growth processes. It was shown that many genes, which are cell cycle regulators, were active in spontaneous

tumours (Figure 10) that link tumour formation with an increased level of cell proliferation. Spontaneous tumours formed on radish taproot are originated from pericycle and cambium cells and consist of undifferentiated parenchyma cells with increased ploidy compared to taproot cells and abnormally organised vascular bundles associated with the plant vascular system (Ilina et al., 2006, Lebedeva et al., 2015). The periphery of young growing tumours contains numerous meristematic foci that resemble RAM and are characterised by increased intensity of cell proliferation, auxin response maxima and the *RsWOX5* expression (Lebedeva et al., 2015).

Genetic analysis has shown that spontaneous tumour formation in radish inbred lines is polygenically controlled, but can be inherited as a monogenic recessive trait in some crosses (Matveeva et al., 2004).

The association of tumour formation in radish inbred lines with a disturbance in the balance of phytohormones and with the expression of genes regulating cell proliferation has been demonstrated. Increased levels of CK and IAA, as a probable main mechanism of tumour induction, relate tumours in inbred radish lines to pathogen-induced tumours (e.g. *Agrobacterium* crown-gall-induced tumours). One of the reasons for spontaneous tumour formation in inbred radish lines is the increased level of free CKs, which was detected in the roots of tumour-forming radish lines during the transition to flowering (Matveeva et al., 2004). Analysis of the distribution of cell proliferation and differentiation zones in tumours of inbred radish lines revealed meristematic foci located at the periphery of the tumour and resembling meristems of lateral roots, including the presence of maxima of auxin response and expression of *RsWOX5*, which is a RAM regulator (Lebedeva et al., 2015).

At the same time, analysis of the transcriptome of spontaneous radish tumours compared to lateral roots of the same lines at the same developmental stage revealed a sharp increase in the expression levels of a large number of cell cycle genes participating in different stages of its regulation, from the G1-S transition to cytokinesis (Figure 11), as well as decreased expression of genes regulating tissue differentiation, such as regulators of cell wall lignification and regulators of the biosynthesis of secondary metabolites such as glucosinolates (Tkachenko et al, 2021a). At the same time, data on transcriptome analysis of tumours induced by *A. tumefaciens* on radish roots and hypocotyls revealed other differentially expressed genes: in particular, the expression of phytohormone

response genes was increased, while biotic and abiotic stress response genes have a decline in expression levels (Tkachenko et al., 2021b).

Thus, the development of spontaneous tumours is a complex trait, which does not exclude that the reason for their development may lie in mutations in a limited number of regulatory genes that may have a systemic effect. In this context, a comprehensive study of the phenomenon of spontaneous tumour formation in plants, particularly in inbred radish lines, may allow us to identify previously unknown systemic regulators of meristematic cell activity. In order to identify them, we set out to sequence the genomes of related tumour and non-tumour lines of radish.

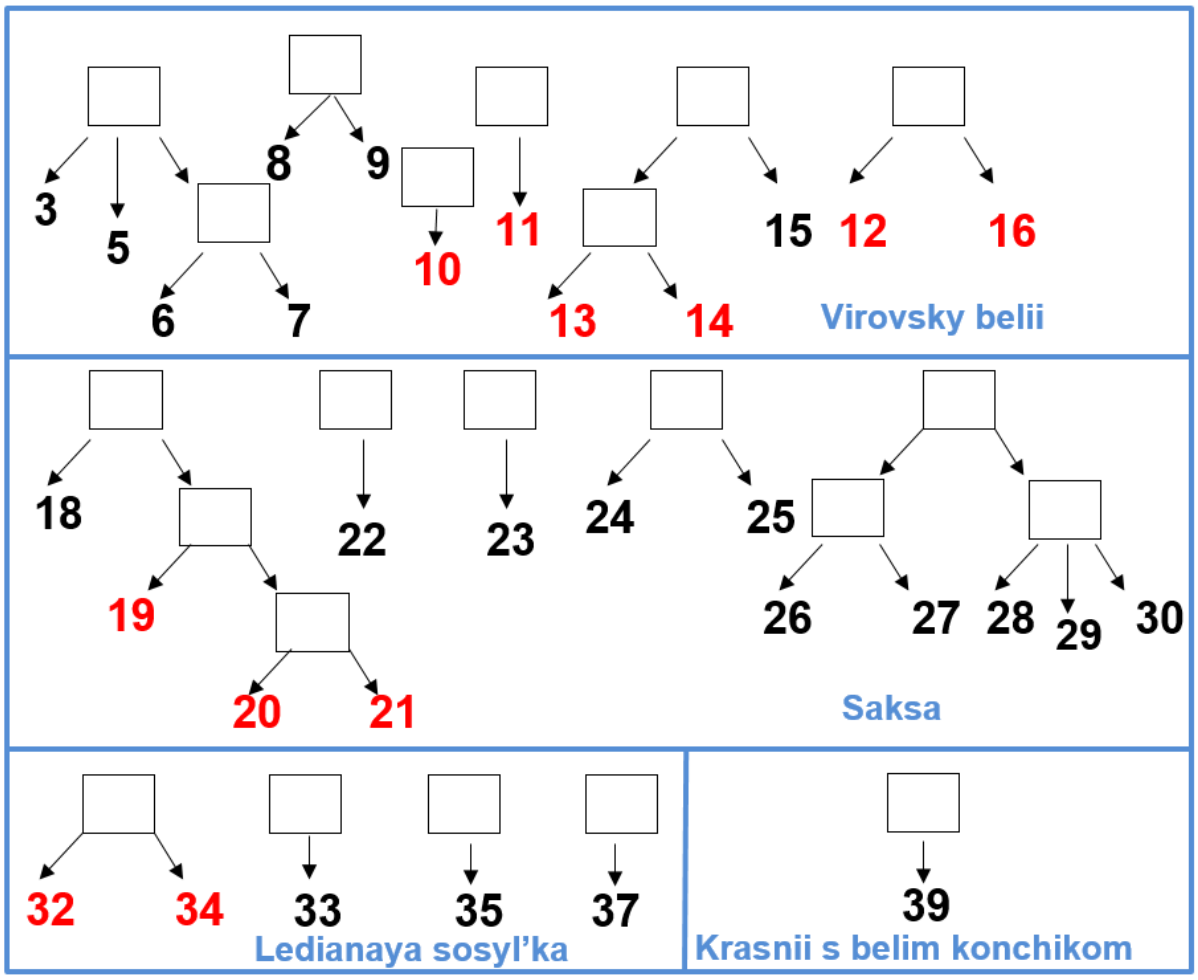

Figure 9. Origin of tumour-forming inbred lines of radish from the SPbSU genetic collection. Lines with spontaneous tumour formation are marked in red (according to Tkachenko et al., 2021a).

Table 2. Lines of the radish genetic collection and their characteristics.

Vari-ety	Lines			
	Non-tumour		**Tumour-forming**	
	№	Characteristics	№	Characteristics
Virovsky belii	3	Coloured storage root, coloured corolla, dissected leaf, round storage root, below average plant height, average shoot formation, average autofertility. **Reduced apical dominance.**	**10**	Uncoloured storage root, coloured corolla, undissected leaf, rounded storage root, below average plant height, late shoot formation, low autofertility
	5	Uncoloured storage root, coloured corolla, undissected leaf, oval storage root, medium plant height, medium shoot formation, medium autofertility.	**11**	Uncoloured storage root, coloured corolla, undissected leaf, rounded storage root, a very high-growing, shoot early, a very low autofertility. **Seed germination in the pod.**
	6	No coloured organs, dissected leaf, long storage root, below average plant height, early shoot formation, medium autofertility. **Reduced apical dominance, no termination of inflorescence meristem (ovary sprouting).**	**12**	Uncoloured storage root, coloured corolla, unbranched leaf, rounded storage root, low plant height, average shoot formation, medium autofertility. **Increased number of flowers in inflorescence, stem fasciation.**
	7	Coloured storage root, coloured corolla, dissected leaf, long storage root, below average plant height, average shoot formation, average autofertility	**13**	Uncoloured storage root, coloured corolla, undissected leaf, rounded storage root, very high-growing, very late shoot formation, low autofertility, sensitive to potassium deficiency
	15	Uncoloured storage root, coloured corolla, undissected leaf, rounded, small storage root, low plant height, late shoot formation, low autofertility, sensitive to potassium deficiency	**14**	Uncoloured storage root, coloured corolla, undissected leaf, rounded storage root, very high-growing, very late shoot formation, very low autofertility, **seed germination in pod,** sensitive to potassium deficiency
			16	Uncoloured storage root, coloured corolla, dissected leaf, rounded, small storage root, below average plant height, early shoot formation, late seed set, very low autofertility. **Shoot bends (*Wsh1*)**
Saksa	18	Coloured storage root, uncoloured corolla, dissected leaf, rounded storage root, medium plant height, late shoot formation , medium autofertility	**19**	Coloured storage root, uncoloured corolla, dissected leaf, rounded, small storage root, medium plant height, medium shoot formation, high autofertility
	22	Coloured storage root, uncoloured corolla, dissected leaf, rounded storage root, medium plant height and shoots formation, low autofertility.	**20**	Coloured storage root, uncoloured corolla, dissected leaf, rounded, small storage root, below average plant height, medium shoots formation, medium autofertility. Light green colour.
	23	Coloured storage root, coloured corolla, dissected leaf, rounded, large storage root,	**21**	Coloured storage root, uncoloured corolla, dissected leaf, rounded, small storage root,

		above average plant height, later shoot formation, average autofertility		below average plant height, medium shoot formation, medium autofertility. Light green colour.
	24	Coloured storage root, coloured corolla, dissected leaf, rounded storage root, above average plant height, medium shoot formation, high autofertility		
	25	Coloured storage root, coloured corolla, dissected leaf, rounded storage root, above average plant height, medium shoot formation, medium autofertility		
	26	Coloured storage root, coloured corolla, dissected leaf, rounded storage root, height medium plant height, shoot formation and autofertility, shoot bends (*Wsh2*), wilting		
	27	Coloured storage root, coloured corolla, dissected leaf, rounded storage root, medium plant height, medium shoot formation, high autofertility.		
	28	Coloured storage root, coloured corolla, dissected leaf, rounded storage root, above average plant height, medium shoot formation, high autofertility.		
	29	Coloured storage root, coloured corolla, dissected leaf, rounded storage root, above average plant height, medium shoot formation, high autofertility.		
	30	Coloured storage root, coloured corolla, dissected leaf, rounded storage root, above average plant height, medium shoot formation, high autofertility.		
Ledianaia sosyl' ka	33	Uncoloured storage root, uncoloured corolla, dissected leaf, long storage root, below average plant height, average shoot formation, low autofertility.	**32**	Uncoloured storage root, uncoloured corolla, dissected leaf, long storage root, medium plant height, medium shoot formation, low autofertility
	35	Uncoloured storage root, uncoloured corolla, dissected leaf, long storage root, medium plant height, late shoot formation, medium autofertility	**34**	Uncoloured storage root, uncoloured corolla, dissected leaf, long storage root, medium plant height, later shoot formation, high autofertility
	37	Uncoloured storage root, uncoloured corolla, dissected leaf, long storage root, medium plant height, late shoot formation, high autofertility		
Krasnii s belim konchikom	39	Uncoloured storage root, uncoloured corolla, dissected leaf, round storage root, late shoot formation, low autofertility. **A dwarf.**		

Figure 10. Spontaneous tumours of radish: 1) spontaneous tumours in different radish lines of the SPbSU genetic collection; 2) main features of the anatomical structure of spontaneous tumours: undifferentiated cells in the periphery of the tumour; vessels in the central part of the tumour; connection between tumour and plant vasculature; 3) visualisation of actively proliferating cells in tumours on radish roots by labelling with 5-ethynyl-2'-deoxyuridine (green fluorescence, dividing cells) and Alexa Fluor-488 fluorescence staining (red fluorescence, nonspecifically coloured cell walls; right picture); 4) cytological analysis of radish roots and tumours in tumour-forming line 19 (staining): tumour cells (lower part of the picture) have an increased number of chromocenters stained with DAPI (according to Lebedeva et al., 2015, Betekhtin et al., 2011)

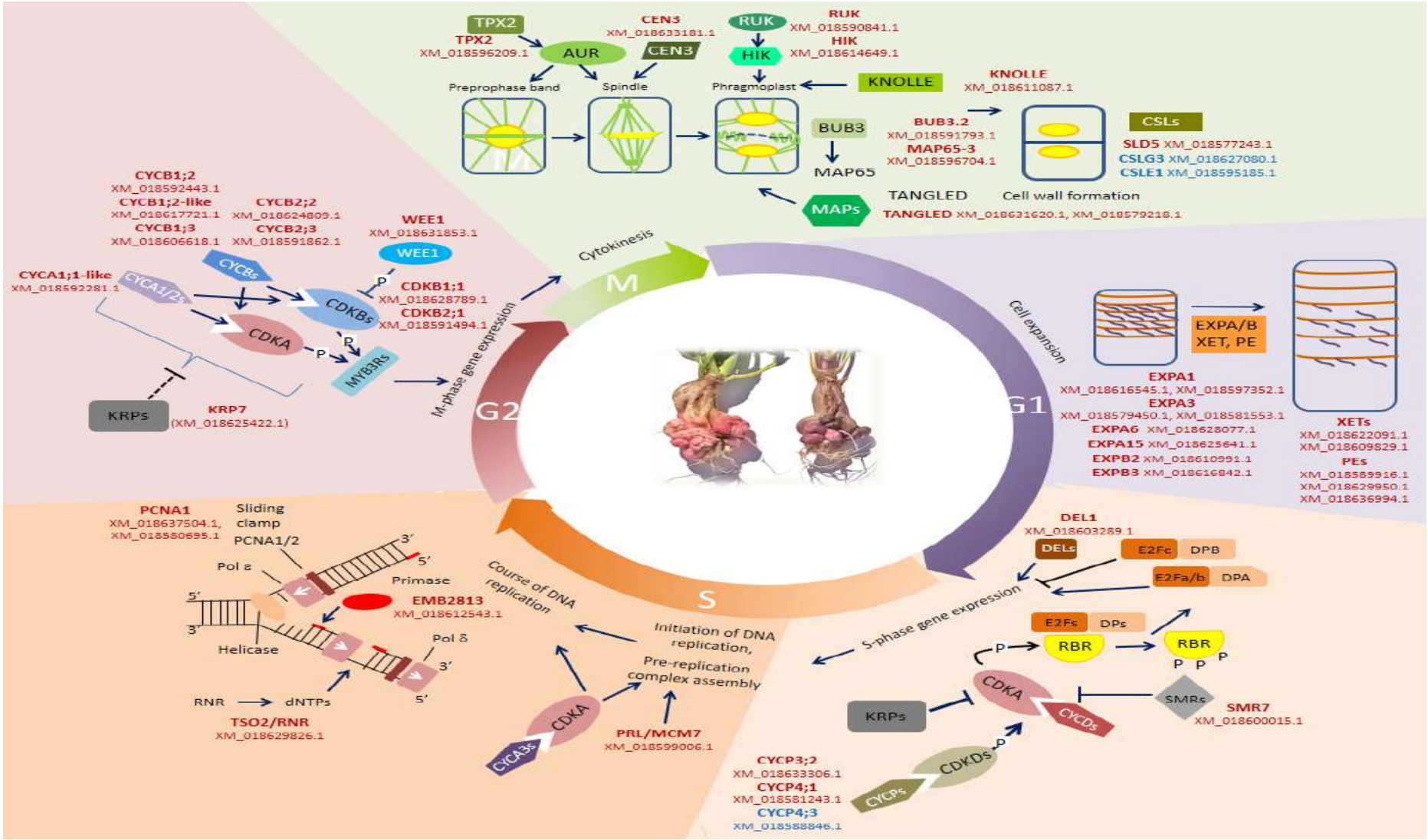

Figure 11. Cell cycle genes with changed expression levels in spontaneous radish tumours compared to lateral roots according to transcriptome analysis (by Tkachenko et al., 2021a). Genes with increased expression levels are highlighted in red, and genes with decreased expression levels are highlighted in blue.

1.5. Conclusion: Radish storage root development as a complex trait

Development of root, including the storage root modified for nutrient storage, is a complex process regulated by many factors, including control of meristem activity, differentiation of different tissue types, and interaction with the external environment (Kuznetsova et al., 2020). The object of our research is radish, an annual root crop related to *Arabidopsis*, whose peculiarity is the presence of highly inbred lines with abnormal storage root development, such as spontaneous tumours, in the SPbU genetic collection.

Tumour formation and storage root development are likely to be under similar genetic control, which, according to our data (Lebedeva et al., 2015; Lutova et al., 2008; Tkachenko et al., 2021a, b), includes meristem regulators involved in the control of meristem activity. According to previous data, the same lateral meristem, the cambium, is involved in the formation of both radish storage root and tumours (Lebedeva et al., 2015). From this point of view, tumours represent a special type of irregular, or facultative, secondary meristems originating from the cambium, and they can be considered as an anomaly of secondary growth leading to the formation of a storage root.

Tumour formation in plants is a consequence of the totipotency of cells and the high plasticity of their developmental programmes. As a result of these features, tumours as ectopic foci of meristematic activity can form under a number of conditions. The development of tumours in plants (as in animals) is caused by a disruption in the systemic control of cell division, which occurs at different levels of regulation. The activation of meristem-specific regulators in plant tumours of different origins demonstrates the meristem-like nature of hyperplasia in plants. Further study of the mechanisms of tumour formation in higher plants, including radish lines, will increase knowledge of the control of plant cell proliferation.

Among events underlying the normal formation of the storage the most important are the formation of a cambium ring from procambium and pericycle, the division of cambium cells and the differentiation of cells at the periphery of the cambium zone into phloem and xylem elements. In radish there is an active xylem cell proliferation, including intensive division of xylem parenchyma cells (Zaki et al., 2012). When studying the mechanisms of radish storage root development, a number of regulatory genes, including genes encoding components of the WOX-CLAVATA systems, have been identified. However, little data is

currently available on the targets of this system in developing the storage root and on the connection between the WOX-CLAVATA system and IAA and CK in the storage root.

Thus, the task of studying root development in radish can be divided into two interrelated parts: 1) investigating the mechanisms of storage root development and 2) searching for regulators of spontaneous tumour formation. Both these parts are presented in this work.

CHAPTER 2. MATERIALS AND METHODS

2.1. Plant material and growing conditions

2.1.1. Plant material

Two closely related inbred inbred lines (18 and 19) of radish (*R. sativus* var. *radicula* Pers.) of St. Petersburg State University radish genetics collection (Narbut, 1966; Buzovkina, Lutova, 2007) were the objects of the study. The genetic collection of radish inbred lines was created in the 1960s years of the XX century (Narbut, 1966) by Stanislava Iosifovna Narbut (Kuznetsova et al., 2023b) and currently includes lines of the 50th-55th generation of inbreeding. Thus, almost pure lines derived from long-term inbreeding were used in this work.

Lines 18 and 19 are descendants of one plant of the Saksa variety (Narbut, 1966; Buzovkina, Lutova, 2007). Saksa is a variety of radish suitable for cultivation in different natural zones, which belongs to the Red oval-round variety bred at the Federal Scientific Centre of Vegetable Growing (according to the State Register of Breeding Achievements Approved for Use). The vegetative period of Saksa variety plants is 32-36 days (medium-ripening variety). Storage roots are red, rounded and oblong-oval, with length of 4,5-5,2 cm and diameter of 2,5-4,0 cm. The surface is smooth, the flesh is white and white-pink. The yield of marketable storage roots is 9,7-13,0 tonnes per ha and the weight is up to 29 g. The rosette is semi-upright, with height of 15-16 cm, and diameter of 17-20 cm. The number of leaves in the rosette is 5-7, leaves are obovate, a petiole with weak anthocyanin colour, the leaf plate is dissected (Sazanova, 1985).

The genetic collection of inbred radish lines of SPbSU includes 13 lines of Saksa variety. All inbred radish lines originating from the Saksa variety have similar morphological characteristics to the mother variety. Some lines of this variety are also characterised by morphogenesis anomalies, such as agravitropic shoot growth (line 26) or chlorophyll deficiency (lines 20 and 21, Buzovkina and Lutova, 2007). In addition, a number of Saxa cultivar lines, such as 19, 20 and 21, are able to form spontaneous formation on storage roots of flowering plants with a frequency of 100% (Narbut, 1967; Narbut et al., 1995; Buzovkina, Lutova, 2007).

Lines 18 and 19, which are the objects of our study, are characterised by dissected leaf, red oblong-oval storage root, white colouring of flower corolla, medium vegetation period and high autofertility in inbreeding. The main morphological characters of lines 19 and 19 are shown in Figure 12.

Lines 18 and 19 contrast in their ability to form spontaneous tumours. In the flowering phase, almost 100% of plants of line 19 form tumours on the root and lower part of the stem, whereas line 18 never forms spontaneous tumours (Narbut et al., 1995; Buzovkina and Lutova, 2007). In addition, crosses between these closely related lines revealed monogenic differences in the ability to spontaneous tumour formation. Tumour formation was characterised as a recessive monogenic trait, controlled by an allele of the *Tur* gene (Narbut et al., 1985; Matveeva et al., 2000; Matveeva et al., 2004).

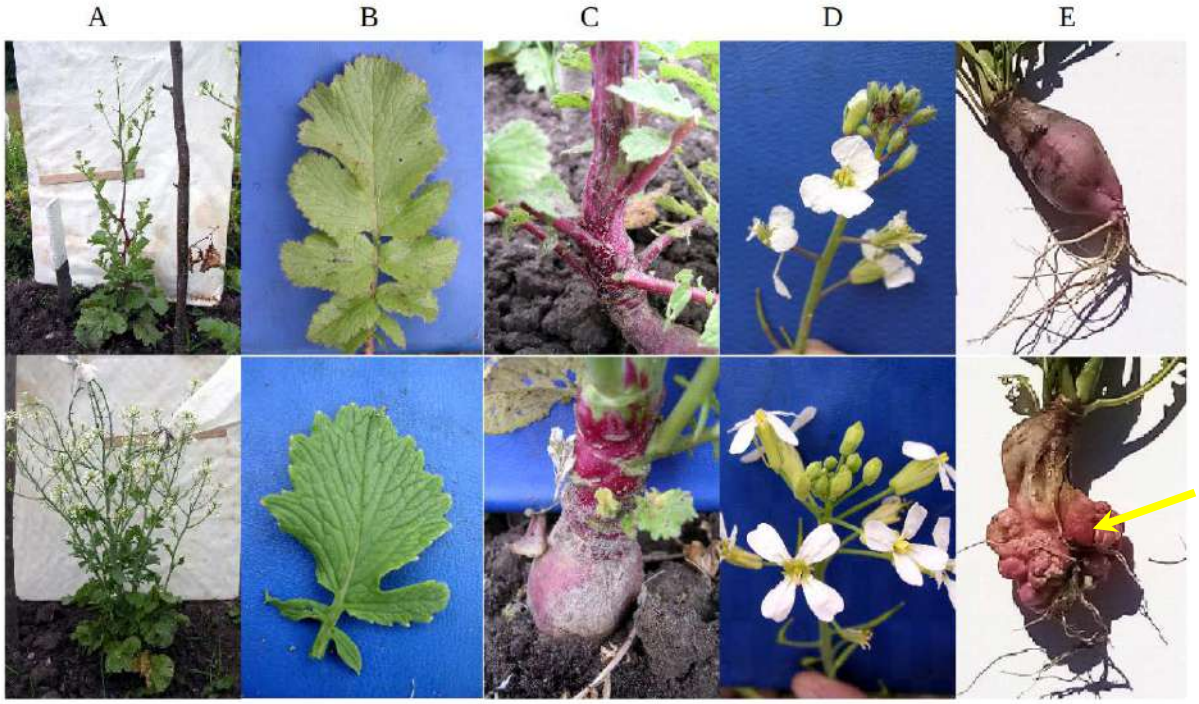

Figure 12. Morphological characteristics of lines 18 (top row) and 19 (bottom row) used in the work: A - appearance. B - leaf, C - stem, D - inflorescence, E - storage root (arrow marks spontaneous tumour).

In addition to diverse spontaneous tumour formation ability, lines 18 and 19 differ in the response of explants to CK *in vitro*. Hypocotyls of line 18 seedlings form root-like overgrowths and leaf explants form calluses on CK-containing medium, whereas line 19 is hypersensitive to CK and its explants form necroses on CK-containing medium (Buzovkina et al., 1993; Ilina et al., 2006). Presumably, the difference in response to CK, as well as the

ability to form tumour, could be explained by an increased level of endogenous CK in plant tissues (Matveeva et al., 2004), or the activation of the cellular response to CK (Lutova et al., 2008), which have previously been detected in tumour lines.

2.1.2. Plant growing conditions

Growing plants *in vitro*

To obtain aseptic seedlings for transformation with the vector constructs (see below), radish seeds were sterilised for 7 min with a mixture consisting of 30% hydrogen peroxide and 95% ethyl alcohol in a 1:1 ratio. The aseptic seedlings obtained were grown on Murashige - Skoog (MS) medium (Murashige and Skoog, 1962) at 23^{o}C and a daylight length of 16 hours.

Growing plants in the ground

The composite radish plants obtained as a result of the transformation were transplanted from sterile conditions into vermiculite, then into soil and grown in a greenhouse under natural light and temperature of 23^{o}C after reaching optimal root system capacity.

2.2. Methods

A variety of molecular genetics and bioinformatic methods were used to solve the problems posed in this thesis. All methods were grouped below according to the tasks to which they were applied.

2.2.1. Analysis of radish lines genome sequencing data

The following sequence of steps was applied to analyse the genomes of related tumour and non-tumor radish lines:

1) Genomic DNA isolation and sequencing using two approaches: a. Oxford Nanopore technology (its advantages are simple sample preparation and obtaining of very long single-end reads, up to 200 kb in length); b. Illumina technology (its benefits are high accuracy and obtaining of short paired-end reads (800 bp), which can be used to refine the data obtained by the first approach);

2) Genome assembly based on sequencing data;

3) Genome annotation using bioinformatics tools that have been validated for analysing dicotyledonous plant genomes;

4) Search for single nucleotide substitutions and insertions/deletions using software used for NGS data of plant genomes. In this paper, SNPs and InDel are also referred to by the general term ‘single nucleotide variants’ (SNVs).

For this purpose, the following standard methods were applied:

Sequencing, assembly, annotation and analysis of the genomes of tumour and non-tumour radish lines

The DNA isolation from 50 etiolated radish seedlings of lines 18 and 19 was performed according to the protocol kindly provided by the Laboratory of Plant-Microbial Interactions of the All-Russian Research Institute of Agricultural Microbiology (unpublished data).

The DNA sequencing of line 19 was performed using Oxford Nanopore technology in the Core Centrum “Genomic Technologies, Proteomics and Cell Biology” at the ARRIAM

using a MinION device (Oxford Nanopore, Cambridge, UK). The genome assembly of line 19 was performed using the Canu v.1.7.1 tool (https://github.com/marbl/canu/releases) with default settings. The sequencing of line 19 was also performed with Illumina technology on the HiSeq2500 sequencer at the Centre of Molecular and Cellular Technologies of Saint Petersburg State University Research Park. The NEBNext® Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) was used for library construction. Dual barcoding was performed using NEBNext® Ultra™ DNA Index Prep Kit for Illumina and NEBNext® Multiplex Oligos® Illumina® (Dual Index Primers Set 1). To improve the quality of the genome assemblies, data were refined to correct possible errors by Pilon v.1.22 (https://github.com/broadinstitute/pilon/releases) tool based on data obtained using two sequencing technologies (Illumina and Oxford Nanopore).

The DNA sequencing of line 18 was performed with Illumina technology only, at the Centre of Molecular and Cellular Technologies of Saint Petersburg State University Research Park using the HiSeq2500 sequencer. The NEBNext® Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs) was used for library construction. Dual barcoding was performed using NEBNext® Ultra™ DNA Index Prep Kit for Illumina and NEBNext® Multiplex Oligos® Illumina® (Dual Index Primers Set 1). The genome assembly of line 18 was performed using the SOAPdenovo v.2.04 tool (https://github.com/aquaskyline/SOAPdenovo2) with maximal read length = 150, average insert size = 100, cutoff of pair number for a reliable connection = 5.

For each assembly, the MultiQC v.1.12 (Ewels et al., 2016) and Trimmomatic v.0.40 (Bolger et al., 2014) with the HEADCROP:15 and CROP:140 options were used for quality control and read correction, respectively. The assemblies were indexed using the bowtie2 tool (https://github.com/BenLangmead/bowtie2). Chromosome-level assemblies of two genomes were generated using the Ragtag tool (https://github.com/malonge/RagTag) and the chromosome-level radish reference genome GCA_019703475.1 (https://www.ncbi.nlm.nih.gov/data-hub/genome/GCA_019703475.1/).

Annotation of the genomes of lines 18 and 19 was performed using the Augustus Gene Prediction Tool (https://github.com/Gaius-Augustus/Augustus) with the –species = arabidopsis parameter.

Alignment of line 19 sequences to the line 18 genome assembly was performed using bowtie2 software (https://github.com/BenLangmead/bowtie2). Identification of candidate genes and differences in the structure of these genes in different radish lines was performed

using the SnpEff (http://pcingola.github.io/SnpEff/), the SnpSift (https://pcingola.github.io/SnpEff/ss_introduction/) tools and the GATK with the HaplotypeCaller option (https://gatk.broadinstitute.org/hc/en-us/articles/360037225632-HaplotypeCaller) with the SelectVariants --select-type SNP or --select-type INDEL parameters.

The GO enrichment analysis was performed based on the list of all genes with SNVs and the list of all radish genes as inputs using the R programming language (v. 4.0.2) based on an unpublished custom R script. The GSEABase v. 1.50 (https://bioconductor.riken.jp/packages/3.11/bioc/manuals/GSEABase/man/GSEABase.pdf) was used for data visualisation. A total of 148 pathways related to different biological processes were identified, all of which were statistically significant (p.val_GO ← 0.01, OddsRatio_GO ← 2).

Single nucleotide substitutions (SNPs) and insertions/deletions (InDel) were searched and sorted by genotype to identify phylogenetic relationships between radish isolates and lines of different origins using Tassel5 (https://tassel.bitbucket.io/).

Visualisation of the sequence alignment per assembly and *in silico* verification of InDel and SNP presence were performed in IGV Genome Browser (https://igv.org/).

To confirm the detected differences in SNVs between line 18 and line 19 as well as between other tumour (12, 13, 14, 16, 20, 21, 32) and non-tumour (3, 5, 6, 8, 9, 23, 25, 26, 27, 28, 29, 30, 37, 39) lines of the radish genetic collection, DNA was isolated from radish seedlings of the listed lines using the CTAB method. PCR was performed under the following conditions: initial DNA denaturation at 98 °C for 3 min; DNA denaturation at 98 °C for 10 s, primer annealing at 52 °C for 30 s, extension at 72 °C for 1 min, repeated 35 times; and final extension at 72 °C for 5 min. Primers were designed using the VectorNTI software v1.1.1 algorithm (Invitrogen, Waltham, Massachusetts, USA) to amplify 300–400-length amplicons and synthesised by Evrogen (Russia). The PCR mixtures were subjected to Sanger sequencing.

The general pipeline of the experiment is presented in Figure 13.

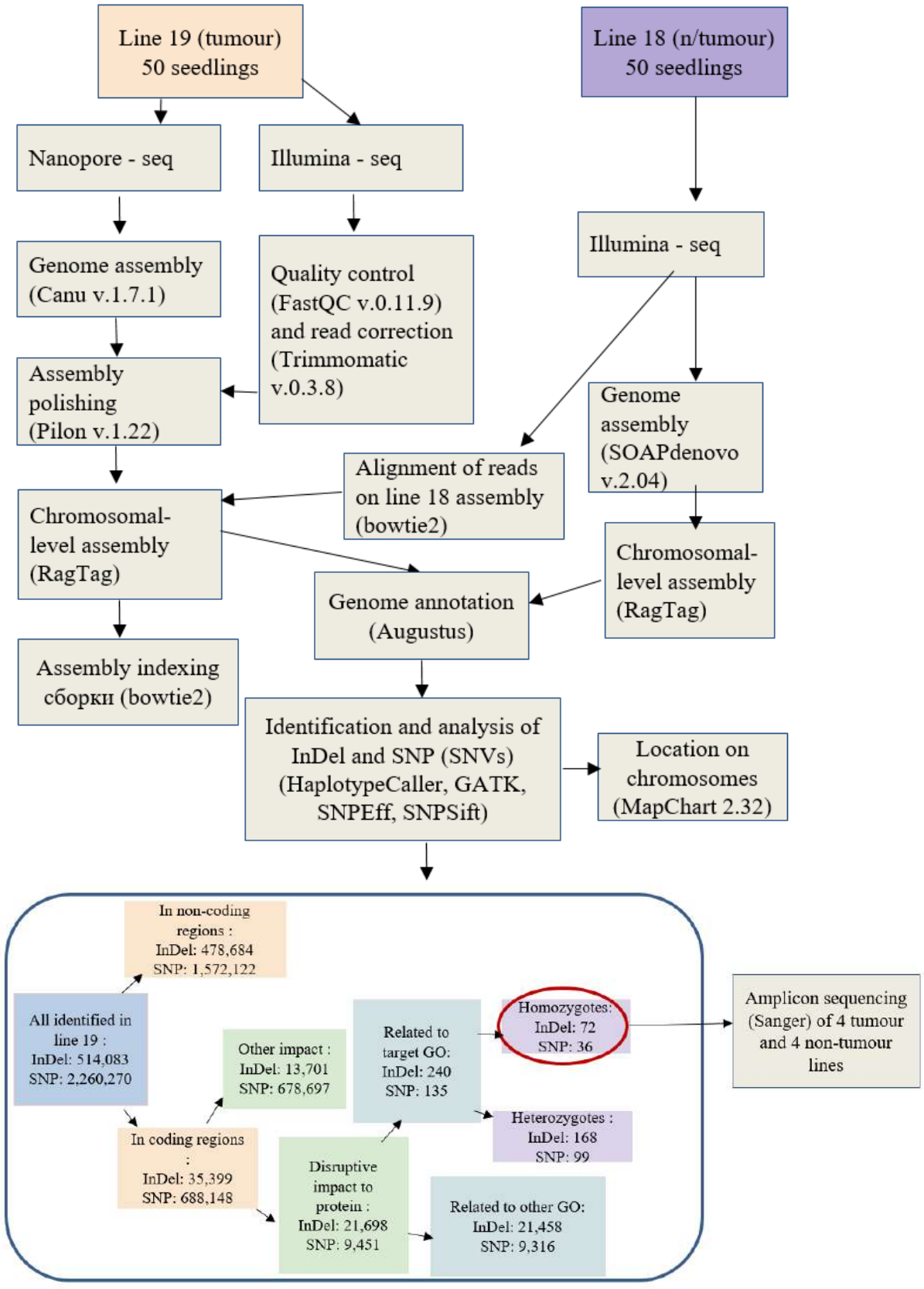

Figure 13. Pipeline of the experiment for the analysis of tumour and non-tumour lines of radish. All SNVs investigated in this work are marked with a red circle.

2.2.2. Study on the WOX-CLAVATA system genes role in root development

The objectives of this work were to identify *CLE* and *WOX* genes in radish and to study the effect of their overexpression on root development. For this purpose, a wide range of methods related to bioinformatic analysis, vector design, transformation and light microscopy were used.

Search for, identification and sequence analysis of *RsCLE* and *RsWOX* genes

The sequences for the *RsCLE* and *RsWOX* genes were searched for in the radish genome assemblies shown in Table 3 using the blastP, blastN and tblastN algorithms of the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi), based on the nucleotide and amino acid sequences of *A. thaliana* and *R. sativus* genes and proteins.

Phylogenetic trees of radish CLE and WOX proteins sequences were constructed based on the alignment of amino acid sequences of *R. sativus* CLE proteins in MEGA7 software (https://www.megasoftware.net/) using the Muscle algorithm (Kumar et al., 2016) by Neighbour joining (Saitou and Nei, 1987) with standard parameters and Bootstrap 1000 (Felsenstein, 1985); the tree was visualised using iTOL software (https://itol.embl.de/). Signal motifs were predicted with the SignalP-6.0 tool (https://services.healthtech.dtu.dk/service.php?SignalP).

Nucleotide and amino acid sequences were analysed using the following programs: ApE (https://jorgensen.biology.utah.edu/wayned/ape/, v.3.1.0), SnapGENE (https://www.snapgene.com/; v.6.0.2), UGENE (http://ugene.net/ru/; v.33), MEGA7 (https://www.megasoftware.net/; v. 10.2).

The localisation of genes on radish chromosomes was visualised using the MapDrawJZ macro for Excel (https://github.com/pinbo/MapDrawJZ).

The MEME software (http://meme-suite.org/) was used to visualise the domains of the RsCLE proteins. The domain structure of RsWOX4 and RsWOX14 proteins was analysed using the Conserved domains tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

The networks on putative interactions of proteins were constructed using the String web resource (https://string-db.org/).

DNA extraction

For amplification of individual *RsCLE* and *RsWOX* genes, total DNA was extracted from 7-day-old etiolated radish seedlings using the modified CTAB method (Aboul-Maaty et al., 2019). Plant material was ground with a pestle in liquid nitrogen, DNA was extracted with 2x CTAB buffer (2 g CTAB, 8.18 g NaCl, 10 ml Tris-HCL 1 M, pH 8, 4 ml EDTA 0.5 M) for 40 min, followed by purification with a mixture of phenol and chloroform (1:1) and phase separation by centrifugation at 17940 g for 20 minutes. The aqueous phase was transferred to a clean microtube and DNA was precipitated with isopropanol for 30 min followed by centrifugation at 17940 g for 20 minutes. The DNA precipitate was washed with cold 70% ethanol and dried under air current in a laminar flow hood, then dissolved in 100 µl sterile distilled water. The concentration of isolated DNA was measured using a Nanodrop-2000 spectrophotometer (Thermo Scientific) at a wavelength of 260 nm.

PCR

Primers for amplification of coding and promoter regions of genes were selected using the Vector NTI (Invitrogen) and Primer3 (Untergasser et al., 2012) software with subsequent quality assessment in the Ugene programme based on *R. sativus* gene sequences presented in the NCBI database. The primer sequences are presented in Table 1 in the Appendix.

The PCR reactions were carried out under the following conditions.

Amplification of the coding parts of the genes:

- 3 min. at 95^{o}C, 1 cycle;
- 60 sec. at 95^{o}C, 30 sec. at 54^{o}C, 30 sec. at 72^{o}C, 35 cycles;
- 5 min at 72^{o}C, 1 cycle.

Amplification of promoter regions of the genes:

- 3 min at 95^{o}C, 1 cycle;
- 20 sec. at 95^{o}C, 20 sec. at 45^{o}C and 2 min at 72^{o}C, 5 cycles;
- 20 sec. at 95^{o}C, 20 sec. at 65^{o}C and 2 min at 72^{o}C, 30 cycles;
- 5 min at 72^{o}C,1 cycle.

After completing all the PCR cycles, electrophoresis was performed on a 1% agarose gel with 0,1% SYBR Safe intercalating dye (Invitrogen) to visualise DNA under blue light. A 100+ or 1kb DNA length marker (Eurogen) was used to determine the approximate size of

the PCR fragments. Target fragments were separated from the gel using the Cleanup Mini reagent and column kit (Eurogen) according to the attached protocol.

Vector construction

The Gateway system (Invitrogen, USA) was used for cloning the DNA sequences. PCR fragments obtained by amplification of radish genomic DNA with primers listed in Appendix 1 were cloned into input vectors using BP-clonase viral recombinase mix (Invitrogen) and then recloned into destination vectors using LR-clonase viral recombinase mix (Invitrogen). Recombination reactions were performed according to the manufacturer's protocol.

To obtain vectors for overexpression, the coding sequences of genes were cloned into the pDONR221 entry vector (Figure 14) and then recloned into the pB7WG2D destination vector (Figure 14) containing the 35S promoter of cauliflower mosaic virus.

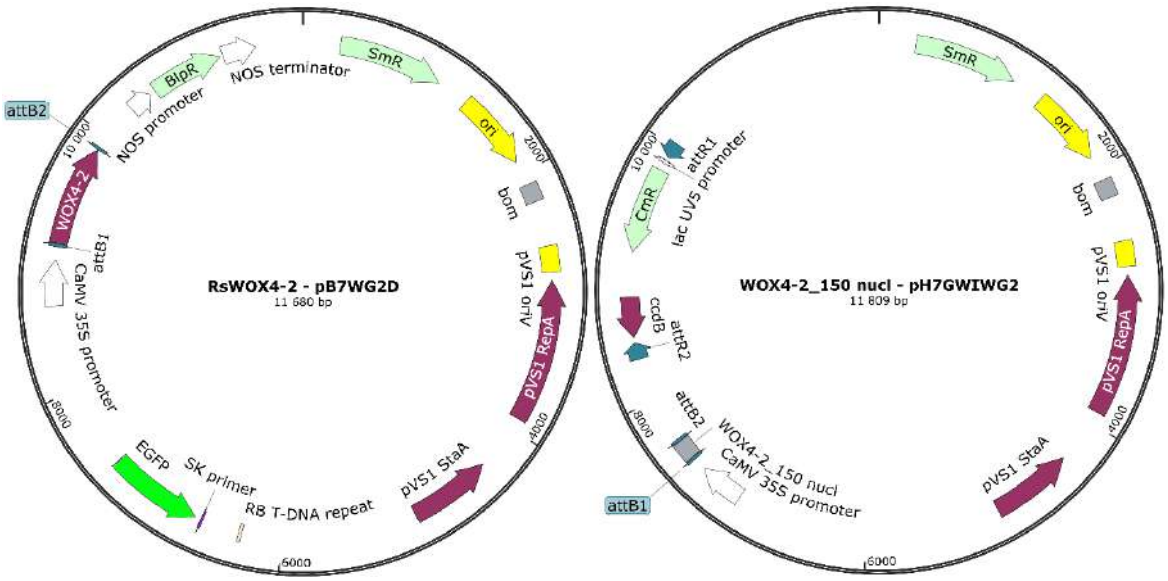

Figure 14. Destination vector-based plasmid maps for overexpression (left) and for gene silencing (right).

To obtain a vector for the silencing of *RsWOX4*, a region corresponding to the first 150 nucleotides of the coding part of the gene outside the homeodomain was amplified, cloned into the pDONR221 entry vector, and then recloned into the pH7GWIWG2 destination vector (Figure 14).

For the yeast one-hybrid assay, the promoter regions of the genes were cloned into the pDONR207 entry vector followed by recloning into the pHisLeu vector containing markers

for selection on histidine and leucine. The coding sequence of the WOX4 TF homeodomain, whose interaction with promoters was studied in the yeast one-hybrid assay, was cloned into the pDONR207 vector and then recloned into the pDEST22 vector containing a marker for selection on tryptophan.

The genetic constructs obtained in this work are listed in Appendix Table 2.

Bacterial transformation

The acquired constructs were used to transform chemically competent *Escherichia coli* cells of the DH10B strain, obtained according to the protocol of Sambrook et al., 2006. Transformation of *E. coli* was performed according to the protocol described by Inoue et al., 1990, after which the bacteria were cultured on LB solid medium with selective antibiotics depending on the vector.

Plasmid DNA was isolated from individual bacterial colonies using the Plasmid Miniprep reagent kit (Eurogen), and plasmids were then screened for the presence of the target insert by PCR with primers to genes of interest.

To obtain plants with overexpression of the *RsCLE41, RsWOX4, RsWOX14* genes or the glucuronidase (*GUS*) gene as a control, transformation of chemically competent cells of *Agrobacterium rhizogenes* strain Arqua with plasmids with the target insertions was performed according to the protocol of Inoue et al., 1990. The obtained *A. rhizogenes* strains were used for plant transformation.

Transformation of plants with *Agrobacterium rhizogenes*

To obtain composite plants with transgenic roots, *A. rhizogenes* strains with vectors for overexpression of *RsCLE41*, *RsWOX4*, and *RsWOX14* genes that regulate cambium development and a strain with a vector for the *GUS* reporter gene overexpression as a control were used in this work. These plasmids were obtained using the pB7WG2D vector (Figure 14).

For transformation of aseptic radish seedlings, the lower part of the hypocotyl was cut off and *A. rhizogenes* suspension was applied to the wounded area. The transformed plants were placed for cocultivation with bacteria on MS medium for 3 days, and then the transformants were transferred to MS medium with cefotaxime (500 mg/L) to kill the bacteria. The first roots induced by *A. rhizogenes* were removed because they were

nontransgenic or chimeric. After a large number of adventitious roots formed, plants were transferred to a sterile filter in a Petri dish with MS0 and cefotaxime.

After 3 weeks, transgenic roots were selected by detecting the luminescence of the GFP (Green Fluorescent Protein) reporter protein in blue light. Plants with transgenic roots were transplanted into pots with vermiculite and grown in the greenhouse, after 2 weeks of growing on vermiculite, plants were transplanted into pots with soil. After 30 days (at the stage of commercial ripeness of storage root; Sazanova, 1985) plants were harvested for further analysis.

Light microscopy

For the preparation of microdissections, plant material (roots of control and transgenic plants, roots of plants at different developmental stages) was incubated in a fixation buffer (3% paraformaldehyde; 0,5% glutaraldehyde; 0,2% Tween20; 0,2% TritonX-100; 10% DMSO in TBS buffer (50 mM PIPES, 5 mM MgSO4, 5 mM EGTA, pH 6.9)), followed by destaining with ethyl alcohol in 10% to 90% increments for 30-60 min, after that followed by destaining with alcohol in 90% to 10% increments, and then roots were placed in TBS buffer.

Roots were then fixed in 3% agarose to obtain sections. Transverse and longitudinal root sections were obtained from the fixed material using a Leica VT1200S vibrating blade microtome. The thickness of the sections obtained was 50 μm with a step of 1 μm and an amplitude of 1 mm.

Root sections were stained with 2% toluidine blue and then examined using a LEICA DM500 semi-automatic microscope at 10x, 20x and 40x magnification.

2.2.3. Quantitative analysis of gene expression

To study the effect of overexpression of *RsCLE41*, a cambial regulator, we performed RNA sequencing and analysed the transcriptomes of radish plants with *RsCLE41* overexpression and control plants with *GUS* overexpression.

In addition, to study the effect of overexpression of *RsCLE41* and its target *RsWOX4*, quantitative analysis of expression of individual genes using qPCR was also performed.

RNA sequencing of the radish transcriptome and analysis of transcriptome data

For RNA sequencing, total RNA was isolated from radish plants and seedlings using a modified phenol-chloroform method (Siebert and Chenchik, 1993). The cDNA was synthesised using the Mint-2 kit (Eurogen, Russia) and sequencing libraries were prepared using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina (New England Biolabs).

Purification of fastq raw reads from radish mitochondrial, plastid and ribosomal DNA was performed using the bbduk tool from the bbtools package (v. 38.96) (https://jgi.doe.gov/data-and-tools/bbtools/). Purified and quality-filtered reads were trimmed using Trimmomatic v. 0.40 (Bolger et al., 2014) with the parameters 'HEADCROP:15 CROP:95'. Quality control of the reads was performed using MultiQC v.1.12 (Ewels et al., 2016).

The filtered reads were aligned to the *R. sativus* reference genome (https://www.ncbi.nlm.nih.gov/assembly/GCA_000801105.2) using HISAT2 v. 2.01.2 (Kim et al., 2015). Reads were then counted using Stringtie (Pertea et al., 2015) tool. Differential expression between experimental and control samples was analysed using the DESeq2 package (v. 1.28.1) for R v. 3.6.2 (Love et al., 2014). Genes with p-value adjusted <0.05 and logFoldChange >1 were considered to be differentially expressed. The GSEABase v.1.50 package for R (https://rdrr.io/bioc/GSEABase/) was used for GO gene enrichment analysis.

qPCR

Total RNA was extracted from radish roots to analyse gene expression using qPCR. Plant material was ground in liquid nitrogen, RNA was extracted using Trizol reagent (Invitrogen) for 5 min at 25ºC, the mixture was purified from tissue residues by centrifugation at 17940 g and +4ºC, followed by chloroform purification and RNA precipitation with isopropanol for 20 min. The precipitate was washed three times with cold 80% ethanol, dried under airflow in a laminar and dissolved in sterile deionised water.

RNA was purified from genomic DNA impurities using DNAase A enzyme (Thermo Fisher Scientific) for 30 min at 37ºC, then DNAase was removed using DNA Removal Reagent (Thermo Fisher Scientific).

RNA concentration was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific) at 260 nm. According to the obtained data, the required amount of RNA for reverse transcription was calculated, assuming that the RNA concentration in the samples

should be approximately equal. Approximately 500 ng of RNA was used per reverse transcription reaction.

RNA reverse transcription was performed using dT-18 primers (Eurogen) and RevertAid reverse transcriptase (Thermo Fisher Scientific); 100 units of reverse transcriptase and 10 μM of dT-18 primers were used per reaction. The resulting cDNA samples were diluted with sterile deionised water to a final volume of 100 μl and stored at -20ºC.

Quantification of expression levels was performed by measuring the relative expression level of the gene of interest in relation to the expression level of the reference gene using the qPCR method. The ubiquitin *(UBI)* and glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)* genes, which are characterised by stable expression levels in different tissues, were used as reference genes (Dodueva et al., 2013).

A set of reagents for qPCR in the presence of Eva Green dye (Syntol) was used. PCR was performed under the following conditions: 1 cycle - 10 sec at 94^{o}C; 45 cycles - 15 sec at 94^{o}C, 30 sec at 52^{o}C, and 30 sec at 72^{o}C, followed by a stepwise increase in temperature from 72^{o}C to 95^{o}C in 5 sec steps of 0.5^{o}C. The primer sequences are listed in Table 1 of the Appendix. qPCR reactions were performed on a CFX96 amplifier with a C1000 thermocycler (BioRad); data on threshold cycles (Ct) calculation were obtained using CFX-Manager software (BioRad).

Gene expression for each sample was assessed in three analytical replicates, and the data were then averaged. The results were processed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittigen, 2001). Gene expression quantification data were presented in relative units calculated by comparison with the expression levels of *UBI* and *GAPDH* reference genes. The specificity of PCR amplification was confirmed by the melting curve (72-95^{o}C). Statistical processing of the obtained results was performed using the RStudio integrated development environment for R (http://www.rproject.org/).

2.2.4. Analysis of TF-DNA interactions

To search for WOX4 TF targets, the yeast one-hybrid assay (Davies, 2013) was used to study the interaction between DNA and protein, i.e. the possibility of TF homeodomain to bind to the promoter of the putative target gene.

Yeast transformation

For application of the yeast one-hybrid system, yeast transformation was performed according to the protocol described by Gietz and Schiestl (2007).

The production of competent *Sacharomyces cerevisiae* cells of Y2H Gold (Clontech) strain, kindly provided by colleagues at Oklahoma State University (USA), was carried out as follows. Cells were cultivated on solid YPD medium (pH 6.0) (10 g yeast extract, 20 g peptone, 20 g glucose, 20 g agar per 1 litre dH_2O) and grown at 30°C for 3 days. A single colony was then placed in liquid YPD medium (without agar) and incubated at 30°C on a shaker (200 rpm) overnight. The next day, 1 ml of overnight culture was diluted in 10 ml of liquid YPD medium and incubated at 30°C on a shaker (200 rpm) for 3 hours. The culture was precipitated by centrifugation for 2 minutes at 4000 rpm, the supernatant was drained, then the precipitate was washed with sterile water. The precipitate was then resuspended in 450 µl of 0.1 M lithium acetate and the obtained suspension was incubated at 30°C for 20 min.

Then, for transformation, plasmids (500 ng each) containing TF homeodomain sequence and promoter regions of genes, 20 µl of ballast DNA (5 mg/ml) and 276 µl of transformation solution (240 µl 50% polyethylene glycol, 36 µl 1M lithium acetate) were added to 50 µl of *S. cerevisiae* cell suspension, thoroughly resuspended and incubated for 30 min at 30°C. The tubes with suspensions were then placed in a 42°C water bath for 20 minutes. Centrifugation was then carried out at 5000 rpm for 30 seconds. The supernatant was then taken from the tubes, resuspended in 100 µl of sterile water, and cultivated on a dish with DDO selective medium (pH=5.9) (1,74 g Yeast nitrogen base mix (Sigma), 5 g $(NH_4)_2SO_4$, 20 g glucose, 20 g agar, 0,68 g Yeast Synthetic Drop-out Medium Supplements without leucine and tryptophan (Sigma, Y0750) in 1 litre of distilled water). The dishes were incubated for 3 days at 30°C.

Yeast one-hybrid assay

The yeast one-hybrid assay was applied as described by Davies (2013). Yeast colonies obtained after transformation were resuspended in 50 µl of water, the density of the suspension was measured using an iMark™ Microplate Absorbance Reader (Bio-Rad), then diluted with water to achieve the same optical density and cultivated on selective media, DDO (1.74 g yeast nitrogen base (Sigma), 5 g $(NH_4)_2SO_4$, 20 g glucose, 20 g agar, 0,68 g Yeast Synthetic Drop-out Medium Supplements without Leucine and Tryptophan (Sigma,

Y0750) and TDO (1,74 g yeast nitrogen base (Sigma), 5 g $(NH_4)_2SO_4$, 20 g glucose, 20 g agar, 0,68 g yeast synthetic drop-out medium Supplements without leucine, tryptophan and histidine mixture (Sigma, Y2146) with different concentrations of 3-amino-1,2,4-triazole (3-AT). To analyse each interaction variant, 4 colonies of yeast carrying the plasmids were seeded onto the media.

The growth of yeast colonies transformed with plasmids with sequences of promoter and homeodomain binding sites and control plasmids (not containing these sequences) was analysed. In the presence of TF homeodomain interactions with the promoters of candidate genes, colonies grew better in the experiment than in the control.

2.2.5. Statistical analysis

Statistical processing, as well as graphing, was performed using the RStudio integrated development environment v. 4.0.2 (http://www.rproject.org/) for the R programming language. The ggplot2 package (Wickham, 2016) was used for data visualisation. Quantitative indicators were compared using Student's t-test (Clogg et al., 1995).

CHAPTER 3. RESULTS

The general scheme of the experiment to analyse the role of meristem regulators during root development in radish is shown in Figure 15.

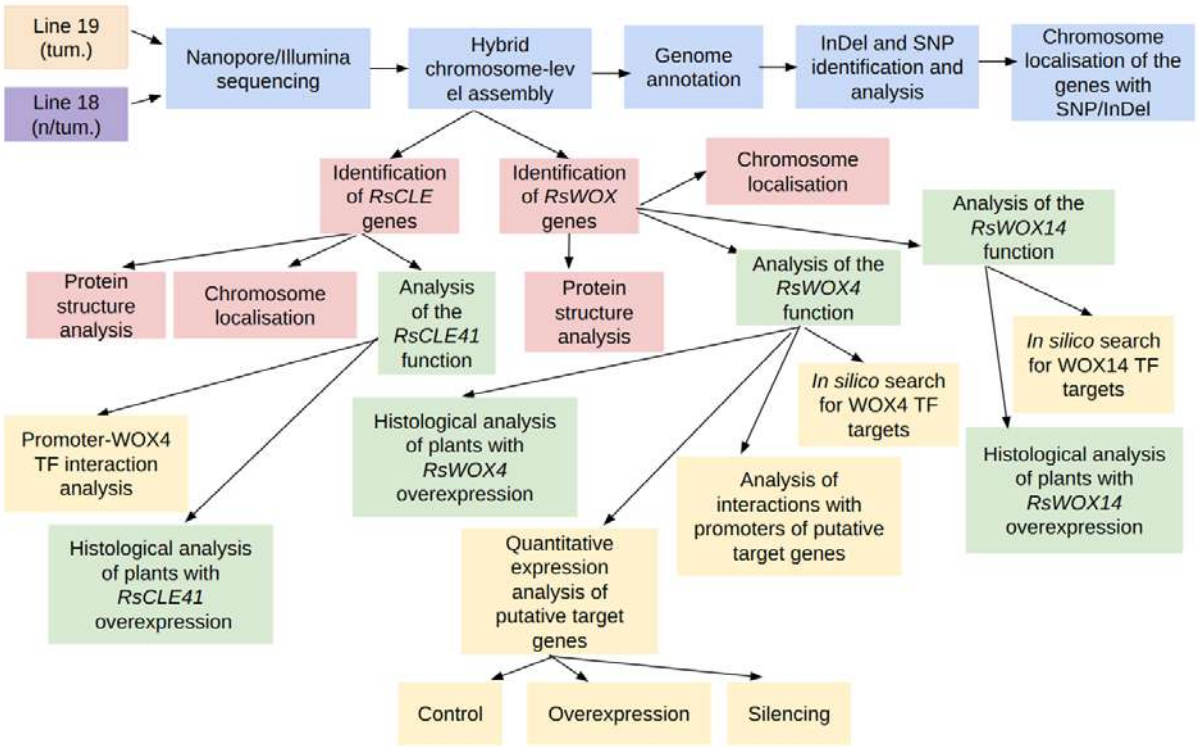

Figure 15. Main stages of the work. Different colours highlight the stages performed to solve different tasks of the work: No. 1 (blue), No. 2 (red), No. 3 (green), No. 4 (yellow).

3.1. Genome analysis of two lines of the radish genetic collection with different ability to spontaneous tumour development

To date, the radish genome has been sequenced and annotated (Kitashiba et al., 2014; Xu et al., 2023). Assemblies of radish genomes (see Table 3), as well as raw sequencing data of individual radish genotypes, are available in the NCBI database.

Radish genome sequencing has previously been performed for several Asian and European cultivars and two isolates (Kitashiba et al., 2014, Kim et al., 2016, Li et al., 2021, Zhang et al., 2021, Xu et al., 2023). We have sequenced and assembled, for the first time, the genomes of two related highly inbred lines of European radish with different ability to

spontaneously form tumours (Kuznetsova et al., 2024a). The lines also differ in other traits such as storage root shape, growth, leaf hairiness, etc, so comparative sequencing data can be further used to investigate genetic control of these traits. In order to identify differences in gene sequences between tumour and non-tumour lines, we carried out the following stages of analysis: quality control of the genome assembly; identification of genes with InDels and SNPs; and obtaining data on the functions of genes with significant SNPs and InDels in tumour line of radish.

3.1.1. Quality control of radish lines genome assemblies

In order to identify SNPs and InDel in genes that are candidates for involvement in the tumour development process, we performed a hybrid assembly (assembly combining data obtained by Illumina and Oxford Nanopore sequencing methods) of the genomes of two closely related lines with different ability to form tumours (lines 18 and 19 from the SPbSU radish genetic collection).

The following indicators were obtained as a result of the assembly quality assessment using the BUSCO programme (https://busco.ezlab.org/):

1) Genome of line 19: number of single copies of nuclear genes 92.4%, number of duplicated sequences 6.4%, total score 98.8, indicating a low content of fragmented or incomplete sequences and absence of contamination by sequences from other phylogenetic domains,

2) Genome of line 18: number of single gene copies 92.2%, number of duplicated sequences 6.4%, total score 98.6, indicating low content of fragmented or incomplete sequences and absence of contamination by sequences from other phylogenetic domains.

In addition, the genome assemblies of lines 18 and 19 were compared with other radish genome assemblies (from the NCBI datasets database) in terms of quality parameters. They were found to range from 93.8 to 98.9 BUSCOs, thus the genomes assembled in this study are of the same quality as those available in the database (Table 3).

Table 3. Features of radish genome assemblies available in the NCBI database. Assemblies obtained during the present work are highlighted in green.

№	GCA/GCF	Year	Assembly method	Assembly level	Sequencing technology	BUSCOs
1	SPbSU Line 19	2022	Canu/RagTag	Chromosomes	Nanopore; Illumina	98.8
2	SPbSU line 18	2022	SOAPdenovo v. 1.05/ RagTag	Chromosomes	Illumina	96.8
3	Cultivar GCF_000801105.1	2015	Newbler v. 1.2.3	Scaffolds	Illumina; PacBio; Sanger; 454	97.6
3	Cultivar GCA_002197605.1	2017	SOAPdenovo v. 1.05	Chromosomes	Illumina HiSeq	97.4
4	Isolate GCA_019705855.1	2021	MECAT v. 1.0	Chromosomes	PacBio RSII; Illumina	98.7
5	Isolate GCA_019705875.1	2021	MECAT v. 1.0	Chromosomes	PacBio RSII; Illumina	98.5
6	Isolate GCA_019705955.1	2021	MECAT v. 1.0	Chromosomes	PacBio RSII; Illumina HiSeq X Ten	98.9
7	Monoisolate GCA_019705955.1	2020	No data	Chromosomes	PacBio и Hi-C	98.7
8	Cultivar Aokubi GCA_001047155.1	2015	Newbler v. 2.7; SSPACE v. 2.0	Scaffolds	454; Illumina	96.7
9	Cultivar Aokubi S-h GCA_000715565.1	2014	SOAPdenovo2 r223; GapCloser v. 1.10; SSPACE v. 2.0	Scaffolds	Illumina HiSeq2000	93.8

3.1.2. Identification of genes with nucleotide substitutions, insertions and deletions in tumour line

When analysing the genome sequences of lines 18 and 19 of the radish genetic collection, numerous (hundreds of thousands) SNPs and InDel were identified in line 19 (Figure 16). We have selected for analysis those SNPs and InDels that 1) Were in the homozygous state; 2) Were located in the coding regions of genes or in positions 1–20 of the 5'-UTR; 3) Caused a frameshift and were likely to lead to non-functional gene products. Those were SNPs and InDel of our interest. Most of the identified SNPs and InDels are located at the beginning or in the middle of the corresponding genes, resulting in impaired function of them. All genes with substitutions in line 19 are functional, as confirmed by data on transcriptome analysis of radish tumours (Tkachenko et al., 2021a, b).

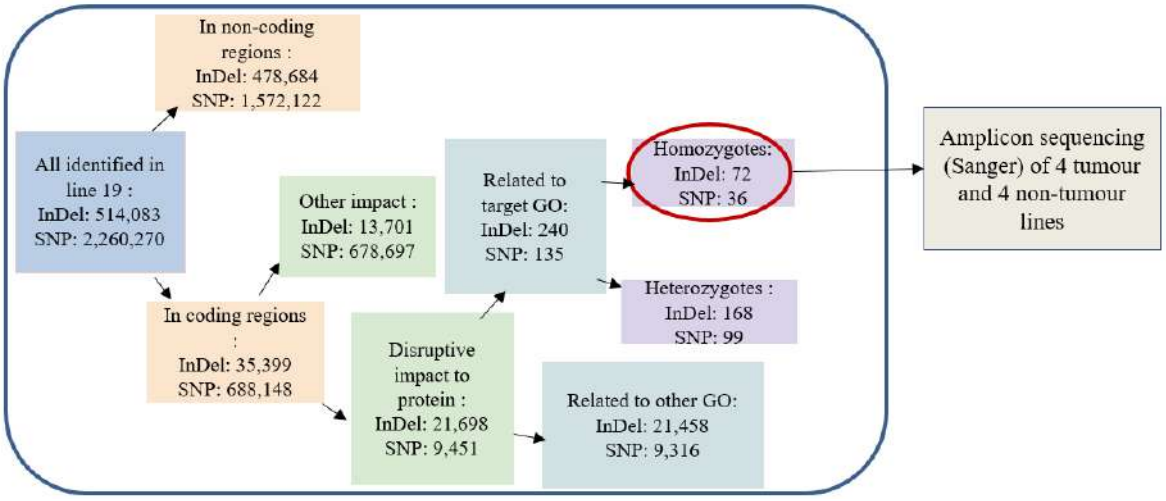

Figure 16. SNVs identified at different stages of the present work. Significant SNVs investigated in this study are marked with a red circle.

In total, 72 genes with InDel in the tumour radish line were of our interest. **InDels** in these genes are in their coding regions resulting in different frameshift variants, such as: frameshift (57 genes), frameshift and loss of start codon (10 genes), frameshift and gain of stop codon (5 genes). The results of functional characterisation of the genes with significant InDel is summarised in Table 4.

In addition, 36 genes with **SNPs** in the tumour radish line, resulting in different frameshift variants, such as: stop codon gain (23 genes), stop codon loss (10 genes) and start

codon loss (3 genes) were also the objects of our interest. The characteristics of genes with single nucleotide substitutions in the tumour line are shown in Table 5.

Moreover, SNP analysis of different radish genotypes showed (Figure 17) that the lines of the genetic collection clustered phylogenetically closer to the European radish lines (Dongby102, Long Scarlet and WK10024) available for analysis in the NCBI database, as we expected when analysing the lines isolated from the European Saxa variety.

We have localised the genes with InDel and SNPs on the radish chromosomes (Figures 18, 19) and, in general, their distribution was fairly even.

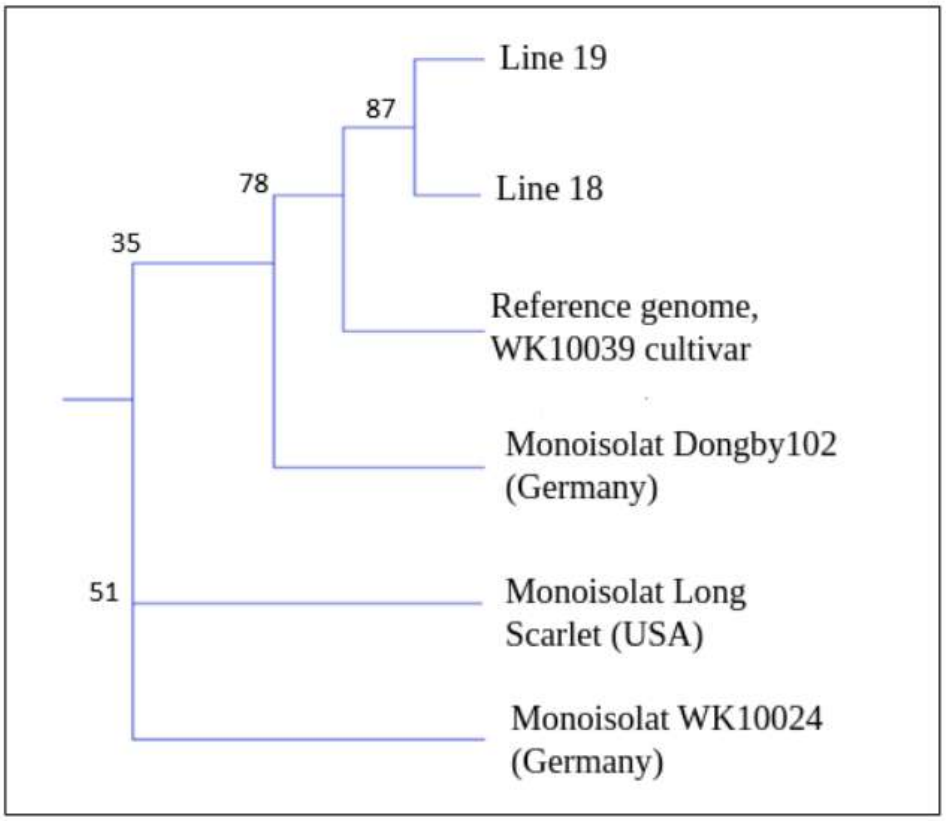

Figure 17. Phylogenetic tree of different radish varieties with sequenced genomes constructed by Neighbor-joining algorithm.

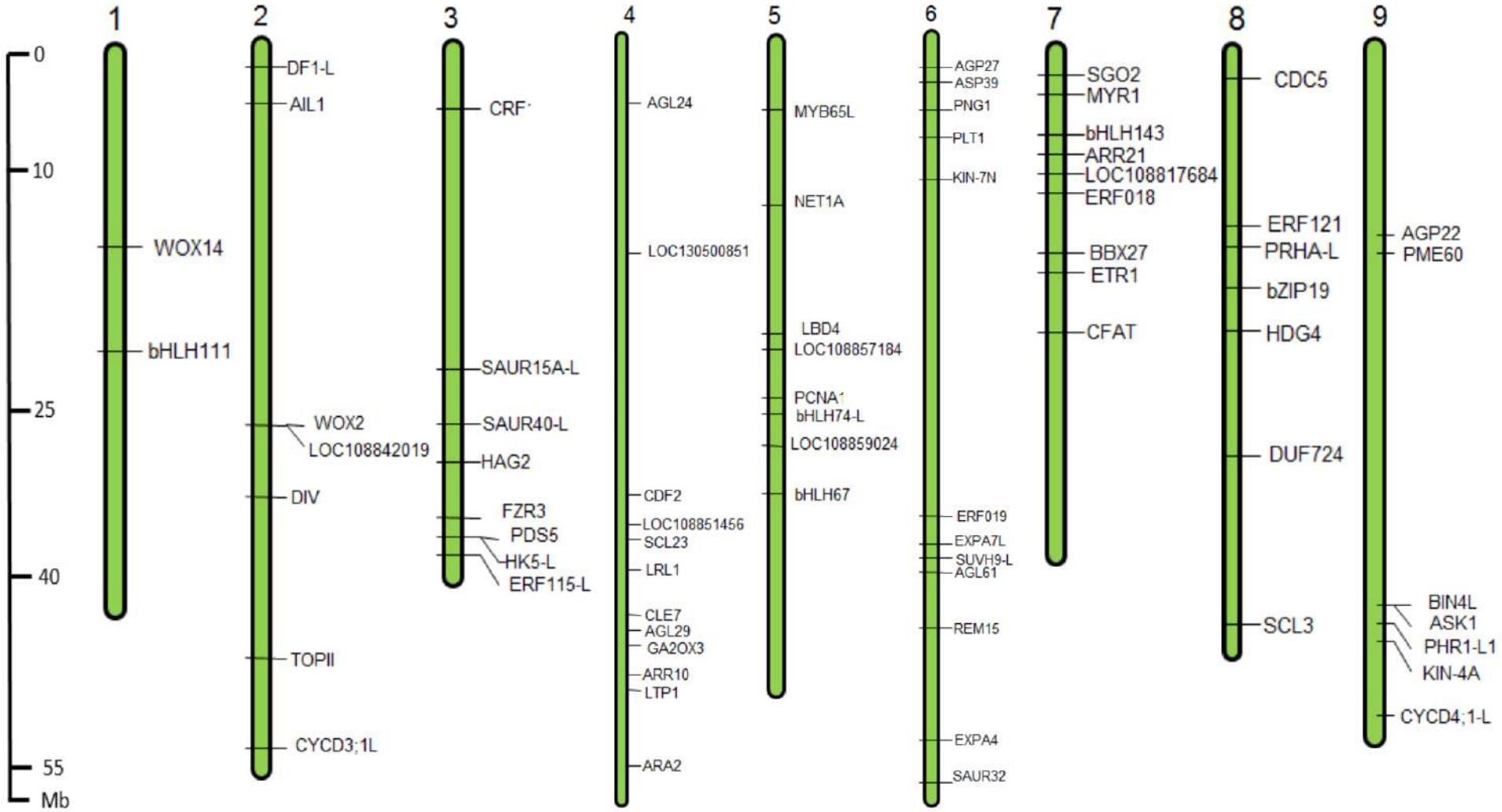

Figure 18. Location of genes with InDel in tumour line 19 on radish chromosomes.

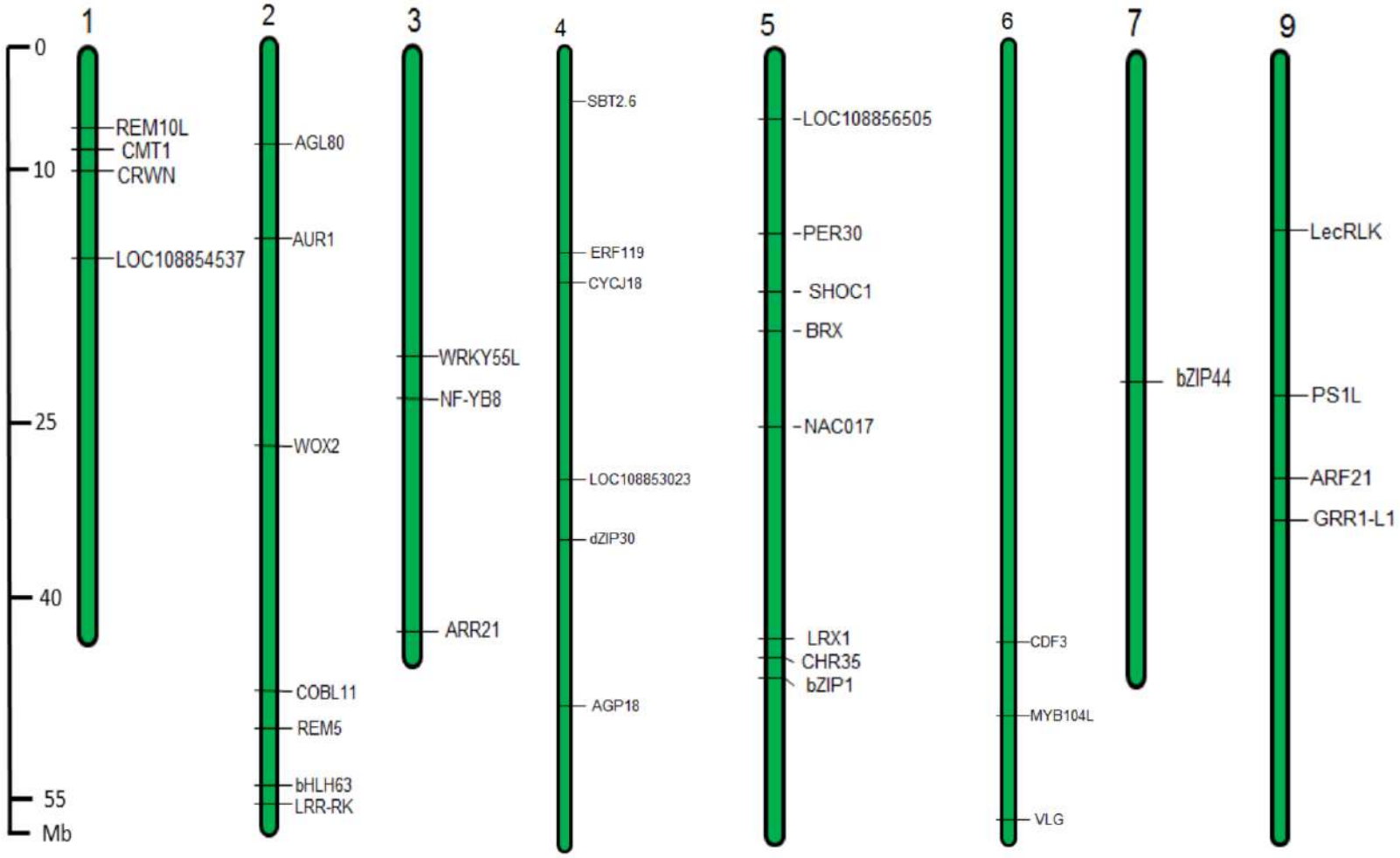

Figure 19. Location of genes with SNPs in tumour line 19 on radish chromosomes. No genes with SNPs were located on chromosome 8, so it is not shown in the figure.

3.1.3. Verification of the SNPs and InDel in the tumour line by amplicon sequencing

Among all the genes with significant SNPs and InDel, we selected genes that belong to the following Gene Ontology (GO) pathways for further study:

1. **Associated with cell cycle as well as control of cell divisions and cell growth:** “proliferation of cell populations” (GO:0008283), “cell cycle regulation” (GO:0007346; GO:0010564; GO:0000278; GO:0051726), “replication of DNA on DNA template” (GO:0006261), “DNA endoreduplication” (GO:0042023), “cytokinesis” (GO:0000910; GO:0000911), “cytoskeleton organisation” (GO:0000226), “component of cell wall” (GO:0009828; GO:0009505), “multidimensional cell growth” (GO:0009825), “anchored component of membrane” (GO:0031225).

2. **Associated with control of meristem activity:** “meristem initiation” (GO:0010014), “regulation of meristem growth” (GO:0010075), “regulation of meristem structural organisation” (GO:0009933);

3. **Associated with phytohormonal metabolism and signalling:** “CK-activated signalling” (GO:0009736), “CK metabolism” (GO:0009690), “gibberellin biosynthesis” (GO:0009686), “gibberellin catabolism” (GO:0045487), “auxin-activated signalling” (GO:0009734), “auxin response” (GO:0009733), “CK response” (GO:0009735), “gibberellin response” (GO:0009739);

4. **Associated with gene expression regulation:** “DNA-binding TFs” (GO:0003700), “DNA methylation" (GO:0006306), "histone methylation by lysine" (GO:0034968), "histone methylation by H3-K9" (GO:0051567).

5. **Associated with organogenesis:** “root development” (GO:0048364), “lateral root development” (GO:0048527), “cotyledon boundary formation” (GO:0090451).

The effects of mutations of several genes of our interest on plant genotype/phenotype have been described in the literature. These effects are summarised in Tables 4 and 5.

Thus, mutations in genes related to the pathways of cell cycle regulation and cell growth had the following effects in *Arabidopsis*:

Mutation in the *CDC5* gene controlling cell cycle regulation led to accelerated cell death and increased susceptibility of plants to pathogens (Palma et al., 2007; Lin et al., 2007).

The *NET1A* mutant, which normally controls cytoskeletal organisation, showed accelerated root cell growth (Deeks et al., 2012).

Plants with knockdown of *sgo2*, which in wildtype regulates sister chromatid cohesion and segregation in meiosis, showed no meiotic or vegetative defects and reduced fertility (Cromer et al., 2013; Zamariola et al., 2013). The *sgo1* and *sgo2* double mutants had premature sister chromatid separation in anaphase I and were sterile (Zamariola et al., 2014).

The *PDS5* gene controls DNA repair, and the *psd5* mutant exhibited impaired homologous recombination and DNA repair, resulting in developmental defects and reduced fertility (Pradillo et al., 2015).

Mutations in genes associated with control of meristem activity:

A mutant in the *KIN-4A* gene, which controls cell wall development, had shorter inflorescence roots and stems and altered orientation of cellulose microfibrils in the fibre walls, resulting in reduced mechanical strength of the fibre. The lower rate of inflorescence stem growth along with reduced wall thickness resulted in the formation of mechanically weaker stems (Zhu et al., 2015). The line 19 has been reported to have lower plant growth compared to line 18 (Buzovkina and Lutova, 2007), which may be due to a substitution in this gene, but additional studies are needed to clarify whether this gene is related to tumour formation or plant growth.

The *MYB65* mutant, which normally regulates tissue growth by lowering cell proliferation, showed reduced levels of gene expression in the seed aleurone layer (Alonso-Peral et al., 2010). The *myb33 myb65* double mutant was characterised by defects in anther development resulting in male sterility (Millar et al., 2005).

Mutations in genes of phytohormone metabolism and signalling:

Mutation in the *HK5* gene, which normally negatively regulates ETR1-dependent ABA signalling, determines root hypersensitivity to ABA and ethylene, reduced closure of

stomata in response to various stresses, and increased sensitivity to pathogens and increased tolerance to high salinity (Iwama et al., 2007; Desikan et al., 2008; Pham and Desikan, 2012).

The *ETR1* mutant showed no apparent phenotype in response to ethylene, while *Ers1 etr1* double mutants exhibited a constitutive phenotype of ethylene response (Qu et al., 2007).

A mutant for the *AGL24* gene controlling the response to gibberellins exhibited delayed flowering (Yu et al., 2002).

In *Arabidopsis*, mutations in genes which regulate expression levels have also been described.

For example, a mutation in the gene encoding the histone methyltransferase *suvh9* led to impaired gene silencing due to decondensation of chromocentres and subsequent de-repression of DNA-methylated genes and mobile elements (Liu et al., 2016).

Mutation in *GT-2* (radish *DF1-L* homologue), which suppresses root hair growth, did not result in a visible phenotype under normal growth conditions, but *gtl1-1 df1-1* double mutants had increased root hair length (Shibata et al., 2018) and less mucus produced by them (Vasilevski et al., 2012, Voiniciuc et al., 2015).

Seedlings mutant in the *bZIP19* gene, which normally controls zinc accumulation in roots, formed shorter roots when growing on zinc-depleted medium (Inaba et al., 2015).

Mutations in *MYR1* and *CDF2* genes regulating photoperiodic control of flowering (Fornara et al., 2009, Zhao et al., 2011) caused early flowering.

In addition, there is also data on the disruption of genes with SNPs (data presented in Table 5). Most of them encode transcription factors.

For example, the *bZIP44* mutant showed a decrease in seed germination rate after swelling, which did not affect the overall germination rate (Iglesias-Fernandez et al., 2013). Mutants for the *CDF3* gene were responsible for photoperiodic control of flowering (Fornara et al., 2009), as well as for the *ARR21* gene that activates the A-type ARR in response to CK, had no apparent phenotype (Horak et al., 2003).

In addition, a mutant for the *CRWN* gene encoding for histone methylase was characterised by a phenotype of moderate dwarfism and leaf curling. It also formed smaller leaf rosettes (Choi et al., 2019).

A mutant for the division orientation regulator gene *PS1* formed diploid pollen grains due to defects in division II of meiosis (d'Erfurth et al., 2008).

A mutant for the *LRX1* gene regulating lateral root growth had uneven development of root hairs (Schaufelberger et al., 2019).

Thus, a number of functionally significant abnormalities in important regulatory genes were identified in the radish tumour line. Genes with InDels or loss-of-function SNPs in tumour line 19 are important for different aspects of plant development regulation, and their loss of function may lead to abnormalities of morphogenesis or impaired response to environmental factors. To date, there is no literature data on mutations in the investigated genes that can lead to ectopic cell divisions and thus to tumour formation. It is possible that these mutations individually do not cause tumours, but collectively they probably can. Most of the identified genes are presented in a triplicated genome of radish by several copies that also do not allow us to confidently consider them to be regulators of tumour formation. This part of the work represents a basis for further research in this field. The next stage of these studies could be verification of the possible connection of certain InDel and SNPs with the tumour formation trait by genetic analysis, as well as investigation of the functions of identified genes by their overexpression, silencing, and editing.

Table 4. Genes with InDel in line 19.

№	Gene	Chr	InDel	The homologue ID in the NCBI database	Function	Number of homologues in radish	Reference
Cell cycle control, cell growth and division							
1	***CFAT***	7	c.868_871delAGGA|p.Arg290fs	LOC108815961	Lignin biosynthesis.	2	Dexter et al., 2007
2	***CYCD3;1L***	2	c.967delC|p.Arg323fs	LOC130508373	Cell cycle control during the G1/S transition: activation in response to CK; proliferation of leaf epidermal cells and stomata density; regulation of secondary root growth.	2	Dewitte and Murray, 2003; Menges et al., 2006; Dewitte et al., 2007; Elsner et al., 2012; Randall et al., 2015; Han et al., 2022
3	***SGO2***	7	c.391_392insCT|p.Phe131	LOC108814355	Cohesion and segregation of sister chromatids in meiosis.	1	Cromer et al., 2013; Zamariola et al., 2013
4	***CYCD4;1-L***	9	c.591_592delCA|p.Lys199fs	LOC108828557	Activation of the G1/S transition in response to CK; mitotic cell division; cell cycle activation in RAM; germinal root promotion.	3	Kono et al., 2003; Masubelele et al., 2005; Strzalka et al., 2015
5	***KIN-7N***	6	c.234_237delGATT|p.Ile79fs	LOC108807218	ATP binding; motor function of microtubules.	2	Day et al., 2009
6	***PDS5***	3	c.1delA|p.Met1fs	LOC108845796	DNA repair; cohesion of sister chromatids in mitosis.	1	Pradillo et al., 2015

7	***EXPA7L***	6	c.375_376insTAAT|p.Gly126fs	LOC108810873	Weakening of cell walls due to disruption of the bond between cellulose microfibrils and matrix glucans; elongation of root hairs.	3	Cho et al., 2002; Lin et al., 2011
8	***EXPA4***	6	c.1delA|p.Met1fs	LOC108813883	Weakening of cell walls due to disruption of the bond between cellulose microfibrils and matrix glucans; formation of syncytium.	3	Jamil et al., 2019; Liu et al., 2021
9	***LTP1***	4	c.1dupA|p.Met1fs	LOC108853879	Lipid transport across membranes; deposition of wax and cutin in cell walls and secretory tissues; binding of calmodulin.	1	Potocka et al., 2012; Wang et al., 2016b
10	***LOC108817684***	7	c.148dupT|p.Cys50fs	LOC108817684	Biosynthesis of xyloglucans.	1	Sampedro et al., 2017
11	***PME60***	9	c.2861_2862insCCTC|p.Thr955fs	LOC108826705	Modification of cell walls by demethylesterification of pectin.	1	Coculo and Lionetti, 2022
12	***KIN-4A***	9	c.61dupA|p.Ile21fs	LOC108827664	Deposition of cellulose microfibrils; cell wall mechanics during cell elongation.	2	Zhong et al., 2002; Kong et al., 2015; Zhu et al., 2015
13	***LOC108859024***	5	c.2410delG|p.Glu804fs	LOC108859024	A gene with unknown function - probably a cell cycle regulator	1	Heyndrickx and Vandepoele, 2012
14	***FZR3***	3	c.353dupA|p.Gln119fs	LOC108844659	Control of ubiquitinligase activity and substrate specificity of anaphase-stimulating complex.	3	Larson-Rabin et al., 2009; Dangarh et al., 2020
15	***NET1A***	5	c.1664_1665delTT|p.Val555fs	LOC108862024	Association with F-actin at plasma membrane and plasmodesmata; response to pathogen-associated stress; inhibition of root growth.	1	Deeks et al., 2012; Hawkins et al., 2014

16	***PCNA1***	5	c.154_155insGC|p.Leu52fs	LOC108832540	Control of DNA replication by increasing polymerase processivity by leading chain elongation.	3	Strzalka and Aggarwal, 2013; Qian et al., 2019
17	***WOX14***	1	c.135_136insTC|p.Phe46fs	LOC108853278	Functions in the organising centre of the shoot meristem, maintaining stem cells in an undifferentiated state. Together with WOX4 act downstream of PXY, independently regulating plant vascular proliferation.		Hirakawa et al., 2010; Etchells et al., 2013; Zhang et al., 2016b
18	***HAG2***	3	c.909_910delGG|p.Ala304fs	LOC108847241	Histone modification; control of cell cycle, flowering time, response to environmental conditions, hormone signalling and epigenetic processes.	2	Chen and Tian, 2007; Pfluger and Wagner, 2007
19	***BIN4L***	9	c.96_100delTTCAT|p.Cys32fs	LOC130499962	Chromatin organisation and endoreduplication cycling.	3	Breuer et al., 2007
20	***MYB65L***	5	c.195_196insAGGCTGGGTGCCT|p.Leu66fs	LOC108862100	Inhibition of tissue growth by reduced cell proliferation; programmed cell death of protein-storing vacuoles in the aleurone layer during seed germination; anther and tapetum development.	1	Gocal et al., 2001; Millar et al., 2005; Alonso-Peral et al., 2010.
21	***DUF724***	8	c.25dupA|p.Thr9fs	LOC108820138	Polar cell growth.	2	Cao et al., 2010
22	***AGP22***	9	c.1_1delGA|p.Met1fs	LOC108826274	Differentiation, embryogenesis and programmed cell death.	3	Schultz et al., 2000; Pereira et al., 2014
23	***AGP27***	6	c.111_112delTC|p.Pro38fs	LOC108809107	Differentiation, embryogenesis and programmed cell death.	1	Pereira et al., 2014
Control of meristem activity							
24	***LBD4***	5	c.17dupG|p.Gly7fs	LOC108857433	Phloem/xylem histogenesis; regulation of radial growth through control of cell size and cambium cell divisions.	1	Smit et al., 2020; Ye et al., 2021; Turley

							and Etchells, 2022
25	***ASK1***	9	c.535_536dupTT|p.Leu179fs	LOC108827710	Regulation of meristem development; response to osmotic stress.	2	Dornelas et al., 2000; Dong et al., 2020
26	***LOC130500851***	4	c.654delT|p.Ala219fs	LOC130500851	Lateral root initiation and shoot regeneration.	2	Johnson et al., 2011
27	***REM15***	6	c.2917dupT|p.Cys973fs	LOC108808545	Flower development.	1	Mantegazza et al., 2014
28	***PLT1***	6	c.177_178insTA|p.Asp60fs	LOC108829093	Specification of the resting root and columella centre; stem cell niche formation during embryogenesis; modulation of polar auxin transport through regulation of PIN gene distribution. A component of the ethylene-activated signalling pathway. Telomere maintenance.	1	Shimotohno et al., 2018; Xiong et al., 2020
Regulation of gene expression							
29	***WOX2***	2	c.2593delG|p.Glu865fs	LOC108841333	Embryonic pattern formation.	1	Chung et al., 2016; Hassani et al., 2022
30	***ERF121***	8	c.428_429delAG|p.Lys143fs	LOC108837093	Regulation of gene expression by stress factors and components of signal transduction pathways under stress.	6	Zlobin et al., 2021
31	***ARR21***	7	c.599delT|p.Leu200fs	LOC108815221	B-type response regulator involved in the phosphorelay-type signal transduction system from His to Asp. Activates the A-type RR in response to CK.	2	Horák et al., 2003; Brenner et al., 2012; Hill et al., 2013
32	***SCL23***	4	c.97delT|p.Ser33fs	LOC108848776	Formation of vascular bundle sheath cells.	1	Cui et al., 2014
33	***ERF115-L***	3	c.232_235delAG	LOC108846562	Activation of phytosulfokine; rate-limiting of resting	3	Heymann et al.,

			CT|p.Ser78fs		centre cell division, active when surrounding stem cells are damaged. Proteolytic target of the APC/C-FZR1 complex.		2016; Zhang et al., 2016a; Johnson et al., 2018; Canher et al., 2020; Hoermayer et al., 2020; Lakehal et al., 2020
34	***bHLH143***	7	c.182_183dupAA|p.Val62fs	LOC108817725	Vascular development.	1	Jorgensen et al., 2012
35	***MYR1***	7	c.291_294delCCCC|p.Pro98fs	LOC108815809	Suppresses flowering and organ elongation when light intensity is reduced; suppresses GC-dependent responses and affects gibberellin levels.	1	Thelander et al., 2002; Zhao et al., 2011; Zhao and Beers, 2013
36	***PHR1-L1***	9	c.262_263insTG|p.Ser88fs	LOC108824019	Response to phosphorus deficiency; carbohydrate transport.	1	Sun et al., 2016; Spies et al., 2022
37	***CDF2***	4	c.378delA|p.Glu127fs	LOC108849692	Control of photoperiodic flowering response through repression of *CONSTANS*.	2	Imaizumi et al., 2005; Fornara et al., 2009; Sun et al., 2015
38	***ARR10***	4	c.865_866delGC|p.Ala289fs	LOC108830348	Activation of RR A-type in response to CK. Callus formation, maintenance of SAM identity, primary root development, regulation of anthocyanin metabolism, chlorophyll biosynthesis, seed growth; response to water deficit.	1	Cortleven et al., 2016; Nguyen et al., 2016; Meng et al., 2017; Zubo et al., 2017; Rong et al., 2018; Xie et al., 2018a

39	***bZIP19***	8	c.763_764dupGA|p.Asp255fs	LOC108818944	Promotes expression of the zinc transporters ZIP3, ZIP4, ZIP5 and ZIP9 during growth under zinc-deficient conditions.	2	Assunção et al., 2010; Inaba et al., 2015; Lilay et al., 2019; Lilay et al., 2020
40	***DIV***	2	c.38_39dupTG|p.Lys14fs	LOC108840441	Suppression of ABA biosynthesis; response to gibberellins; response to salicylic acid; response to salinity.	4	Fang et al., 2018
41	***AGL61***	6	c.278_279delTG|p.Met93fs	LOC108808389	Control of cell differentiation during female gametophyte development.	2	Steffen et al., 2008
42	***AGL29***	4	c.1dupA|p.Met1fs	LOC108850931	Pollen development.	2	Verelst et al., 2007
43	***bHLH67***	5	c.632_633insT|p.Thr212fs	LOC108860648	Adaptation to cold.	2	Gao et al., 2017
44	***HDG4***	8	c.1189_1190delGC|p.Ala397fs	LOC108820851	Identity of subepidermal cells during postembryonic development.	2	Nakamura et al., 2006; Bhatia et al., 2021
45	***SCL3***	8	c.100delA|p.Ile34fs	LOC130499224	Response to gibberellins.	1	Zentella et al., 2007; Zhang et al., 2011
46	***AGL24***	4	c.246_247insC|p.Tyr83fs	LOC108852002	Transition to flowering in response to jarrowisation; determination of inflorescence fate in apical meristems; signal transduction via receptor-like kinases. Upon binding to SOC1, mediates the effect of gibberellins on flowering under short-day conditions and regulates LFY expression.	1	Michaels, et al., 2003; Yu et al., 2004; Lee et al., 2008; Torti and Fornara, 2012

47	***bHLH74-L***	5	c.1020delT|p.Tyr340fs	LOC108856918	Cell elongation; binds to the chromatin region of FT, which promotes its expression and flowering in response to blue light. A component of miR396a, a bHLH74 module that regulates root formation in *Arabidopsis* seedlings.	2	Bao et al., 2014
48	***AIL1***	2	c.1039_1040insTT|p.Tyr347fs	LOC130498742	Control of shoot and flower meristems; flowering initiation; seed pod development; flower organ identity; cell proliferation; regulation of gene expression by stress factors.	4	Horstman et al., 2014
49	***ERF018***	7	c.-1_1insCAG	LOC108835321	Cell division; response to insect exposure; ethylene-activated signalling pathway; phloem/xylem histogenesis; response to wounding; activation of jasmonate biosynthesis gene expression.	3	Etchells et al., 2012; Nieminen et al., 2015; Huang et al., 2021
50	***DF1-L***	2	c.709_710delCC|p.Pro237fs	LOC108848832	Suppression of root hair growth; mucilage synthesis in seed coat.	4	Vasilevski et al., 2012; Voiniciuc et al., 2015; Shibata et al., 2018
51	***LRL1***	4	c.206delG|p.Gly69fs	LOC108852215	Lipid accumulation under nutrient-deficient conditions; elongation of root hairs.	2	Karas et al., 2009; Lin et al., 2015; Hidayati et al., 2019
52	***ERF019***	6	c.458_459delAA|p.Lys153fs	LOC108809887	Response to drought; ethylene-activated signalling pathway; response to fungal infection.	2	Scarpeci et al., 2017; Huang et al., 2019; Lu et al., 2020
53	***bHLH111***	1	c.1delA|p.Met1fs	LOC108829884	Anthocyanin biosynthesis; expressed in response to nitrogen deficiency	1	Silva et al., 2019; Li et al., 2021

54	***CRF1***	3	c.252_253insG|p.Thr85fs	LOC108846019	Probable target of CLE41; component of CK signalling; seedling, leaf and embryo development; regulation of gene expression by stress factors and components of stress signalling pathways.	1	Cutcliffe et al., 2011; Wickramasuriya and Dunwell., 2015; Huo et al., 2020
55	***CDC5***	8	c.1494_1495insTT|p.Ala499fs	LOC108830943	Regulation of defence responses through control of transcription; mRNA splicing; cell cycle control; SAM development; control of plant innate immunity.	4	Lin et al., 2007b; Palma et al., 2007; Zhang et al., 2013
56	***PRHA-L***	8	c.714dupG|p.Ser239fs	LOC108821801	Response to auxin; response to pathogens.	2	Plesch et al., 1997
57	***SUVH9-L***	6	c.865delG|p.Glu289fs	LOC108835120	Transposon silencing; RNA-directed DNA methylation.	2	Johnson et al., 2008; Kuhlmann and Mette, 2012; Johnson et al., 2014; Liu et al., 2014; Liu et al., 2016
58	***TOPII***	2	c.1680delG|p.Arg561fs	LOC130508706	Control of DNA topological states by temporary breakage and subsequent reunion of DNA strands.	1	Martinez-Garcia et al., 2018; Martinez-Garcia et al., 2021
59	***BBX27***	7	c.690dupT|p.Ser231fs	LOC108816763	Photoperiodic control of short-day flowering.	1	Gangappa et al., 2014; Lyu et al., 2020
Organogenesis							
60	***ARA2***	4	c.926_927insTG|p.Arg310fs	LOC108854961	Establishment and development of lateral roots independent of auxin and ABA.	1	Coates et al., 2006; Nibau et al., 2011;

							Gibbs and Coates, 2014
Metabolism and signalling of phytohormones							
61	***CLE7***	4	c.1_2delAT|p.Met1fs	LOC108853737	Signal peptide, synthesised in response to nitrogen deficiency. Expressed in the vascular network and pericycle, inhibits lateral root formation.	1	Araya et al., 2014; Kane et al., 2022
62	***SAUR15A-L***	3	c.1dupA|p.Met1fs	LOC108846677	Formation of lateral and adventitious roots in response to auxin.	1	Yun et al., 2020; Li et al., 2022
63	***SAUR32***	6	c.365_366delAA|p.Lys122fs	LOC108809074	Response to auxin; inhibits hypocotyl cell growth.	2	Park et al., 2007
64	***SAUR40-L***	3	c.543delA|p.Lys181fs	LOC108846468	Response to auxin.	1	Qiu et al., 2013
65	***GA2OX3***	4	c.827_834delCACCATTG|p.Ala276fs	LOC130510497	A component of the GC signalling pathway.	1	Sakai et al., 2003
66	***LOC108857184***	5	c.72dupT|p.Pro25fs	LOC108857184	A gene of unknown function, probably CK-activated	1	
67	***HK5-L***	3	c.2573delA|p.Asn858fs	LOC108844370	Negative regulation of ETR1-dependent ABA and ethylene signalling pathway; inhibits root elongation; opening/closing of stomata in closing cells by ABA-independent pathway; control of AFC production in response to stress.	3	Desikan et al., 2008; Mira-Rodado et al., 2012; Pham and Desikan, 2012; Huo et al., 2020
68	***ETR1***	7	c.77dupT|p.Leu26fs	LOC108817715	A two-component negative regulator of ethylene signalling. Control of seed germination, control of RAM.	1	Qu and Schaller, 2004; O'Malley et al., 2005; Bisson and

							Groth, 2010; Bakshi et al., 2015; Bakshi et al., 2018; Harkey et al., 2018; Li et al., 2019; Zdarska et al., 2019; Schott-Verdugo et al., 2019
Other functions							
69	***LOC108842019***	2	c.716_717insAT|p.Leu240fs	LOC108842019	A putative receptor kinase.	2	Rodriguez-Furlan et al., 2022
70	***PNG1***	6	c.1773_1779delGCCGCCA|p.Pro592fs	LOC108813526	Protein quality control.	2	Diepold et al., 2007
71	***LOC108851456***	4	c.383_384delTT|p.Phe128fs	LOC108851456	Binding of metal ions; DNA replication.	1	Ronceret et al., 2005
72	***ASP39***	6	c.387_388insC|p.Lys130fs	LOC108812392	Synthesis of phytochelatins and homophytochelatins that bind heavy metals. Degradation of glutathione conjugates.	1	Vatamaniuk et al., 2004

Table 5. Genes with SNPs in line 19.

№	Gene	Chr	Homologue number in the database	SNP	Function	Number of homologues in radish	Reference
Cell cycle control							
1	***CYCJ18***	4	LOC108830438	c.718T>C| p.Ter240Argext*	Cell division; regulation of cyclin-dependent proteinserine/threonine kinase activity; phase transition of the mitotic cell cycle.	1	Abrahams et al., 2001
2	***PS1L***	9	LOC108861742	c.1297G>T| p.Glu433*	Orientation of the division spindle in the second division of meiosis, formation of microspore tetrads.	1	d'Erfurth et al., 2008
3	***AUR1***	2	LOC108819297	c.1168C>T| p.Arg390*	Cytokinesis; formation of microtubule organisation centres.	2	Kawabe et al., 2005; Van Damme et al., 2011
4	***bZIP1***	5	LOC108825955	c.64C>T| p.Arg22*	Spatial control of cytokinesis by proper assembly of the phragmoplast.	1	Müller et al., 2019; Romanowski et al., 2021
5	***LRX1***	5	LOC108836486	c.1638C>A| p.Tyr546*	Cell morphogenesis, including root hairs, through cell wall formation and assembly and/or growth polarisation.	3	Baumberger et al., 2003; Draeger et al., 2015; Schaufelberger et al., 2019; Herger et al., 2020
6	***AGP18***	4	LOC108807296	c.578C>A| p.Ser193*	Differentiation, embryogenesis, and programmed cell death.	3	Demesa-Arévalo,Vielle-Calzada, 2013;

							Farquharson, 2013
7	***COBL11***	2	LOC108841217	c.574A>T| p.Arg192*	Pollen tube formation and development.	1	Brady et al., 2007;Li et al., 2013
Metabolism and signalling of phytohormones							
8	***GRR1-L1***	9	LOC108825013	c.665A>T| p.Ter222Leuext*	Reception of auxin; response to auxin.	3	Thelander et al., 2002; Dharmasiri et al., 2005
Regulation of gene expression							
9	***CHR35***	5	LOC108862654	c.2327T>A| p.Leu776*	Epigenetic control of RNA-directed DNA methylation.	1	Cho et al., 2016
10	***CMT1***	1	LOC108807343	c.902G>C| p.Ter301Ser*	CpXpG methylation; gene silencing.	1	Yadav et al., 2018
11	***WOX2***	2	LOC108841333	c.1204A>T| p.Arg402*	Embryonic pattern formation.	1	Chung et al., 2016; Hassani et al., 2022
12	***bZIP44***	7	LOC108814136	c.415T>C| p.Ter139Glnext*	Regulation of seed germination by loosening of micropylar endosperm and seed coat rupture.	2	Iglesias-Fernández et al., 2013
13	***ARF21***	9	LOC108822118	c.487A>T| p.Lys163*	Response to auxin.	1	Hagen and Guilfoyle, 2002
14	***NAC017***	5	LOC108856330	c.2T>C|p.Met1	Response to stress.	1	Meng et al., 2019
15	***MYB104L***	6	LOC108810575	c.448C>T| p.Gln150*	Microgametogenesis, development of polarised microspores.	1	Oh et al., 2020
16	***NF-YB8***	3	LOC108844481	c.566A>T| p.Ter189Leuext*	Control of flowering time; flavonoid biosynthesis.	1	Wei et al., 2017; Wang et al., 2021

17	***WRKY55L***	3	LOC130509746	c.363G>A| p.Trp121*	Regulation of leaf senescence through control of salicylic acid and AFC accumulation.	2	Wang et al., 2020b
18	***ERF119***	4	LOC108833429	c.841T>G| p.Ter281Gluext*	Regulation of gene expression by stress factors and components of stress-mediated signal transduction pathways; formation of secondary cell wall.	3	Kavas et al., 2015; Seyfferth et al., 2018
19	***ARR21***	3	LOC108846809	c.1694C>G| p.Ser565*	B-type response regulator involved in a phosphorelay-type signal transduction system from His to Asp. Activates A-type RR in response to CK.	2	Horák et al., 2003; Brenner et al., 2012; Hill et al., 2013;
20	***REM5***	2	LOC130494531	c.392G>T| p.Ter131Leut*	Vesicular transport.	2	Paul et al., 2014
21	***bHLH63***	2	LOC108824102	c.487A>T| p.Lys163*	Epigenetic regulation of flowering: activation of FT expression, flowering in response to blue light. Suppression of the innate immune response.	2	Radoeva et al., 2019
22	***AGL80***	2	LOC108815229	c.432A>G| p.Ter144Trp*	Endosperm cell proliferation.	1	Wolff et al., 2011; Sun et al., 2020a
23	***SHOC1***	5	LOC130500734	c.20T>A|p.Leu7*	Biosynthesis of gibberellins; seed germination; differentiation of xylem elements; lignification of secondary cell wall.	1	Frei dit Frey et al., 2010; Liu et al., 2010; Yan et al., 2014; Gutierrez-Beltran et al., 2015a,b, 2021.
24	***CDF3***	6	LOC108813543	c.984G>A| p.Trp328*	Photoperiodic control of flowering through suppression of CONSTANS; enhances nitrogen use efficiency; increases biomass production and yield under salinity stress.	1	Renau-Morata et al., 2017; Krahmer et al., 2019; Domínguez-

							Figueroa et al., 2020
25	***dZIP30***	4	LOC108848413	c.240T>A| p.Cys80*	TF, a repressor of reproductive development, meristem size and plant growth. Interacts with regulators of meristem and gynoecium development (WUS, HEC1, KNAT1, KNAT2, HAT1, BEL1 and NGA1).	1	Lozano-Sotomayor et al., 2016
Control of meristem activity							
26	***REM10L***	1	LOC108857973	c.604A>T| p.Lys202*	Flower development.	2	Mantegazza et al., 2014; Yamasaki et al., 2017
27	***BRX***	5	LOC108850063	c.946T>A| p.Ter316Arg*	Proliferation and elongation of root and shoot cells; control of the protophloem; interactions between ISC and brassinosteroid signalling; CK-mediated inhibition of lateral root initiation.	1	Mouchel et al., 2004; Rodriguez-Villalon et al., 2014; Breda et al., 2017
Other functions							
28	***PER30***	5	LOC108862038	c.2T>C|p.Met1	Oxidation of toxic reducing agents; lignin biosynthesis and degradation, suberisation, auxin catabolism, response to exogenous stresses.	1	Showalter et al., 2010
29	***CRWN***	1	LOC108806851	c.8G>A|p.Trp3*	Organisation of core structure.	1	Wang et al., 2013a; Choi et al., 2019
30	***LRR-RK***	2	LOC108842145	c.1526T>A| p.Leu509*	Encodes protein with serine/threonine/tyrosine kinase activity.	1	Xun et al., 2020
31	***LecRLK***	9	LOC108824077	c.107G>T| p.Ter36Leuext	Plant development; response to stress.	1	Wang et al., 2014, Sun et al., 2020b
32	***SBT2.6***	4	LOC108849971	c.2442C>G|	Proteolysis; activated in response to eoCLE40.	1	Stührwohldt et al.,

				p.Tyr814*			2020
33	***LOC108853023***	4	LOC108853023	c.87T>A|p.Tyr29*	Ubiquitin-dependent catabolic processes.	2	Kosarev et al., 2002
34	***VLG***	6	LOC130496156	c.309C>G|p.Tyr103*	Gametogenesis	1	D'Ippólito et al., 2017
35	***LOC108856505***	5	LOC108856505	c.1354C>T| p.Arg452*	Response to hypoxia.	1	Giuntoli et al., 2014
36	***LOC108854537***	1	LOC108854537	c.3G>A|p.Met1	No data	1	

Due to the large number of identified InDels and SNPs, it is currently not possible to make clear assumptions about the role of each identified substitution in spontaneous tumour development. According to data on transcriptome analysis of radish storage roots and spontaneous tumours of an inbred radish line, all 108 genes with SNVs identified in tumour line 19 were expressed in radish storage roots (Tkachenko et al., 2021a). Moreover, five genes with such SNVs identified in this study were among those differentially expressed: the *RsPCNA1*, a cell cycle regulator, and the *LOC108817684*, a gene of unknown function, were upregulated in tumours, whereas the expression levels of homologues of the auxin response gene *RsSAUR32*, the cambium-associated ethylene response genes *RsERF018* and *RsERF019*, and *RsLRR-RK*, a gene encoding a receptor-like protein kinase, were downregulated (Tkachenko et al., 2021a).

In order to investigate the identified InDels and SNPs in more detail, the results obtained using bioinformatics tools were confirmed by sequencing the coding parts of genes, containing SNPs and InDels, in different tumour (12, 13, 14, 16, 19, 20, 21, 32) and non-tumour (3, 5, 6, 8, 9, 18, 23, 23, 25, 26, 27, 28, 29, 30, 37, 39) lines of the radish genetic collection. For this purpose, the corresponding genes were amplified and sequenced using the Sanger technology. The genes initially selected for such screening were 1) those whose homologues play a role in meristem development control; 2) those localised on chromosomes outside the telomeric regions; and 3) genes with SNVs, which were located closer to the start of the gene.

As a result, the presence of InDel in the *RsERF018* gene was confirmed in the majority of tumour lines (Figure 20). For the other genes, it was shown that either InDel or SNPs were identified only in line 19, or there was a polymorphism not associated with the tumour formation trait.

The *RsERF018* gene, whose homologue in *Arabidopsis* controls the response to ethylene and cambium cell division (Etchell et al., 2012), was shown to have a CAG insertion upstream of the start codon in six out of eight tumour lines (12, 13, 14, 19, 20, and 21) as well as in two non-tumour lines (26 and 27), whereas tumour lines 16 and 32 as well as most non-tumour lines (10 out of 15) lacked this insertion (Figure 20). Without the insertion, the 5'-UTR region contained the AAA sequence immediately upstream of the start codon, resulting in high translation efficiency (Kim et al., 2014). Therefore, insertion of the CAG between the start codon and the AAA sequence (Figure 20) could result in a significant

decrease in the amount of protein translated, as has been shown in *Arabidopsis* (Kim et al., 2014).

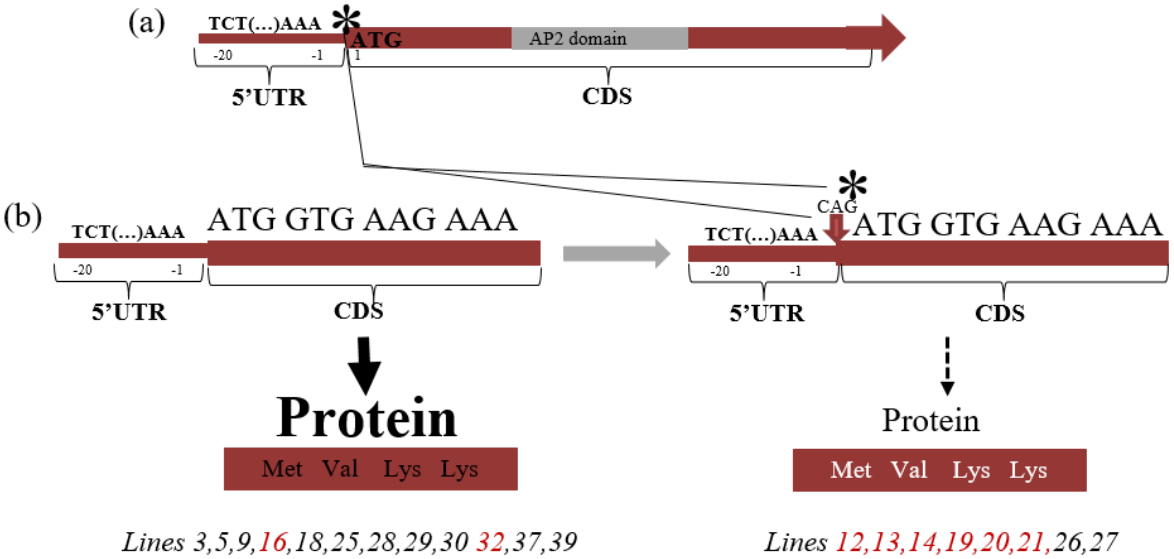

Figure 20. Schematic representation of the insertion (marked with an asterisk) detected in the *RsERF018* gene. (a) The scheme of the *ERF018* gene. The insertion is located on the border of the 5'-UTR and the start codon. (b) The 5'-UTR insertion of the *RsERF018* gene in radish inbred lines and its possible consequences. The amino acid content of the protein synthesised during translation of the normal sequence is marked in black, and the protein synthesised during translation in the case of the CAG insertion is marked in white. Radish tumour line numbers are highlighted in red.

According to literature data, the WOX4 TF represses the activity of several TFs of the ERF family (RsERF018, RsERF019, and RsERF1), which are activated by ethylene and are involved in cell proliferation (Etchell et al., 2012). Since the *RsERF018* is one of the putative targets of the WOX4 TF in radish and an important cambium regulator, further investigation of its functions and verification of the hypothesis of its role as a possible conserved regulator of spontaneous tumour formation is an important and promising goal for further research in this field.

Another interesting result of this study is the detection of InDels and SNPs in the genes of the WOX-CLAVATA system, namely the *RsWOX14*, *RsWOX2* and *RsCLE7* (Figure 21).

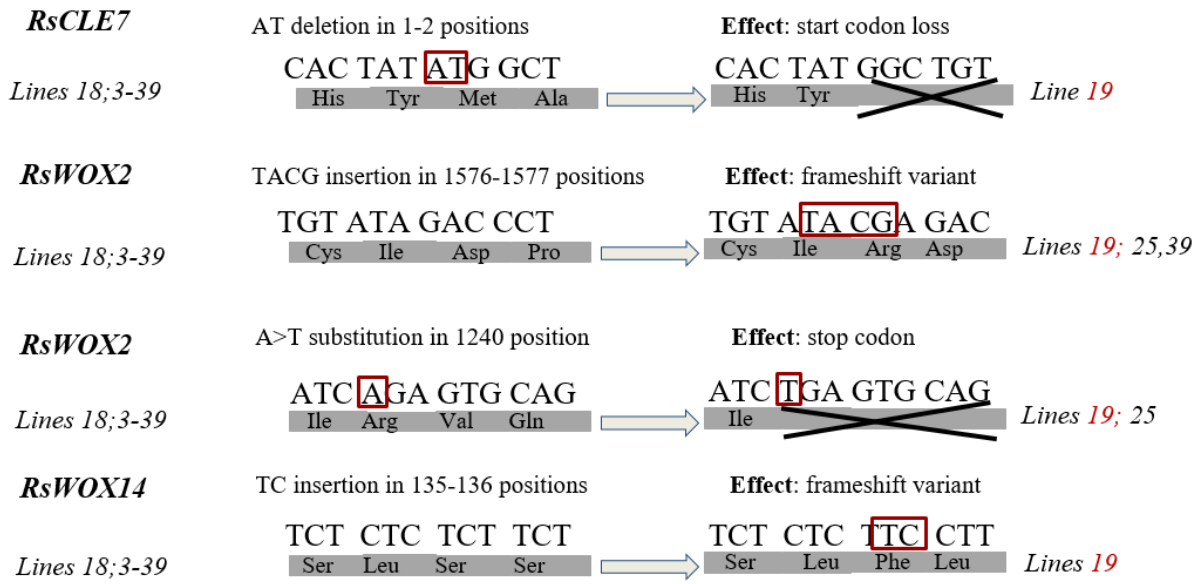

Figure 21. Schematic representation of the *RsCLE7*, *RsWOX2*, *RsWOX14* without SNVs (left parts of the figure) and with SNVs (right parts of the figure) and their likely products. For each SNV, its type and effect on the gene product are indicated. SNVs are highlighted in the red box. Radish tumour line numbers are highlighted in red.

In *Arabidopsis*, the *WOX14* gene is a regulator of cambium and xylem balance and it acts in concert with the *WOX4* (Etchells et al., 2013). The *WOX2* gene is known to regulate early embryogenesis and callus formation (Hassani et al., 2022). The *Arabidopsis CLE7* gene is also a regulator of callus formation and regeneration (Kang et al., 2022). Since spontaneous tumours on radish roots arise from the cambium and develop as undifferentiated callus-like structures (Lebedeva et al., 2015), we considered these genes as promising candidates for the role of regulators of tumour formation. However, the results obtained for these genes were not very encouraging: bioinformatic data verification using amplicon sequencing allowed us to confirm the presence of substitutions/insertions in line 19 and their absence in line 18, but the presence of InDel and SNP data in other tumour lines was not observed and no correlation of these InDels with the tumour formation trait was found (Figure 21).

The *RsWOX14*, *RsWOX2*, and *RsCLE7* genes are represented by a single copy in the radish genome, but homozygosity for loss-of-function mutations in these genes does not result in reduced viability of the radish line 19. It has been reported in *Arabidopsis* that a single mutation in each of these genes does not cause severe developmental abnormalities in mature plants (Etchell et al., 2013).

Thus, of all the identified genes with SNPs and InDel, the *RsERF018* is the most promising candidate for a role as a conservative regulator of tumour formation. Further

sequencing of less promising genes for which functionally significant SNPs and InDel were identified in line 19, which we did not include in the initial analysis, will be required in other tumour lines of the radish genetic collection.

3.2. Identification and sequence analysis of the *RsWOX* and *RsCLE* genes and study of the domain structure of the corresponding proteins

The WOX-CLAVATA system in plants is an example of a complexly organised conserved module that regulates meristem activity and a range of other processes in the plant organism. This system includes CLE signalling peptides, their receptors, which are leucine-rich repeat protein kinases, and CLE peptide targets, which are homeodomain-containing WOX TFs (Stahl and Simon, 2009; Lee and Torii, 2012). Our work focuses on the role of meristem regulators in storage root development, where the WOX-CLAVATA system plays an important role in regulating the activity of the lateral meristem, the cambium, and its derivatives, and is thought to control the formation of ectopic foci of meristematic activity in tumours. A crucial part of this work was therefore to identify and analyse the sequences of the genes belonging to this system.

As the radish genome is three times larger than the *Arabidopsis* genome (Jeong et al., 2016), there are typically 2-3 homologues for each *AtWOX* or *AtCLE* gene in radish: for example, there are three *AtWUS* homologues and five *AtWOX13* homologues in the radish genome. Within each of these groups, the genes are characterised by high similarity: for instance, the similarity of the *RsWOX4-1* and *RsWOX4-2* genes reaches 95%.

3.2.1. *RsWOX* genes identification and chromosomal localisation; analysis of RsWOX protein structure

In total, in radish genomes we have identified 24 *RsWOX* genes and 52 *RsCLE* genes. It should be mentioned that a significant proportion of *RsWOX* genes were previously identified in the radish reference genome by Aliaga (2019). Our special attention was paid to *RsWOX4* (2 genes), *RsWOX14* (1 gene) and *RsCLE41* (2 genes), whose homologs in

Arabidopsis play a central role in the regulation of cambium activity.

The phylogenetic tree of the radish *WOX* genes is shown in Figure 22. The *RsWOX4* genes are coloured in blue and the *RsWOX14* genes are coloured in red.

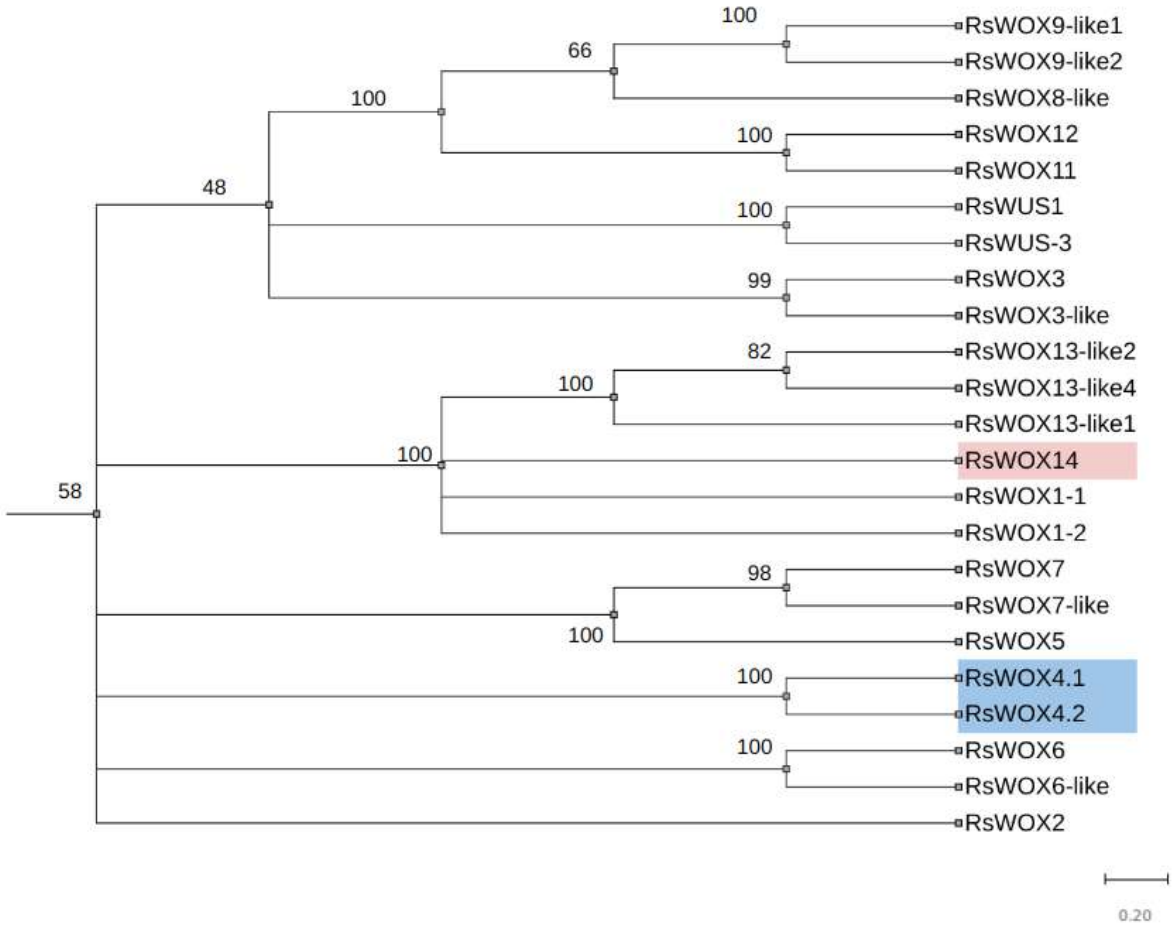

Figure 22. Phylogenetic tree of *RsWOX* genes constructed using the Neighbour-joining algorithm. Genes of interest for this work are marked in colour.

We have also studied the sequence structure of RsWOX4-1, RsWOX4-2, and RsWOX14 proteins of radish (Figure 23). These proteins were found to contain multiple domains. Firstly, the homeodomain-DNA-binding domain of all homeodomain-containing TFs was identified in them. In addition, a number of other domains that influence the regulation of target gene expression were identified in RsWOX4-1 and RsWOX4-2, which belong to the modern branch of WOX proteins (Haecker et al., 2004). In particular, RsWOX4-1 and RsWOX4-2 contain the WUS box, which is required for transcriptional repression of target genes and which, in *Arabidopsis* WOX proteins, binds to the TOPLESS corepressor (Martin-Arevalillo et al., 2017). In contrast, the second repressive domain, the

EAR domain (van der Graaff et al., 2009), that many WOXs have is absent in RsWOX4 proteins, as well as in the corresponding *Arabidopsis* WOX TFa. The RsWOX4-1 and RsWOX4-2 proteins also have an acidic domain, which is conserved for eukaryotic TFs and is required for transcription activation (Ma and Ptashne, 1987) and is also used by some WOX proteins to interact with other TFs (Su et al., 2020). The PEST sequence, a target for ubiquitination and proteolysis, which is conserved for all eukaryotes, has also been identified in WOXes of our interest as a feature of short-lived proteins.

Although the *WOX4* and *WOX14* genes belong to different clades: ancient (*WOX14*) and modern (*WOX4*, see Tvorogova et al., 2021 for a review), the sequences of their homeodomains are conserved, suggesting that they could bind to the same targets. It is likely that the presence of the homeodomain alone is sufficient to positively regulate the expression of their targets. The presence of the WUS-box and EAR-domain in WOX4 in contrast to WOX14 is important for negative regulation by attracting corepressors, indicating that WOX4 may have unique targets. Thus, WOX4 and WOX14 are expected to have a partial overlap in function rather than a complete overlap.

In addition, we have identified and localised *RsWOX* genes, which are targets of *RsCLE* genes and regulators of meristem development, on chromosomes. Data on the localisation of *RsWOX* genes, of which 24 were identified in radish, on chromosomes is presented in Table 6 and Figure 24.

Table 6. *RsWOX* genes and their chromosomal localisation.

№	Gene	Id	Chromosome	Location
1	*RsWUS1*	LOC108826423	9	25391638..25393659
2	*RsWUS-2*	LOC108860672	5	32134429..32136395
3	*RsWUS-3*	LOC108810506	6	25374911..25376943
4	*RsWOX1-1*	LOC108859792	5	7390678..7389734
5	*RsWOX1-2*	LOC108862713	5	7388589..7391154
6	*RsWOX2*	LOC108841333	2	21341442..21342606
7	*RsWOX3*	LOC108853017	4	36182105..36183709
8	*RsWOX3-like*	LOC108837685	3	11436310..11437644
9	*RsWOX4-1*	LOC108857708	5	28767860..28769449
10	*RsWOX4-2*	LOC108820732	8	22263942..22265330
11	*RsWOX5*	LOC108860345	5	3995650..3996669
12	*RsWOX6*	LOC108825323	9	33082008..33082440
13	*RsWOX6-like*	LOC108808383	6	37791476..37794151
14	*RsWOX7*	LOC108816831	7	5878921..5879361
15	*RsWOX7-like*	LOC108841429	2	25782570..25783384
16	*RsWOX8-like*	LOC108808083	6	20653111..20655047
17	*RsWOX9-like1*	LOC108810630	6	48266564..48268968
18	*RsWOX9-like2*	LOC108846840	3	18165462..18167903
19	*RsWOX11*	LOC108812707	6	2916296..2919135
20	*RsWOX12*	LOC108815129	7	1948016..1952302
21	*RsWOX13-like1*	LOC108843060	2	38230366..38231894
22	*RsWOX13-like2*	LOC108854890	4	1228814..1230202
23	*RsWOX13-like3*	LOC108820866	8	8201772..8202881
24	*RsWOX14-like*	LOC108853278	1	9049502..9051017

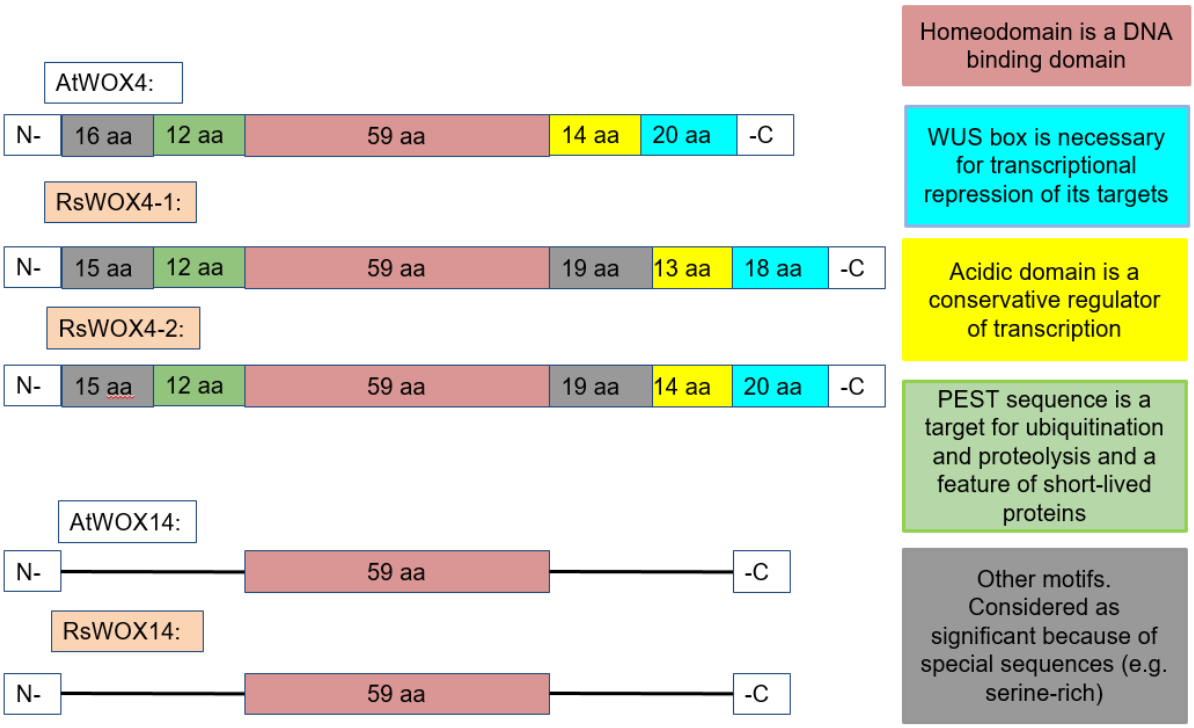

Figure 23. Domains of WOX4 and WOX14 proteins of radish and *Arabidopsis*.

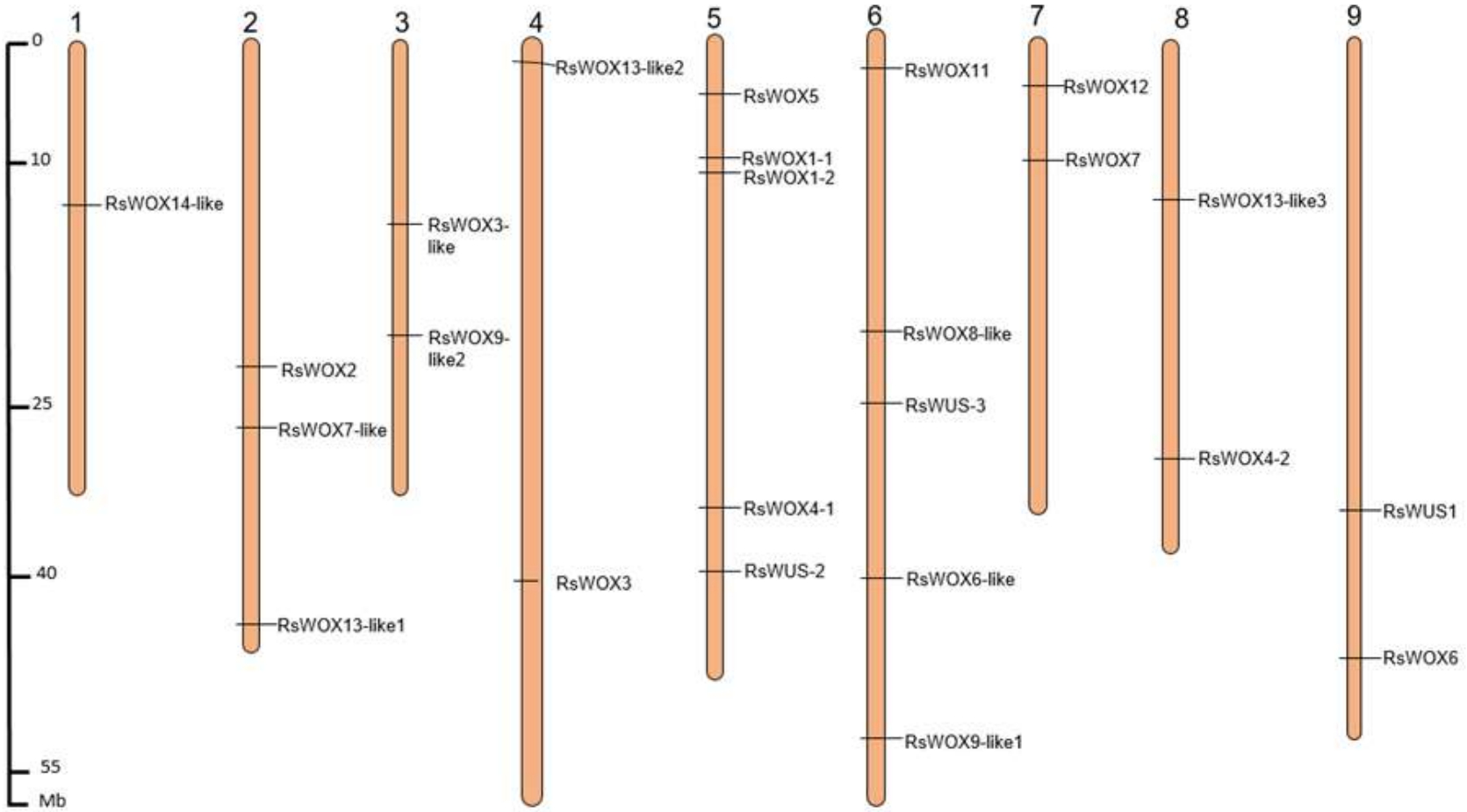

Figure 24. Location of *RsWOX* genes on radish chromosomes.

3.2.2. *RsCLE* genes identification and chromosomal localisation; analysis of RsCLE protein structure

In addition, 52 *RsCLE* genes, including genes belonging to the TDIF group, have been identified in radish. There are 33 *CLE* genes in *Arabidopsis* (Strabala et al., 2008), and, for each *AtCLE* gene, we have indentified two *RsCLE* homologues. Some of the identified *RsCLE* genes were annotated earlier by Gancheva (2016).

Interestingly, in addition to *AtCLE* homologues, we have identified 2 unique multidomain *RsCLEs* (Figure 25) with unknown functions (Kuznetsova et al., 2024a), which homologues were also previously found in *Brassica* (Han et al., 2020) but were not in *Arabidopsis*.

Among all *RsCLE* genes, the *RsCLE41* and the *RsCLE42* from the TDIF group (Figure 25) were objects of our interest.

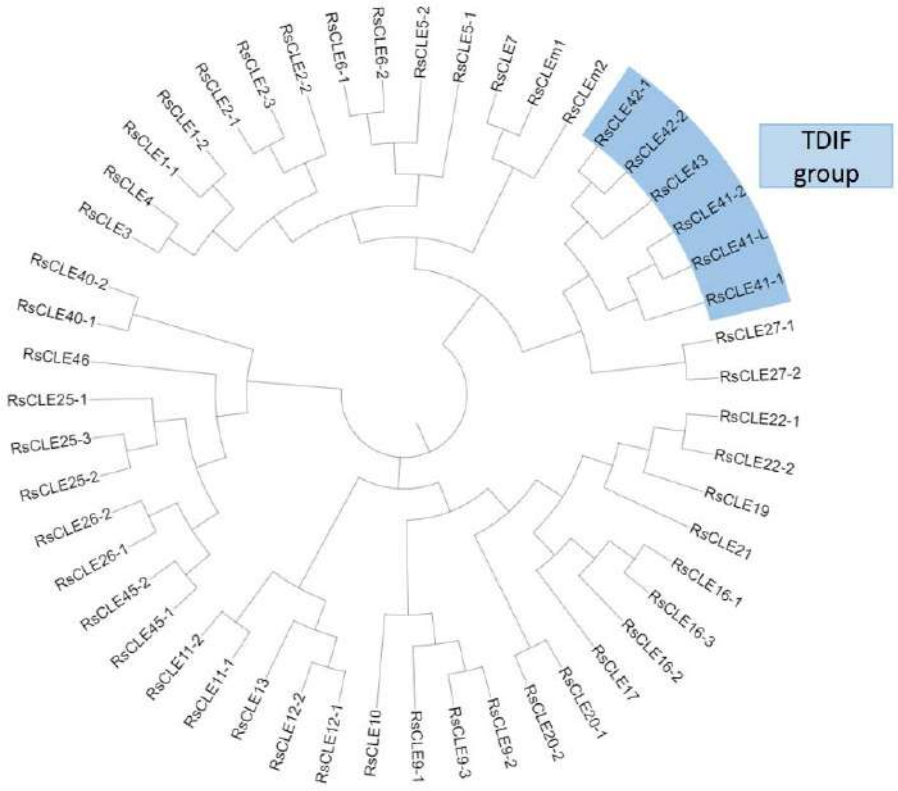

Figure 25. Phylogenetic tree of radish *CLE* genes constructed using the Neighbour-joining algorithm. The colour indicates the sector corresponding to the genes of the TDIF group which are regulators of cambium development.

The TDIF group, or the B group, which involved in the regulation of cambium activity, includes 3 genes in *Arabidopsis*: the *AtCLE41*, the *AtCLE42*, and the *AtCLE44* (Ito

et al., 2006; Yaginuma et al., 2011), while there are 6 genes *(RsCLE41-1*, *RsCLE41-2*, *RsCLE41-L*, *RsCLE42-1*, *RsCLE42-2*, and *RsCLE43*) to have been identified in radish. Peptides of this group differ from other CLE peptides in the amino acid composition of the CLE domain (Figures 26, 27): unlike peptides of group A, there are serine, isoleucine, and serine at conserved positions 5, 10, and 11 of the CLE domain, respectively.

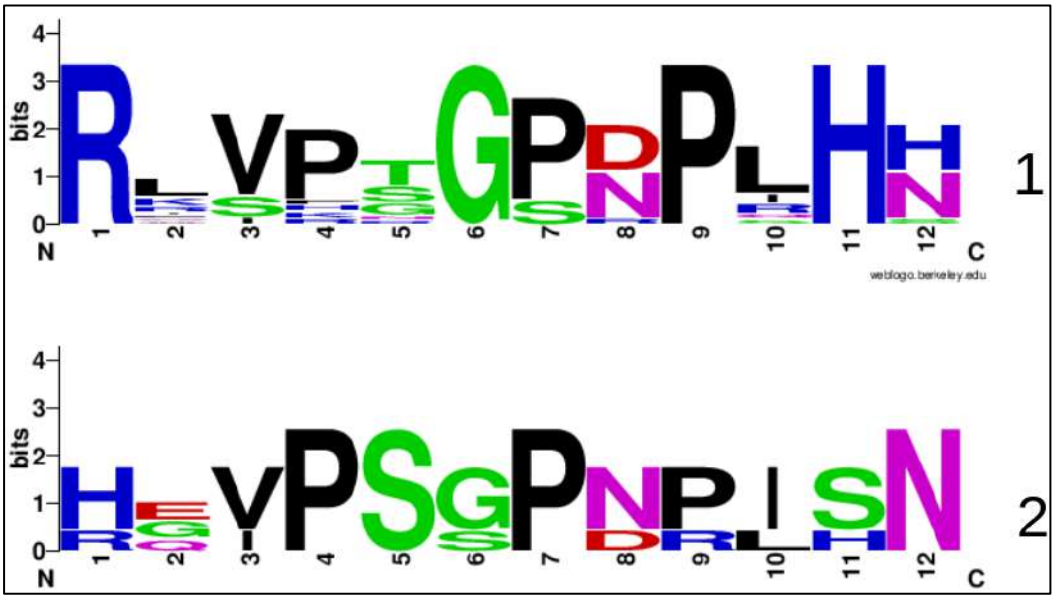

Figure 26. CLE-domain amino acid sequence motifs of peptides in 1) group A; 2) group B.

After analysing the CLE domain sequences of all identified B group peptides, we have found that the CLE domain composition of the RsCLE43 peptide differs from the CLE domain composition of the other members of this group by amino acids at the following positions: arginine-1, glutamine-2, isoleucine-3, serine-6, aspartic acid-8, leucine-9 and histidine-10 (Figure 27). In *Arabidopsis*, the *CLE41/44* and *CLE42* genes encode functional TDIF group peptides that regulate cambium activity and vascular development, whereas the CLE43 peptide is non-functional.

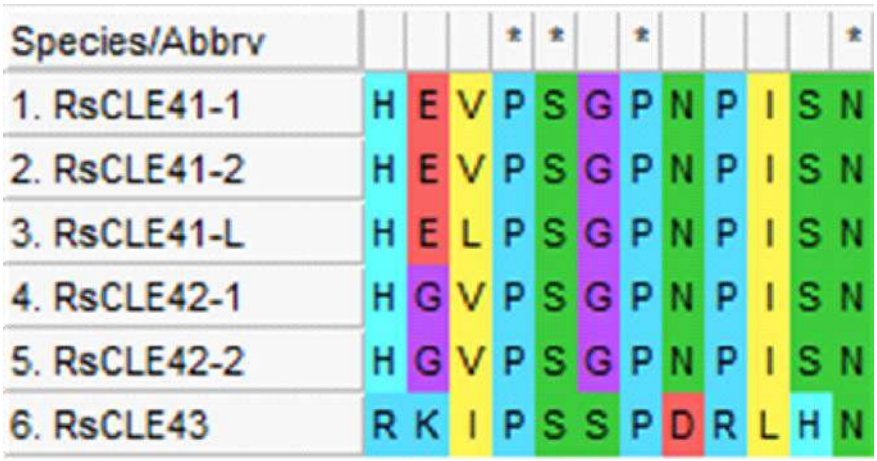

Figure 27. CLE domains of RsCLE proteins of the TDIF group.

In radish, we have also identified two genes that are not present in *Arabidopsis*, which we named *RsCLEm1* and *RsCLEm2* because they encode multidomain CLE peptides: each of them has 8 CLE domains. The genes encoding multidomain CLE peptides were also previously identified in *Brassica* napus (Han et al., 2019). It was not the aim of this work to study the functions of these genes but this is an important issue for future research.

In addition, we have localised *RsCLE* genes on radish chromosomes: the data is presented in Table 7 and Figure 28. What is curious about these results is that a cluster of *RsCLE* genes was detected on chromosome 9, which allows us to speculate about probable duplications occurring here during the course of evolution.

Table 7. *RsCLE* genes and their chromosome localisation.

№	Gene	Id	Chromosome	Location
1	*RsCLE1-1*	LOC108841390	2	3191415..3192147
2	*RsCLE1-2*	LOC108843041	2	3197975..3198516
3	*RsCLE2-1*	LOC108821700	8	13271037..13271432
4	*RsCLE2-2*	LOC108853569	4	6882809..6883161
5	*RsCLE2-3*	LOC108853568	4	6891416..6891719
6	*RsCLE3*	LOC108824362	1	607138..607689
7	*RsCLE4*	LOC108853505	4	37230512..37230914
8	*RsCLE5-1*	LOC108846750	3	19875811..19876403
9	*RsCLE5-2*	LOC108811152	6	47131282..47131923
10	*RsCLE6-1*	LOC108846750	3	19875811..19876403
11	*RsCLE6-2*	LOC108853458	4	37280842..37281463
12	*RsCLE7*	LOC108853737	4	37245967..3724644
13	*RsCLE9-1*	LOC108831381	8	4812048..4812772
14	*RsCLE9-2*	LOC108821573	8	4812324.. 4812844
15	*RsCLE9-3*	LOC108810044	6	35921594..35922686
16	*RsCLE10*	LOC108826236	9	2423342..2424998
17	*RsCLE11-1*	LOC108857705	1	22097302..22097985
18	*RsCLE11-2*	LOC108821365	8	24037054..24037712
19	*RsCLE12-1*	LOC108840313	2	6241832..6242663
20	*RsCLE12-2*	LOC108826002	9	2066133..2067263
21	*RsCLE13*	LOC108826513	9	5380674..5381240
22	*RsCLE16-1*	LOC108850391	4	15636111..15636434
23	*RsCLE16-2*	LOC108808008	6	19256776..19257099
24	*RsCLE16-3*	LOC108825322	9	33079361..33080497
25	*RsCLE17*	LOC108846339	9	3546645..3549159

26	*RsCLE19*	LOC130503403	6	9074418..9074865
27	*RsCLE20-1*	LOC108860579	5	21410155..21410896
28	*RsCLE20-2*	LOC108858161	1	2412807.. 2413362
29	*RsCLE21*	LOC108860201	5	32912076..32912755
30	*RsCLE22-1*	LOC108818245	7	3709257..3710007
31	*RsCLE22-2*	LOC108846157	3	26138832..26139918
32	*RsCLE25-1*	LOC108833148	9	32051111..32054680
33	*RsCLE25-2*	LOC108851777	4	12718828..12720994
34	*RsCLE25-3*	LOC108809875	6	16707003..16708424
35	*RsCLE26-1*	LOC108840632	2	5203217..5205554
36	*RsCLE26-2*	LOC108836893	9	2944703..2946116
37	*RsCLE27-1*	LOC108810763	6	17738367..17739088
38	*RsCLE27-2*	LOC108837461	4	13849353..13849910
39	*RsCLE40-1*	LOC108816739	7	3541867..3542573
40	*RsCLE40-2*	LOC108846890	3	26030539..26031690
41	*RsCLE41-1*	LOC108857555	5	12821672..12822454
42	*RsCLE41-2*	LOC108857305	5	34479291..34479891
43	*RsCLE41-L*	LOC130512782	5	35284491..35285276
44	*RsCLE42-1*	LOC108855008	4	39036056..39036736
45	*RsCLE42-2*	LOC108837535	6	48711791..48712456
46	*RsCLE43*	LOC130511469	1	11388890..11389614
47	*RsCLE45-1*	LOC108841040	2	5633117..5633932
48	*RsCLE45-2*	LOC108826373	9	2629261..2630076
50	*RsCLE46*	LOC108826060	9	5928323..5929081
51	*RsCLEm1*	LOC108807713	6	6757266..6757976
52	*RsCLEm2*	LOC108858878	5	35811497..35812612

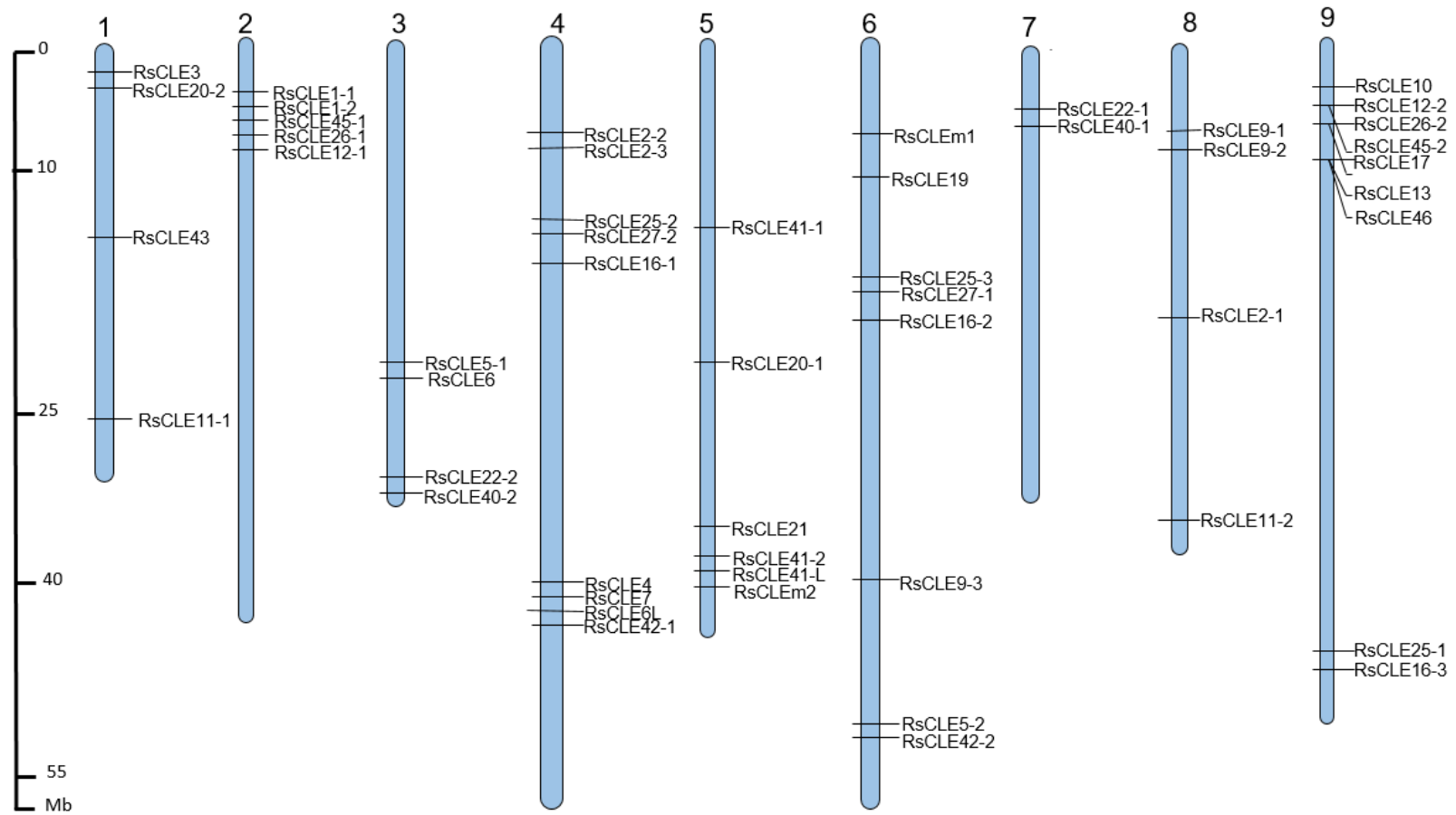

Figure 28. *RsCLE* genes on radish chromosomes.

3.3. The *RsWOX4*, the *RsWOX14* and the *RsCLE41* genes in the storage root development and gene expression in radish

To study the role of components of the WOX-CLAVATA systems in the development of storage root in radish, we investigated the functions of the *RsWOX4*, *RsWOX14* and *RsCLE41* genes, whose homologues in *Arabidopsis* and woody plants regulate cambium activity and vascular system formation. For this purpose, we studied the effects of overexpression of these genes on root phenotype and the expression profile of putative target genes.

3.3.1. Transcriptome analysis of roots with the *RsCLE41* overexpression

According to previous data obtained in our laboratory, overexpression of *RsCLE41* affects the phenotype of radish plants, causing an increase in root and lower stem diameter and a decrease in the number of lignified stem elements (Gancheva et al., 2016; Gancheva et al., 2018). Since CLE41 peptide, like other CLE peptides of the TDIF family, is an important regulator of vascular meristem (pro)cambium cell division and prevents xylem differentiation, we further investigated the role of this gene and performed transcriptome analysis of radish roots with *RsCLE41* overexpression. We analysed differential gene expression in primary structure radish roots transformed by *p35S:RsCLE41-1* using RNA sequencing. Analysis of transcriptome data revealed a total of 62 genes differentially expressed (Figure 29) in radish roots with overexpression of *RsCLE41-1* compared to control roots with overexpression of *GUS* gene, among which 38 genes were upregulated and 24 were downregulated (Kuznetsova et al., 2022). Figure 30 shows a schematic depicting the effect of *RsCLE41-1* overexpression on the expression of target genes and the presence of different interactions between components of the gene network. Lines show the predicted interactions between components of the gene network. If network components are not connected by lines, it means that they probably do not interact and are not regulated together.

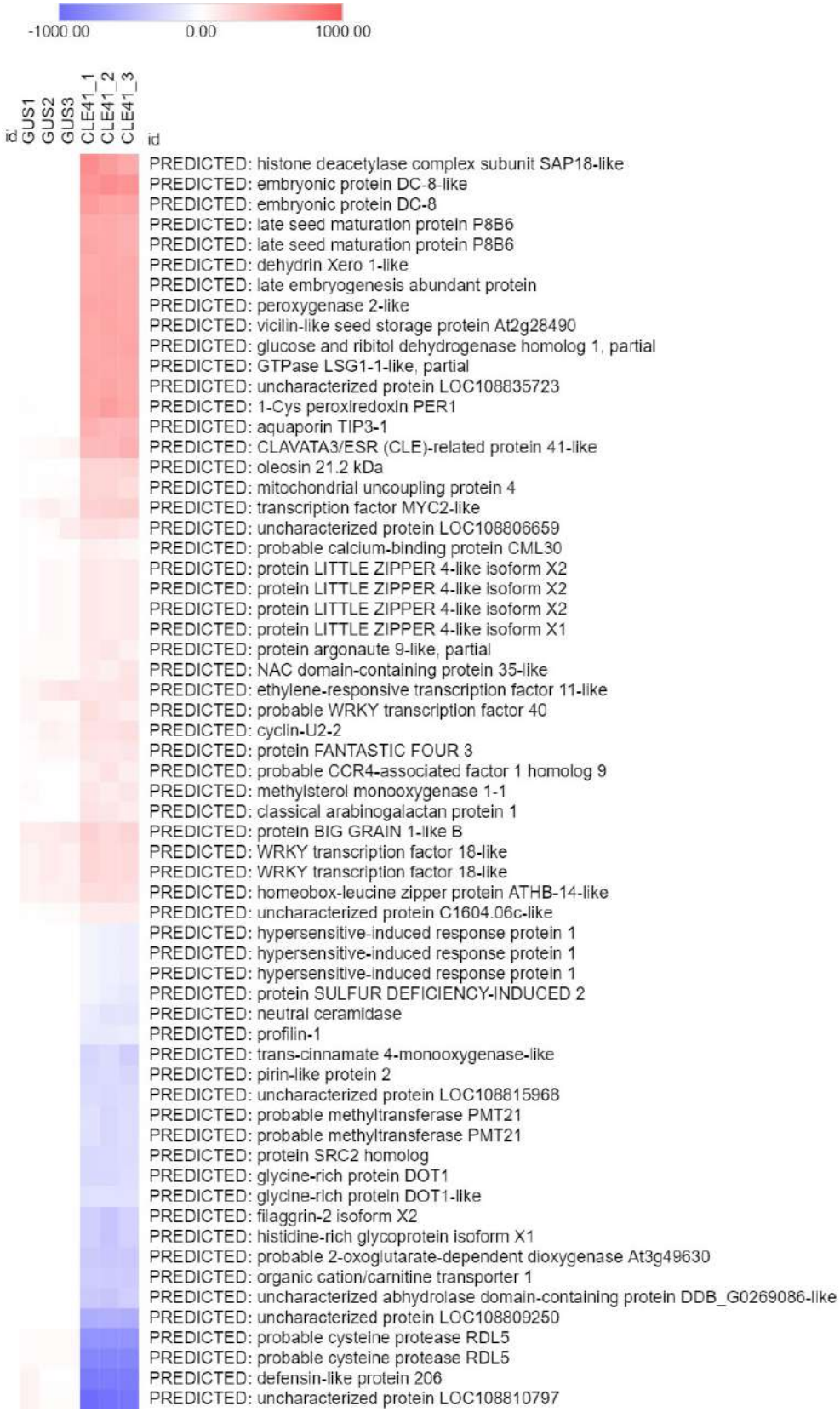

Figure 29. Genes are differentially expressed in *35S:RsCLE41-1* roots compared to control.

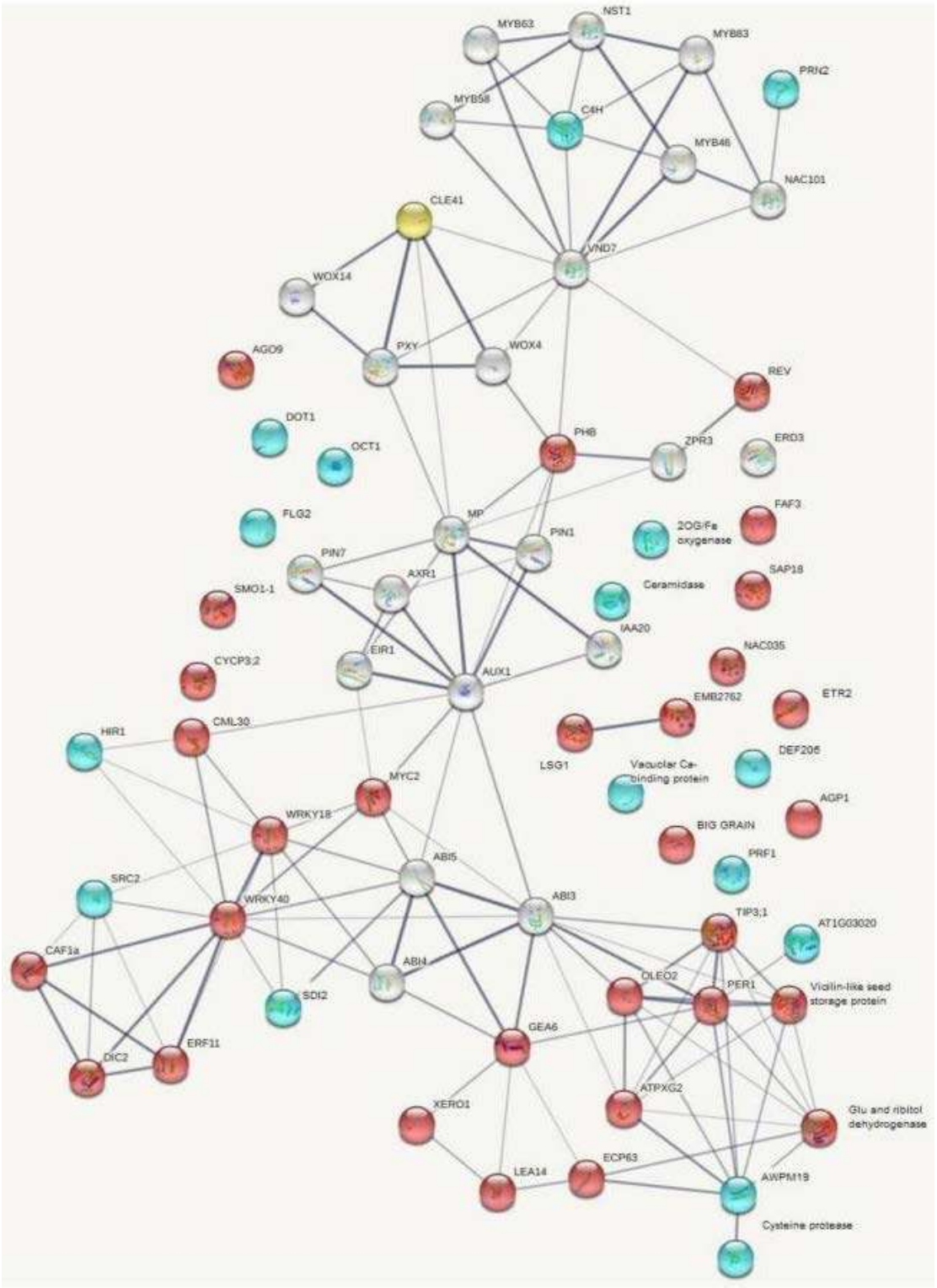

Figure 30. Schematic showing changes in expression levels of RsCLE41-1 target genes in plants with *RsCLE41-1* overexpression. Target genes upregulated are highlighted in red, downregulated ones are in blue. Other participants of the signalling network are in white and the *RsCLE41-1* is in yellow. Lines indicate predicted protein-protein interactions for the protein products of these genes. The figure was generated using the String tool (https://string-db.org/).

Among all genes, we were interested in those that control xylem specification, as a large number of genes of this group demonstrated differential expression. The data on genes that reduced their expression levels were expected, as *RsCLE41* is known to repress vascular differentiation. Thus, stress-regulated genes, especially pathogen response genes (*RsDEFL206*, *RsHIR1*), as well as some genes related to xylem cell determination (*RsC4H*, *RsPRN2*) and drought defence (*RsRDL5*, *RsDOT1*) were predominant among the downregulated. As for upregulated genes, dehydration tolerance genes associated with late embryogenesis, response to ABA and auxin-dependent xylem cell fate were identified. We speculate that this unexpected result could be due to the fact that overexpression of *RsCLE41* leads to an altered vascular specification programme, the consequence of which is dehydration.

Thus, based on these data, we hypothesised that overexpression of *RsCLE41-1* probably leads to the activation of pathways that help plants to increase their drought tolerance (Kuznetsova et al., 2022). These pathways are more characteristic of late embryogenesis, but we suggest that under conditions of *RsCLE41-1* overexpression they may also be activated, in particular, by reducing the expression levels of genes regulating the vascular development programme.

Among the set of genes differentially expressed in roots of plants with overexpression of *RsCLE41-1*, we have not identified *WOX4* or *WOX14* which are well-known targets of TDIF group CLE peptides. A possible explanation for this might be that radish, unlike *Arabidopsis*, has different pathways operating to regulate them. This discrepancy could be attributed to the assumption that signalling pathways in different parts of the plant may be directed to different targets. The transcriptomic data we obtained reflect changes in gene expression in roots with *RsCLE41-1* overexpression during primary growth so it is possible that signalling occurs differently in plants at the developmental stage at which secondary growth occurs.

3.3.2. Effect of *RsWOX4* and *RsWOX14* overexpression on root development in radish

The data obtained in the previous sections of the work allowed us to investigate the effect of overexpression of the *RsCLE41* target, RsWOX4, as well as its partner RsWOX14, on root development. In *Arabidopsis*, the *AtWOX4* gene is known to be a central positive regulator of cambium cell proliferation (Wang et al., 2020a; Fisher et al., 2019), so its homologues in radish may influence the development of the storage root, which is formed under the influence of the activity of this meristem.

According to the literature (Ji et al., 2010), overexpression of *AtWOX4* leads to cambial overactivity and proliferation of xylem and phloem. In our experiments, we expected to obtain similar results, which could lead to the enhanced secondary root growth, for radish.

To test this hypothesis, we transformed radish seedlings with *A. rhizogenes* strains carrying genetic constructs for the *RsWOX4-2* and the *RsWOX14* overexpression individually and then analysed the histological structure of the roots in grown composite plants.

In plants with overexpression of *RsWOX4-2*, in contrast to control plants with overexpression of *GUS* (Figure 31), an altered structure of the stele, a complex of tissues located inside the axial organs of vascular plants, under the primary cortex, was observed in the roots: it is triarchic (consisting of three rays of primary xylem) rather than diarchic (consisting of two rays) as in control plants, i.e. it has an increased number of primary xylem poles. Plants with overexpression of *RsWOX4-2* also have an increased number of vessels, probably indicating increased cambial activity (Kuznetsova et al., 2024b). This effect correlates well with data on *WOX4* overexpression in other plants (Kucukoglu et al., 2017; Wang et al., 2020a).

What was surprising being that there was the change in the spatial structure of the stele which may indicate that the establishment and spatial orientation of procambium and provascular tissues in the developing root primary structure is initially dependent on *WOX4* activity.

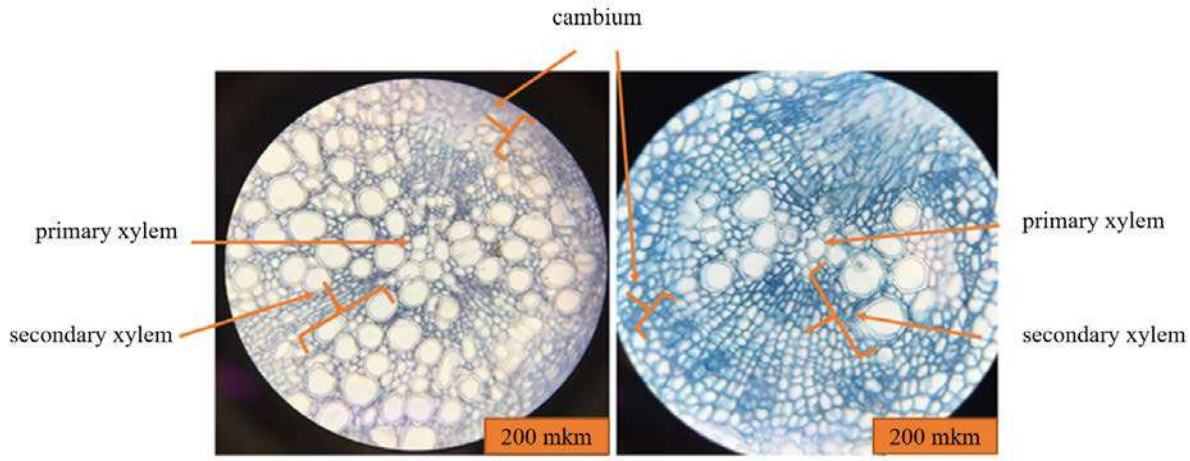

Figure 31. Effect of *RsWOX4* overexpression on root development (left - sector of root with *RsWOX4* overexpression, triarch type of stela; right - sector of root with *GUSoe* overexpression (control), diarch type of stela).

The WOX14 TF is a putative partner of WOX4 TF (Etchells et al., 2013), so we expected that overexpression of *RsWOX14* would have effects on root development similar to that of *RsWOX4* overexpression. In addition, unlike *WOX4*, *WOX14* promotes differentiation and lignification of vascular cells in *Arabidopsis* inflorescence stems (Denis et al., 2017), so its enhanced expression levels in radish may have contributed to an increase in the number of lignified xylem elements.

Figure 32 shows sections of roots with the *RsWOX14* overexpression. Which is consistent with our assumption, here we observed a disruption in the structure of the vascular system and formation of a triarch type of stele instead of a diarch one, which are effects similar to those of *RsWOX4* overexpression. At the same time, we did not observe such a strong increase in the vessel number in the roots of plants with *RsWOX14* overexpression (Figures 32, 33; Kuznetsova et al., 2024b), suggesting that it is the *RsWOX4* which is the central regulator of cambial activity.

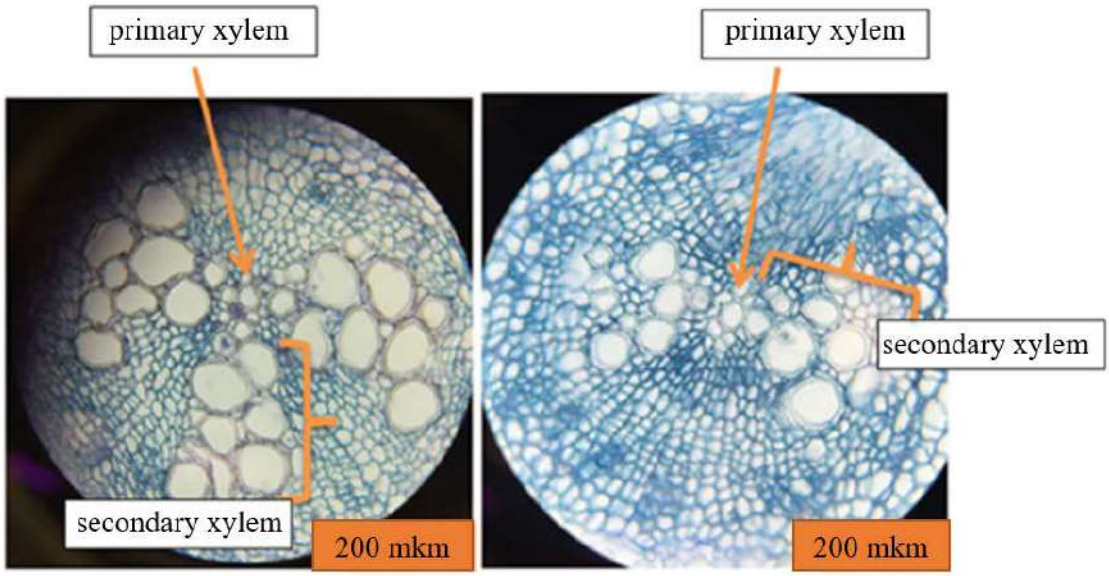

Figure 32. Transverse section of radish roots with overexpression of *RsWOX14* (left) and *GUS* as control (right).

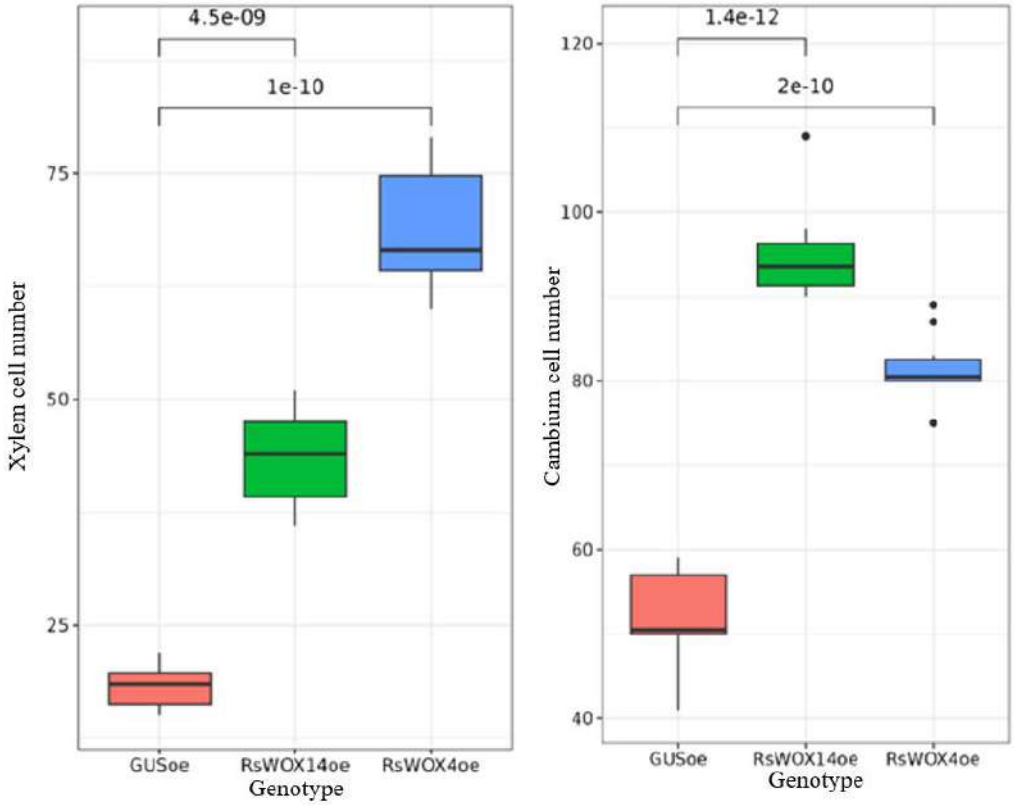

Figure 33. Effect of *RsWOX4* and *RsWOX14* overexpression on the number of xylem (left) and cambium cells in the root stele. Transgenic plants with overexpression of the glucuronidase *(GUS)* reporter gene were used as control. Cell counting was performed in a 200 × 200 μm sector of a root slice. The significance level was assessed using Student's t-test.

In plants with overexpression of the *RsWOX4-2* and *RsWOX14* genes, we have observed an increase in cambial cell number and in cambial cell layers. Besides, according to our data, the size of the cambial cell size was significantly reduced in plants with overexpression of *RsWOX4-2* and *RsWOX14*. All these effects could probably be associated with increased cambial proliferation (see Figures 33, 34; Kuznetsova et al., 2024b).

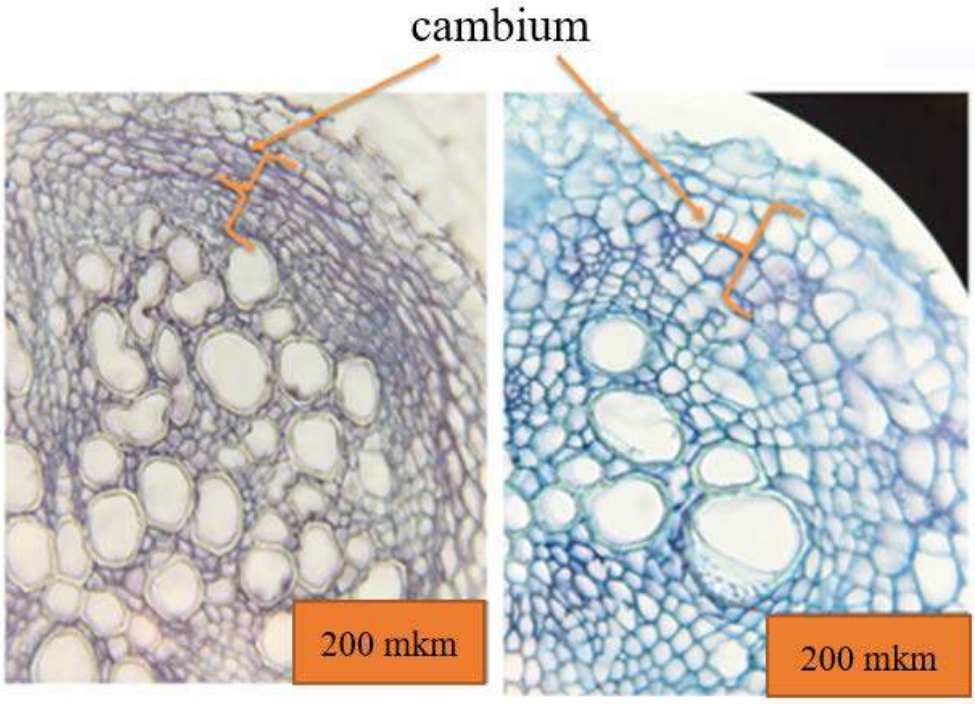

Figure 34. Sectors of root with *RsWOX14* (left) and *GUS* (control; right) overexpression.

Thus, plants with overexpression of *RsWOX4* and *RsWOX14* demonstrated similarities in their root stele structure and features of vessel development. We have observed such similar effects of overexpression as disruption of the radial pattern of tissues and an increase in the number of vessels in case of both *RsWOX4* and *RsWOX14* overexpression. Our data correlate with that on the overexpression of these genes in other plants.

3.4. Search for targets of the RsWOX4 and the RsWOX14 transcription factors

3.4.1. In silico search for putative targets of RsWOX4 and RsWOX14 TFs

To date, binding of WOX4 or WOX14 TFs to the promoters of any target genes has not been reliably confirmed, and in this study we decided to search for such targets. There is literature data on WUS and WOX5 targets, and we have used the available information on conserved binding sites for all WOX TFs for the first stage of our *in silico* analysis. As for the known WUS and WOX5 targets, they are:

1) CK biosynthesis genes (*IPT, LOG*, Guo et al., 2010; Tokunaga et al., 2012);

2) CK response genes (*ARR-A*, Leibfried et al., 2005);

3) Cyclin genes (*CYCD*, Randall et al., 2015);

4) Genes that control the development of the cambium and the vascular system (*HD-ZIPIII, ANT, PEAR*, Smetana et al., 2019, Miyashima et al., 2019);

5) Genes encoding for CLE peptides (Schoof et al., 2000);

6) Genes encoding for other TFs, e.g. columella differentiation regulator *CDF4* (Pi et al., 2015).

According to literature data, in other plant species, particularly trees, which also characterised by an enhanced secondary growth, the *WOX4* overexpression affected the expression levels of phytohormone signalling genes *(ARF2, ARF3, ARF7* and *ARF18*), root-operating TFs (WOX5, LBD29 and SCR) as well as *CYCD3, GRF1* and *TAA1* genes, which are involved in cell division, root development and IAA biosynthesis, respectively (Wang et al., 2020a).

Using an in silico interaction prediction tool (STRING, https://string-db.org), we constructed gene networks representing the likely interactions of the WOX4 and WOX14 TFs with other regulators of cambium activity in *Arabidopsis* (Figures 35, 36; Kuznetsova et al., 2024b.

Based on our literature search, we have compiled a list of candidate genes and searched for the presence of WOX4 TF binding sequences in the promoters of these genes. In total, we have identified several variants of such motifs, including two common

ones TAAT[G/C][G/C] (Lohmann et al., 2001) and TCACGTGA (Busch et al., 2010). Among the genes with such sites in promoters close to the start codon, we have identified *RsCLE41*, *RsCLE42*, *RsCLE22*, *RsHAM4*, *RsLOG3*, *RsLOG6*, *RsRR18*, *RsIAA28*, *RsYUC7* as our genes of interest.

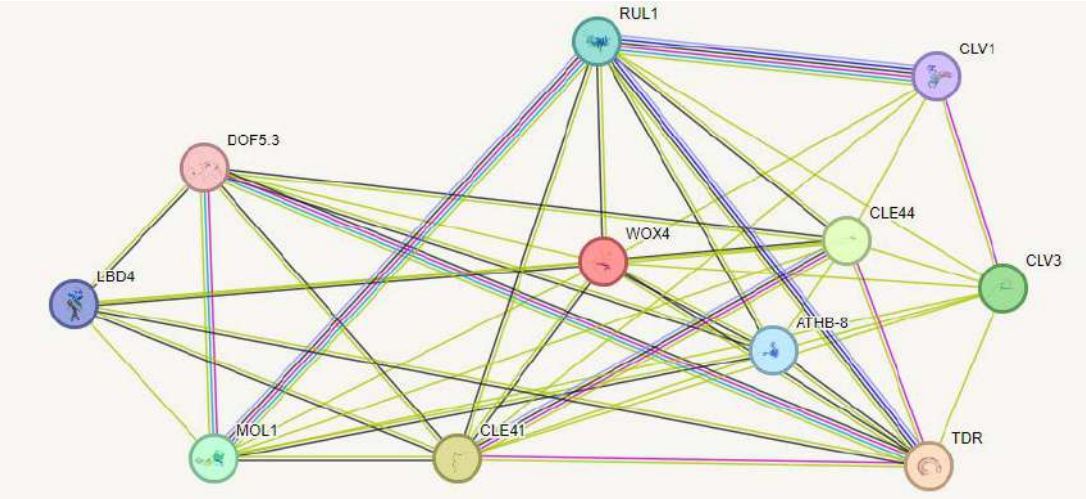

Figure 35. Predicted interactions between WOX4 and its putative targets, plotted using the STRING program (https://string-db.org/). Lines of different colours indicate different types of protein-protein interactions predicted for protein products of these genes: sea-wave colour - known interactions based on information from databases; crimson - known experimentally confirmed interactions; green - predicted gene neighbour interactions; red - predicted interactions based on gene fusion studies; blue - predicted interactions based on gene co-occurrence studies; light green - other types of interactions based on textmining; dark - other types of interactions based on co-expression studies; light purple - other types of interaction based on protein homology. Circles of different colours indicate different protein products of genes; the structure of the corresponding proteins is depicted in the centre of each circle.

Based on the in silico analysis, we speculated that the following genes might be among the targets of the WOX14 TFs:

1) *DOF5.3* which encodes a TF with a zinc finger domain and binds specifically to the consensus sequence 5'-AA[AG]G-3' of genes that promote radial growth of the sieve-like elements of the protophloem (Miyashima et al., 2019).

2) *LBD4*, which controls phloem or xylem histogenesis and, together with other LBDs, regulates radial growth by controlling cell size and cell division in the cambium (Smit et al., 2020; Ye et al., 2021; Turley and Etchells, 2022).

3) *CLE41*, *CLE42*, *CLE44* encode for CLE peptides of the TDIF group which have an identical sequence and inhibit plant stem cell differentiation (Etchells and Turner, 2010).

4) *CLV3* is one of three CLAVATA genes that regulate SAM size and floral meristem development in *Arabidopsis*. The *CLV3* acts in tandem with CLAVATA receptor kinase1 (CLV1) to control the balance between meristem cell proliferation/differentiation (Fletcher et al., 1999; Fiers et al., 2005).

5) *PXY/TDR* encodes a leucine-rich repeat receptor-like protein kinase and acts alongwith CLE41 and CLE44 peptides as a ligand-receptor pair in a signalling pathway involved in the regulation of procambium maintenance and polarity during vascular tissue development. It mediates repression of vascular element differentiation and promotes procambium cell formation and polar division leading to phloem cell formation in leaf veins (Fisher and Turner, 2007; Hirakawa et al., 2008).

6) *MOL1* and *RUL* encode leucine-rich repeat protein kinases which are putative regulators of cambium development (Gursanscky et al., 2016).

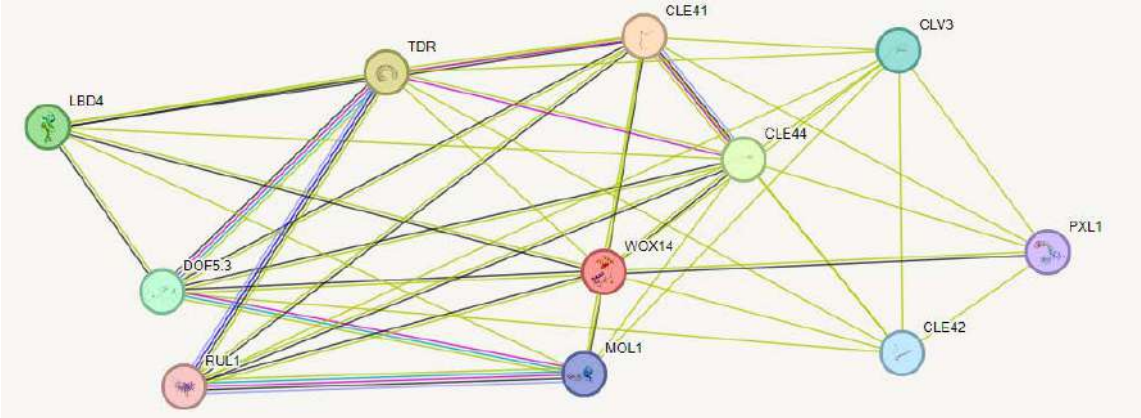

Figure 36. Gene network depicting the interactions between WOX14 and its predicted targets, constructed using the STRING programme (https://string-db.org/). The description is the same as that of Figure 35.

Based on the results obtained, we have continued our search for the WOX4 TF targets among the putative candidate genes, and then performed qPCR and the yeast one-hybrid assay.

3.4.2. Quantitative analysis of candidate gene expression in normal, overexpression and silencing of the *RsWOX4-2* gene

It has already been mentioned above that the targets of TF WOX4, an important cambium regulator, are currently unknown. To validate in silico data on WOX4 targets obtained during the previous stage of our work, we performed a quantitative analysis of the relative expression levels of genes that are probable targets for the RsWOX4 TF. Using qPCR, we evaluated the expression levels of *RsWOX4-2* and its putative targets in roots of composite plants overexpressing and silencing this gene compared to control roots with *GUS* overexpression.

In our experiment, the expression of the following genes was analysed:

1) The *WOX* family genes: *RsWOX5, RsWOX9, RsWOX11, RsWOX12, RsWOX14*;

2) The *CLE* family genes: *RsCLE41*, *RsCLE42*, *RsCLE12*, *RsCLE22*, *RsCLE2*;

3) Genes encoding other TFs involved in the regulation of cambium development: *RsHAM4, RsCYCD3-1, RsREV, RsHB8*;

4) CK biosynthesis genes: *RsIPT1, RsIPT3-1, RsIPT3-3, RsIPT5, RsIPT7, RsIPT8, RsLOG1, RsLOG2*, *RsLOG3*, *RsLOG4, RsLOG5, RsLOG6, RsLOG8*;

5) CK response genes: *RsRR3, RsRR7, RsRR18*;

6) IAA response genes: *RsIAA7*, *RsIAA28*;

7) IAA biosynthesis genes: *RsYUC1, RsYUC3, RsYUC4, RsYUC5, RsYUC7, RsYUC9.*

As a result, we have identified genes whose expression levels were increased upon overexpression of *RsWOX4-2* and decreased upon *RsWOX4-2* silencing. There were *RsCLE41, RsCLE42, RsYUC7, RsLOG3* and *RsRR18* (Figure 37) genes. We have considered them as candidates for further investigation. In addition, we have shown that the expression of several genes, namely *RsHAM4* and *RsIAA28*, was upregulated upon the *RsWOX4-2* overexpression and were not changed upon its silencing (Kuznetsova et al., 2024b).

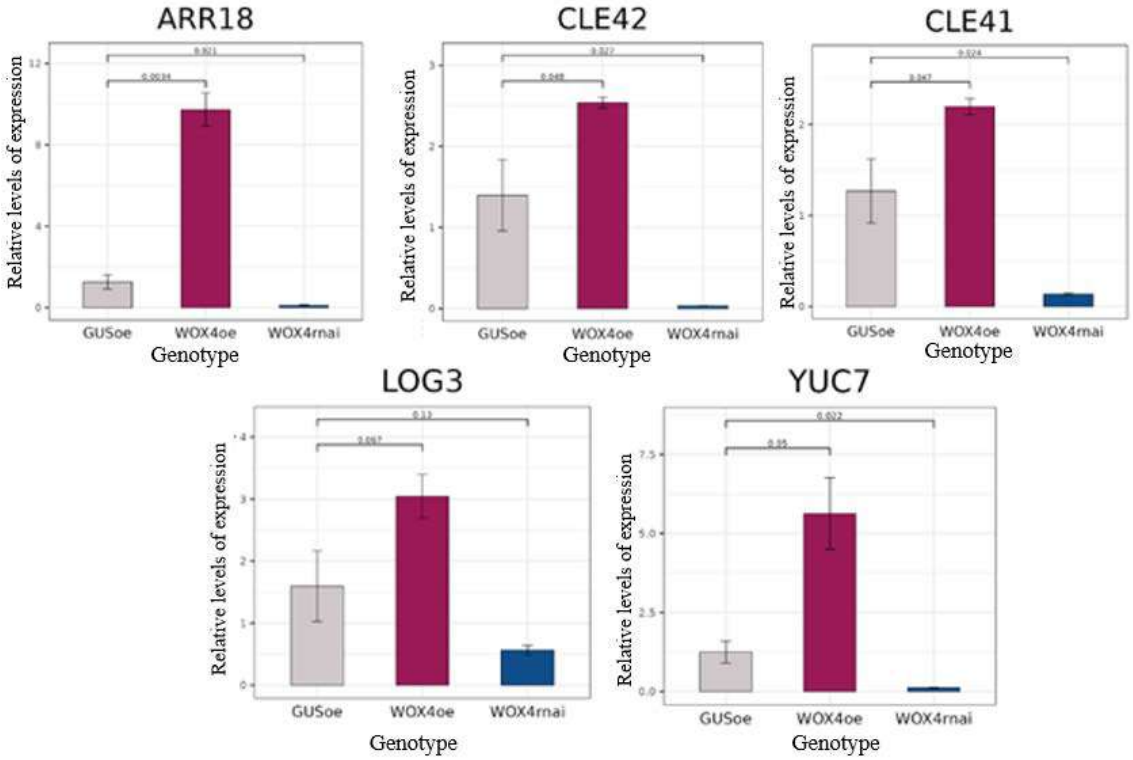

Figure 37. Changes in expression levels of candidate genes upon overexpression (purple colour of columns) and silencing (blue colour of columns) of the *RsWOX4* gene compared to the control plants with *GUS* gene overexpression (grey columns). The experiment was performed in triplicate biological replicates. Error bars indicate the error of the mean. The significance level was assessed by Student's t-test.

Based on literature data on the functions of homologues of the identified genes in *Arabidopsis*, it was supposed that these genes may be involved in the control of cambium activity in radish. In particular, homologues of the *RsCLE41* and *RsCLE42* genes encode TDIF signal peptides, which are regulators of cambium activity (Ito et al., 2006), and the homologue of the *RsHAM4* gene is a partner and cofactor of the WOX4 TF (Zhou et al., 2015). As for others, homologues of the *RsYUC7*, *RsLOG3*, *RsRR18* and *RsIAA28* genes encode a IAA biosynthesis gene (Won et al., 2011; Lee et al., 2012), a CK biosynthesis gene (Kuroha et al., 2009), a CK signalling inhibitor (Mason et al., 2004) and a BER signalling inhibitor (Liscum and Reed, 2002), respectively.

3.4.3. Analysis of the interaction of the RsWOX4 TF homeodomain with the promoters of candidate genes using a yeast one-hybrid system

To verify the data on possible RsWOX4 targets obtained in the previous step, we have examined the interaction of promoter regions of our candidate genes with the RsWOX4 TF homeodomain using the yeast one-hybrid assay. The method of the yeast one-hybrid system (Davies, 2013) consists of testing the interaction of the TF, to which the activation domain is attached, with the promoter of interest which is under the control of the yeast promoter and the reporter gene. When the TF interacts with DNA, the activation domain binds to the yeast promoter and triggers expression of the reporter gene, resulting in yeast growth on selective medium.

In this study, we studied interactions between the homeodomain and promoter sites of the *RsCLE41, RsCLE42, RsYUC7, RsLOG3* and *RsRR18* genes, which contain conserved TAATG/CG/C binding sites for the WOX TFs (Lohmann et al., 2001; Busch et al., 2010). The interaction between the RsWOX4 TF and promoter site was considered to be confirmed if yeast transformed with a combination of plasmids carrying the RsWOX4 homeodomain and promoter sites of the target genes grew more vigorously than controls transformed with empty plasmids.

The putative WOX4 target genes identified in the previous real-time PCR-based experiment perform diverse functions related to the control of phytohormone homeostasis in plant tissues. They are summarised below.

The *Arabidopsis LONELY GUY3 (LOG3)* gene encodes a CK-activating enzyme involved in the final step of CK biosynthesis and is a phosphoribohydrolase that converts inactive CKs to biologically active forms as free bases (Kuroha et al., 2009). Its expression in young xylem cells is known to be directly regulated by TMO5 and LHW, which in turn are targets of the *MP* gene, which regulates, among others, the WOX4 TF (Ohashi-Ito et al., 2013, Brackmann et al., 2018) activity. No direct interaction between WOX4 and *LOG3* has been reported in the literature; however, in our experiments on assessment of quantitative gene expression in roots with *RsWOX4* overexpression, the *RsLOG3* gene demonstrated a statistically significant increase in its expression level and therefore became a candidate for further research as a likely direct target of WOX4 TFs.

The *AtYUC7* gene, encoding indole-3-pyruvate monooxygenase, is involved in

auxin biosynthesis, embryogenesis and seedling development. It is essential for the formation of floral organs and vascular tissues. The *AtYUC7* is known to belong to a set of redundant *YUCCA* genes responsible for auxin biosynthesis in shoots. According to literature data, it could also be induced in response to drought (Won et al., 2011; Lee et al., 2012). In addition, the *YUC7* is known to be a trigger for the IAA biosynthesis in the lateral root meristem through signalling from the brassinosteroid coreceptor BAK1, which is induced under conditions of low nitrogen availability (Jia et al., 2019, 2020, 2021).

The *ARR18* gene encodes TF, a type B response regulator (ARR-B), which activates some of the A-type response regulators (ARR-A), such as ARR22-ARR24, in response to CK.

The TDIF group peptides CLE41 and CLE42, which regulate *WOX4* expression, are components of the WOX-CLAVATA system that controls the balance between cambium and differentiated cells (Hirakawa et al., 2010). It was suggested that there is a feedback loop between CLE41/42 and WOX4 TFs in the cambium, as between WUS and CLV3 in the SAM, where WUS TFs activate the CLV3 gene expression (Schoof et al., 2000).

The yeast one-hybrid assay has confirmed the presence of an interaction only between the WOX4 homeodomain and the TAATCC site in the promoter of the ***RsLOG3*** gene (Figure 38), which regulates the final step of free CK biosynthesis (Figure 39). At the same time, no interaction with the WOX4 homeodomain was shown for the promoters of *RsCLE41, RsCLE42, RsYUC7* and *RsARR18* genes using the yeast one-hybrid system method (Kuznetsova et al., 2024b).

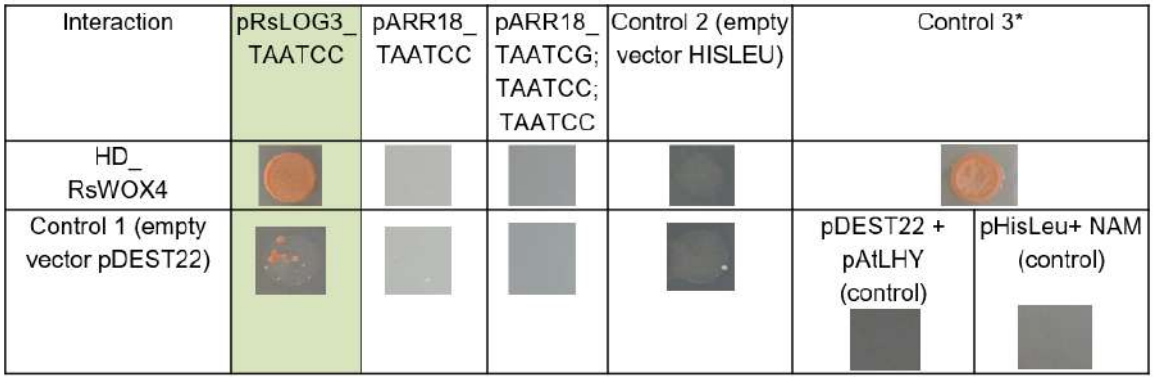

Figure 38. Yeast one-hybrid assay results for the assessment of interactions between the promoter regions of the *RsLOG3* and the *RsARR18* genes and the RsWOX4 TF homeodomain. The interaction was considered to be confirmed if yeast transformed with the combination of plasmids grew better than controls (yeast transformed with plasmids without insertions of the analysed DNA fragments). The experiment was performed in 4 repetitions and representative variants are shown in the figure. Confirmed interaction is highlighted in green. Colonies were grown on media with different concentrations of 3-AT, the series of photos shows the growth of colonies on media with 10 mM 3-AT (maximum concentration for this experiment). * The plasmids pHisLeu-pAtLHY and pDEST22-NAM, with promoter and TF sites, respectively, which are known to interact with each other (Lopato et al., 2006), were used as control 3 (positive control).

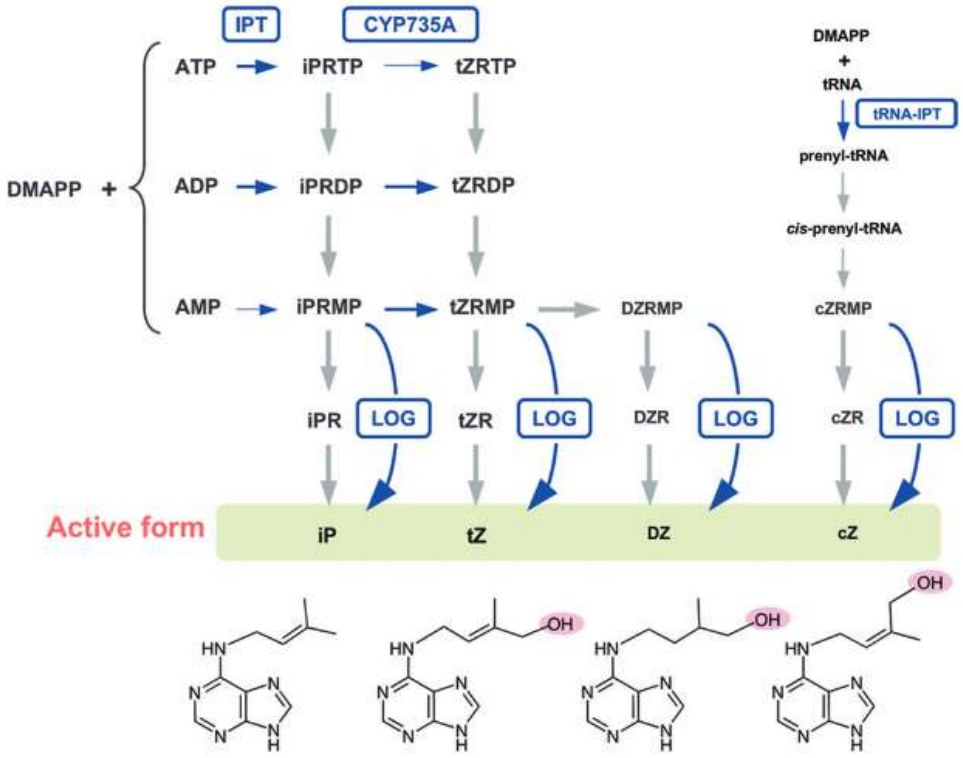

Figure 39. Regulation of cytokinin biosynthesis in plants (by Hirose, 2008).

Thus, this is the first time that we have identified a putative target of TF WOX4 among the CK biosynthetic genes. The CKs are known to play a role in cambium cell proliferation: according to literature data, loss of function of *IPT1, -3, -5* and *-7* genes encoding isopentenyl transferase, an enzyme involved in the first step of CK biosynthesis (upstream of LOG enzymes), leads to reduced cambium activity and root thickening (Matsumoto-Kitano et al., 2008). Nieminen et al. (2008) also highlighted an important role for CKs in controlling cambium activity in poplar: genes encoding CK receptors are preferentially expressed in dividing cambial cells of the poplar stem. It is also known that overexpression of the *CYTOKININ OXIDASE* gene, which encodes a CK-degrading enzyme, leads to suppression of secondary growth. A possible role of the *LOG3* gene in the control of cambium activity has been demonstrated for the first time.

3.5. Conclusion: Identification of regulators of storage root and spontaneous tumour development in radish

In this work, we have sequenced for the first time the genomes of two inbred lines of the radish genetic collection that differ in their ability to spontaneously tumour. A number of differences have been identified between the lines, including those associated with presence of loss-of-function genes that may be involved in the regulation of root development and/or meristem activity. We have performed here the first large-scale analysis of plant genomes with spontaneous tumour formation. Studying the influence of the genes identified in this study on root development and spontaneous tumours will help to provide new data on the mechanisms controlling meristematic cell activity in plants.

The role of components of the cambial WOX-CLAVATA system in the development of the radish storage root has been studied in this work. We have also provided data on the identification, chromosomal location analysis and sequence analysis of genes encoding components of the WOX-CLAVATA system, WOX TFs and CLE signal peptides, in radish. The effects of overexpression on cambium development were analysed for individual members of the radish *WOX* and *CLE* gene families whose homologs in *Arabidopsis* are key regulators of cambium development (i.e. *RsWOX4-2*, *RsWOX14* and *RsCLE41-1*). We have investigated the effects of *RsWOX4-2* and *RsWOX14* overexpression on radish root stele formation and cambium cell proliferation

and have suggested their role as possible regulators of storage root development. Using qPCR and transcriptomic analysis, we have obtained data that show that overexpression of *RsWOX4-2* and *RsCLE41-1* in the roots of radish affects the expression of a number of genes belonging to different gene families. This set of genes, according to our results, could potentially be direct targets of *RsWOX4-2* and *RsCLE41-1* in cambium. We have also used a yeast one-hybrid method to search for targets of the RsWOX4-2 TF, and have detected the binding of the RsWOX4-2 protein to the promoter of the *RsLOG3* gene, which regulates CK biosynthesis. These are the first data on the direct target of the WOX4 TF, allowing us to establish a link between the activity of the WOX-CLAVATA system and the level of CK in the cambium.

Our work allowed us to obtain a set of new data on the mechanisms of storage root development (an economically important trait), in particular linking the WOX-CLAVATA system, which regulates cambium activity and secondary root growth, to CK (through a direct target of the WOX4 TF which is the *LOG3* gene). Our sequencing of the genomes of tumour and non-tumour radish lines allowed us to identify a number of differences, the detailed analysis of which opens up the possibility of elucidating the mechanisms of tumour formation in plants (a feature whose study is important for the identification of hitherto unknown systemic regulators of cell proliferation in plants).

CHAPTER 4. DISCUSSION

Our research was devoted to the study of two different, but related, features of root development in a model root crop, radish: 1. Spontaneous tumours developing on the root of certain inbred radish lines from the SPbSU genetic collection and 2. Storage root formation in radish.

The formation of storage roots and tumour can be governed by similar mechanisms including meristem regulators involved in the control of meristem activity. According to previous data, the same lateral meristem, the cambium, is involved in the formation of storage roots and tumours in radish (Lebedeva et al., 2015). Moreover, the structure of spontaneous tumours in radish possess a number of features characteristic of meristems, such as the demarcation of active cell proliferation zones as well as zones of differentiation; maxima of auxin concentration coinciding with zones of expression of the *RsWOX5*, a regulator of apical root meristems (Lebedeva et al., 2015); increased expression of cell cycle genes and meristem-specific genes (Lutova et al., 2008; Tkachenko et al., 2020). From this point of view, tumours represent a special type of irregular, or facultative, secondary meristems originating from the cambium, and they can be considered as an anomaly of secondary growth and the other side of storage root formation. The presence of numerous tumour lines of radish of different origins in the SPbSU genetic collection indicates that radish breeding process could have selected forms with impaired control of root cell proliferation, which can lead to increased secondary root thickening and, in the presence of a certain genetic background, to tumour formation. Thus, the study of tumour lines can help to understand the direction of selection in the development of root crops (Buzovkina and Lutova, 2007).

A characteristic feature of plant developmental control is the participation of the same so-called regulatory modules consisting of genes and their products in different developmental programmes (see review by Kuznetsova et al., 2023a). Thus, a number of similar mechanisms may be involved in the development of spontaneous tumours and the radish storage root. One of the most important regulatory modules that plays a key role in controlling the activity of various meristems is the WOX-CLAVATA module, which includes the CLE peptide phytohormones, their receptors and their targets, the genes encoding WOX TFs. In this respect, it is the cambial components of the WOX-CLAVATA module that have been of particular interest in our study.

Spontaneous plant tumours develop without the involvement of an infectious agent or external inducer, due to mutations or epigenetic changes (Dodueva et al., 2021). According to the literature, the study of spontaneous plant tumours has revealed a number of new systemic regulators of plant cell division. This is important because, despite the conservatism of the basic mechanisms of cell cycle regulation, the systemic control of plant cells is based on a different principle than in animals, and therefore spontaneous plant tumours are quite rare (Doonan and Sablowski, 2010; Dodueva et al., 2021). Significant progress in identifying new mechanisms of systemic control of plant cell division has been made in the study of multigenic *Arabidopsis* tumour mutants, which have identified genes whose loss of function leads to tumour formation in plants. Among those genes was a number of genes related to cell wall formation and cell adhesion (Krupkova and Schmulling, 2009), cell cycle control (Bellec et al., 2002) or chromatin remodelling (Mao et al., 2006).

The SPbSU genetic collection includes inbred radish lines with spontaneous tumour formation on the storage root (Buzovkina and Lutova, 2007), in crosses of which tumour formation inherits as a monogenic recessive trait (Narbut et al., 1995; Matveeva et al., 2004). Comparative studies of such lines, as well as studies of *Arabidopsis* tumour mutants, can help to identify regulators of spontaneous tumour formation and systemic control of plant cell division. We have included two contrasting related radish lines, one of which forms tumours in contrast to other, in our analyses. At the first stage of our research, we assume a similar nature of tumour formation in different tumour lines of the collection. According to literature data, there are very few mutations leading to tumour formation in plants. In this respect, it could be illogical to suggest that several non-allelic mutations resulting in the same tumour phenotype are found in a one SPbSU collection, whereas no single tumour line was among other world collections of crop root plant. Since the nature of this mutation may be different (it could be insertion, or deletion, or single nucleotide substitution in the coding or promoter part of the gene), searching for such a candidate gene will be a complex task that is unlikely to be solved within the frames of one PhD thesis.

Transcriptome analysis of spontaneous radish tumours revealed activation of a large number of genes participating in all stages of the cell cycle (Tkachenko et al., 2021a), suggesting the presence of an unknown upstream regulator that controls this entire gene network. Identifying genes disrupted in tumour lines could therefore allow us to identify such regulator or regulators.

In this work, we have sequenced and assembled for the first time the genomes of two closely related lines of the SPbSU radish genetic collection, which differ in their ability to spontaneously form tumours, and carried out their comparative analysis, which is one of the most important results of our work. During these studies, 72 genes with insertions or deletions in their coding regions, leading to a frameshift and probably to the synthesis of a non-functional protein product, and 36 genes with single nucleotide substitutions were identified in the tumour line. Among these were a number of important regulatory genes involved in different plant developmental programmes. We tested some of these genes by sequencing PCR fragments and confirmed the differences identified in six of the ten genes assessed. The radish lines studied in this work diverged in the first generation of inbreeding and differ in a large number of traits. The large number of identified genes with sequence differences opens up further perspectives for research and elucidation of which genes play a role in tumour development and which genes are associated with other traits.

In addition to the search for new regulators of the systemic control of cell division, the sequencing of the radish lines used in our work allowed us to perform chromosomal localisation and sequence analysis of genes encoding components of the WOX-CLAVATA systems, which are of our particular interest. A total of 24 *WOX* genes (3 of which, the *RsWOX4-1*, *RsWOX4-2* and *RsWOX14*, are homologues of cambial regulators identified in other plant species) and 52 *CLE* genes (with three of them encoding TDIF group peptides that regulate cambial activity) were found in the radish genome. The chromosomal localisation of the genes of the studied families, based on the obtained assemblies of radish genomes, provides prospects for studying genes that influence cambium and vascular system development, as well as for those that control economically important traits. In the future, these data can be used for genetic analysis and selection of radish genotypes with new combinations of traits.

As the differences between tumour and non-tumour lines were revealed by the sequences of a large number of regulatory genes, which are thought to be involved in the control of meristem activity and root development, the search for candidates for the role of new plant oncogenes is quite wide. In order to narrow this down, further studies, including the analysis of the co-inheritance of the identified molecular traits with morphological disorders, as well as the location of these traits on genetic maps, are needed.

The genetics analysis of co-inheritance with tumour formation seems to be the

only crucial further step of this research that can help to establish the relationship between this trait and the nucleotide differences in genes with insertions or deletions and single nucleotide substitutions that we have identified. This work thus opens up a new area of research in this field, including both the establishment of a link between molecular differences and root size, and the establishment of differences associated with tumour development. Of particular interest for further work are the closely related lines 19, 20 and 21, which are promising for crosses with line 18 and subsequent genetic analysis. Many of the issues initiated in this work by sequencing individual lines of the radish genetic collection require further research. In particular, we consider promising aspects relating to the sequencing of other lines in the collection and the identification of candidate genes involved in other processes determining developmental anomalies such as stem curvature, seed germination in the pod, ovary budding, chlorophyll deficiency and dwarfism.

The next aim of our work was to study the genetic mechanisms underlying the development of the radish storage root. This work has both practical implications, because of the important contribution of root crops to human and animal nutrition, and theoretical significance, because it provides new data on the mechanisms of regulation of lateral and irregular root meristems.

Inbred lines of the genetic collection of radish (*R. sativus* var. *radicula* Pers.), created at the Department of Genetics and Biotechnology of St. Petersburg State University, were used as the object of study. Radish is an appropriate model for studying the development of the storage root because of its short life cycle and close kinship with *A. thaliana*. In addition, it has been reported (Hoang et al., 2020) that different plant species with a storage root are characterised by changes in the expression of the same regulatory genes during root growth, so the data identified in radish can be applicable for breeding other root crops.

The activity of the cambial lateral meristem underlies the development of the storage root, which depends on the activity of specific groups of TFs, the balance of phytohormones (mainly IAA and CK) and the functioning of the cambial WOX-CLAVATA system. The WOX-CLAVATA systems, which include CLE peptide hormones, their receptors and their targets, which are genes encoding homeodomain-containing WOX TFs, represent a highly conserved regulatory module controlling the maintenance of stem cells and various meristems (Kuznetsova et al., 2023a).

At the same time, studies on the functions of the WOX-CLAVATA module in

storage root development are currently in their initial stages. Previously, researchers from the Department of Genetics of St Petersburg State University obtained data on the key role of the CLE41 peptide in the thickening of the radish storage root (Gancheva et al., 2016; Gancheva et al., 2018). In the present work, these studies are continued. The main results of our work include 1) identification of a wide range of RsCLE41 targets by transcriptome analysis of roots with the *RsCLE41* overexpression, 2) investigation of the effect of the cambium TF regulators WOX4 and WOX14 on radish root development, 3) identification of probable direct targets of the WOX4 TF, which is a central regulator of cambium activity, using a yeast one-hybrid assay.

Our findings on novel targets of the CLE41 signalling peptide among genes regulating vascular lignification and drought response were rather unexpected and included, for instance, genes encoding LEA proteins responsible for cell protection against dehydration (Kuznetsova et al., 2022).

Overexpression of *WOX4* and *WOX14* has also been shown to alter root stele structure in radish, inducing an increase in cambium cell layers and the number of primary xylem poles, which may enhance secondary root growth. However, in our experiment, traditional TDIF targets such as WOX4 and WOX14 (Etchells et al., 2013) were not identified as differentially expressed genes: their expression levels were not statistically significantly altered in radish roots overexpressing *RsCLE41* compared to control plants. This result may be explained by the assumption that signalling pathways may be directed to different targets in different parts of the plant, as well as by the stage of plant development at which the transcriptome analysis was performed.

Finally, a probable direct target of TF WOX4 was identified for the first time: this important TF, a cambium regulator, was shown to bind to a specific promoter region of the *RsLOG3* gene. The product of this gene is a phosphoribohydrolase-type enzyme that acts in the final step of CK biosynthesis, releasing active CK from inactive transport forms (Kuroha et al., 2009). Homologues of this gene are expressed in the xylem according to literature data (Ohashi-Ito et al., 2013, Brackmann et al., 2018), but no data are available on its role in cambium development. CKs are important for the regulation of cambium activity (Brackmann et al., 2018) and storage root thickening, including in radish (Jang et al., 2015); thus, the WOX4-LOG3 pathway may be essential for CK-dependent regulation of storage root growth.

Therefore, in this work, we have identified candidate genes involved in processes related to radish root developmental traits which are secondary growth leading to the

development of a storage root and abnormal meristematic activity leading to spontaneous tumour formation. We have investigated the functions of some of these genes, including the WOX-CLAVATA system genes, which were of particular interest to us. The proposed scheme of interaction of the WOX-CLAVATA system with other regulators in the radish storage root, based on our findings and literature data, is shown in Figure 40.

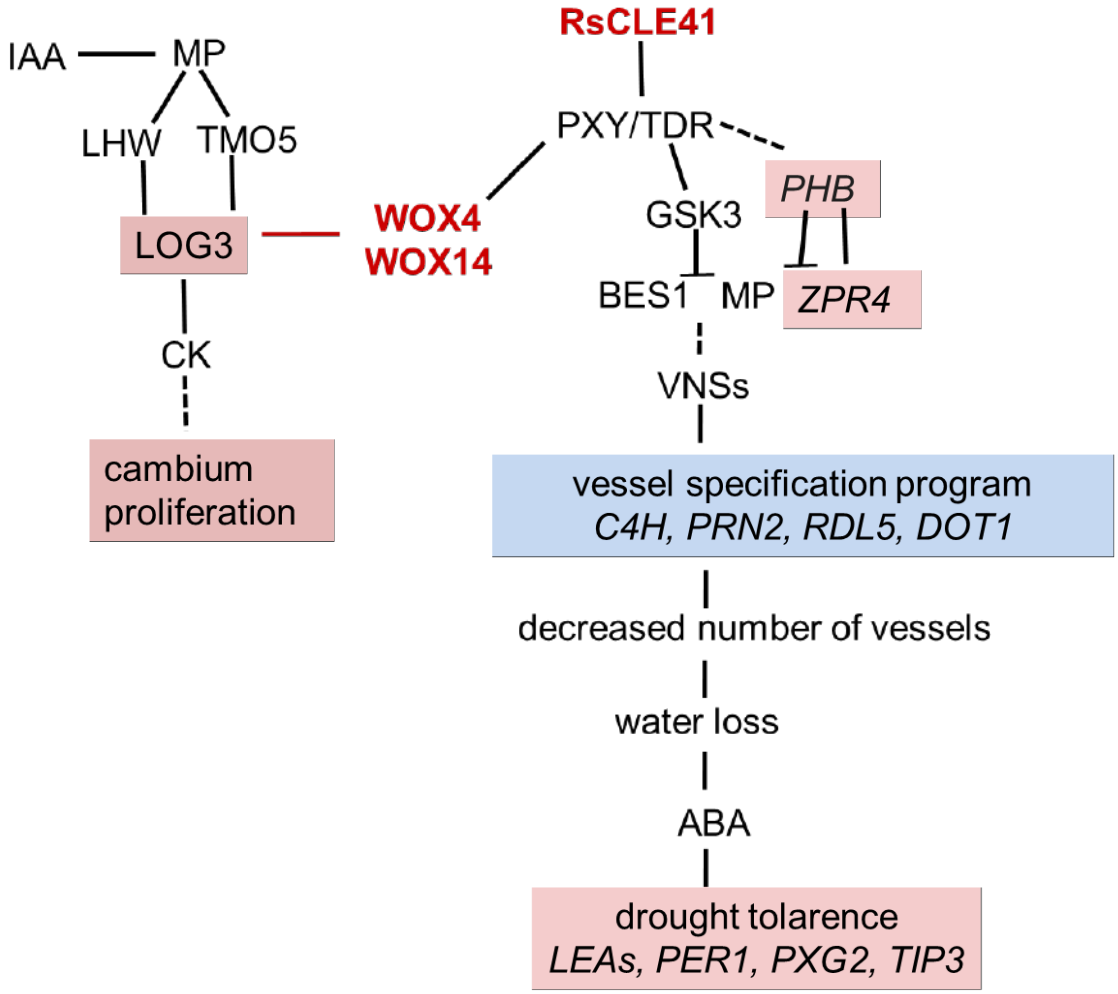

Figure 40. The scheme of the components of the WOX-CLAVATA system role in the development of the radish storage root based on our data (highlighted in colour).

In addition, there would be a fruitful area for further work to answer the questions of whether the meristem regulators identified in radish are conserved during root development in other species of the genus *Brassica*, as well as in other plant genera, and whether they may have played a role in the domestication process of radish and root crops in general.

Thus, the present work is important for understanding the mechanisms of storage root development (an economically important trait) and spontaneous tumours (a trait

whose study is important for identifying hitherto unknown systemic regulators of cell proliferation in plants). Our results are forward-looking for use in selection of root crops for yield.

CONCLUSIONS

1. Genome assemblies and annotations have been obtained for the first time for two SPbSU radish genetic collection inbred lines, which differ in their ability to spontaneously form tumours.
2. In the tumour line of the SPbSU radish genetic collection, 36 genes with single nucleotide substitutions (SNPs) and 72 genes with insertions/deletions (InDel), which are thought to affect the structure of the encoded proteins, were detected compared to the non-tumour line.
3. Twenty four *RsWOX* and fifty two *RsCLE* genes were identified in the radish nuclear genome and located on radish chromosomes.
4. New putative targets of the RsCLE41 signal peptide were identified using transcriptome analysis. Among them are genes associated with late embryogenesis, response to water deficit and auxin-dependent determination of xylem cell fate.
5. Overexpression of the *RsWOX14* and *RsWOX4* genes was shown to alter the root stele structure and to enhance the number of secondary xylem cells.
6. The following putative targets of the RsWOX4 transcription factor have been identified: *RsCLE41*, *RsCLE42*, *RsYUC7*, *RsLOG3* and *RsRR18*. The WOX family transcription factor conserved binding sites were found in the promoters of these genes.
7. The RsWOX4 transcription factor was shown to interact with the TAATCC site in the promoter of the *RsLOG3* gene, which regulates cytokinin biosynthesis.

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SUPPLEMENTARY MATERIALS

Appendix 1. Primers used in the work.

Primer	Sequence
Primers for studying the effects of gene overexpression and loss of function	
RsWOX4 HD F	TGGAGACGCACTCGGGAGG
Rs WOX4 HD R	GTGACGATGTTGTTGTTGTTTTCG
RsWOX4 CDS F	ATGAAGGTAGATGAGTTTTCCAATGGGT
RsWOX4 CDS R	TCAACTACTTTCAGGGTACAATGGG
RsWOX4 RNi F	TGTAGAGAAGGGGGAGTTAGTGGAA
RsWOX4 RNi R	GGAGTAAGAAGGAGGAGGAGAGCA
RsWOX14 CDS F	ATGGAGAAAGAGAGCCAAAACGGTG
RsWOX14 CDS R	TCACAGTCTATACGAATTGAACT
Primers for studying of interactions using the yeast one-hybrid system	
RsCLE41-like F	TATTCTCATACTCTGCTGCTGCTGCT
RsCLE41-like R	ATAGGATTAGGTCCAGAAGGAACTTC
pRsCLE41 s1 F	ATCGGATCGGATATTCGTTTCCCC
pRsCLE41 s2 F	ATCACTGGATCTCTTCTCCCATACAC
pRsCLE41 s2 R	CGCATATTCCCCTTTGGTAAAAG
pRsCLE42 s1 F	GTTCTGACACTATTGCCGTTCTT
pRsCLE42 s1 R	TGCGTTGTCTTATGGTTAGAGCG
pRsCLE42 s2 F	TTCGCTCCACACTCTCTCTTTTG
pRsCLE42 s2 R	TCTCTTCCATGACAAGATTTAACG
pRsCLE42 s3 F	CTCTCTCTCTACCAGGAAGAACACTC
pRsCLE42 s3 R	TGATGTGAGGAGATCGCATCAC
pRsLOG3 s1 F	CGATGACCAGCATGACAAGA
pRsLOG3 s1 R	AACAGTTTTAAGGTTTCTTTGAGCAC
pRsLOG3 s2 F	TTTTTAGGAAAGCCTCGCAAA
pRsLOG3 s2 R	TTCTTGTCATGCTGGTCATCG
pRsYUC7 s1 F	GCACATTTAGAAGTCAAATCCAAGG
pRsYUC7 s1 R	TGCGAGGACGAGTTTTAGGG
pRsYUC7 s2 F	GCGAAGTTGTAGAAAGGTAGAAACGA
pRsYUC7 s2 R	CTAGCAGCGGCATTTGTGAA
pRsARR18 s1-2 F	CCAACAATAAGGAAAGAAAGAGAGG
pRsARR18 s1-2 R	AGGAGGCAGTCCAAAAAGAG

pRsARR18 s3 F	TCCAACACAATGTTTCCGTTTT
pRsARR18 s3 R	TGACCTGATTCATACCACCATCC
pRsARR18 s4-6 F	CCCAATAATCCGCACGAAAA
pRsARR18 s4-6 R	CCCTTTTACGATTGAATGGTTTAGTT
Primers for sequencing gene with InDels/SNPs	
RsCYCD4;1 indel1-2 F	GGTTGGAGATCCTCAGTTTGTGT
RsCYCD4;1 indel1-2 R	CCTTTGGTTGTGCTGGCTATC
RsCYCD4;1 indel 3 F	GCTCAGACTTATGTTCACAAACACC
RsCYCD4;1 indel3 R	TTTTCAACCTCTCATCAGACATCATAC
RsLBD4 indel1 F	TCCTGTTTACGGTTGCGTAGGT
RsLBD4 indel1 R	ATGCCCTTGTTGTCCTGTGG
RsCLE7 indel1 F	TTTTCTTAATTTCATTGTTTCCACATC
RsCLE7 indel1 R	AGATCGTTGCTCTCACCATCTTT
RsCYCD3 indel1 F	GTTGATCCCTTTGACCCTCTTT
RsCYCD3 indel1 R	CGTGACTCTTGCGTTTCTTGG
RsWOX2 indel1 F	TGGACATCAATAAAGAAGGAGGAGA
RsWOX2 indel1 R	TTTGCCACCAAACGACAAC
RsWOX2 indel2 F	GCAGAGGTTGTCGCAGAGG
RsWOX2 indel2 R	TCAGGGTCATGGCAGTTCA
RsBRX indel1 F	CGTCGGGTCAGATTCAGGT
RsBRX indel1 R	GGCTTTGTATTCTCTCTCTGTTCTGCT
RsBRX indel2 F	ATCACATTCGCTTCGCTTCC
RsBRX indel2 R	TCTCTGTTCTGCTCCCACCAC
RsCYCD4;1CDS F	ATGGCAGACAATCTGGAACTGAGA
RsCYCD4;1 CDS R	TTAGGAAAGATGTGTATGGGAAGAAG
RsCYCD3;1 CDS F	GAAAAACCAAACGATACAGAAGATAAA
RsCYCD3;1 CDS R	TACCCTTCACACATGACAGAGCAAAGT
RsCLE2-2 cds F	ATGGCTAAGTTAAGTTTTACTTTCTG
RsCLE2-2 cds R	CTAGTGATGTTGTGGGTCGGGTC
Primers for quantitative assessment of gene expression	
RsWOX5 F	AGGTGGAACCCTACGGTGGA
RsWOX5 R	GAGAGAGGCAAAAACGCAGT
RsWOX9 F	GCCAAGCATGTTCAAGTCCAAA
RsWOX9 R	GAGCCCTAATCCTTCTGATCTCCTC
RsWOX11 F	ATGGACCAAGAACAAACACCACATA
RsWOX11 R	GAGCATCTTTCGTATCCTTACGGT
RsWOX12 F	GGATCGTCACAAAaCCCTACTGG

RsWOX12 R	GCTTCTCTGGAGGCGACAGC
RsCLE42-1 F	ATGAGATCACACAGCACaATTTTACTT
RsCLE42-1 R	CTACCTATTTGAGATGGGATTTGG
RsCLE12-like F	ATTCTGGTTATTGTTCTGTGGCTGTCT
RsCLE12-like R	CTAGGATCAATTTCATCTCCAACATC
RsCLE22-like F	CTAGAAAGCATCTGACTACTGTTGC
RsCLE22-like R	GAGGATTAGGTCCAGTAAAAACTCTTCTC
RsIPT8 R	TCAGGCGGATAAACTCCGAAGTACC
RsIPT8 F	ATTGCTGCTTCCTCTGGGTGGAT
RsLOG2 F	ATGGAAGAGACAAAATCAAGATTCAG
RsLOG2 R	ACGAAATGGTGGGCGAGTTGG
RsLOG7 F	GGTGGGTCTTCTTAACCTGGATGG
RsLOG7 R	CCTCGTGTTGTGGAACCAACTC
RsLOG6 F	GGTTGGTTTATTAAATGTTGATGGATAT
RsLOG6 R	CCTCTAATTTCTCAAACAACTCTTATGC
RsHB8 F	ATGGAGTGTGCCAGAAGTTCTTCG
RsHB8 R	ACTGCTTCGTTGAATCCTTTGCT
RsIPT1 F	CCGAGCGATACGATCCGAAG
RsIPT1 R	ATCGTACGCCGCTCTCCTC
RsIPT3-1 F	GGCAAACCTCTCAGTTGAATCTGTT
RsIPT3-1 R	ACAATTCTTCTCTGTCTCCCACATTC
RsIPT3-3 F	GGCGAATCTCTCAGTTGAAAGCAT
RsIPT3-3 R	TGTCTCTCATGTTTAAGAACGGTTC
RsIPT7 F	ATGAAGTTCTCAATCTCAGCAATGAAGC
RsIPT7 R	GAGCAAGTGGTGAGGCACGC
RsLOG1 F	GTTCAAGAGAATATGTGTGTTTTGTGG
RsLOG1 R	ACAATGATATGACGAGCAGTTGGTG
RsLOG3 F	TGTGTCTTCTGTGGAAGCAGCCAAG
RsLOG3 R	GCAGAGAGTTGTAGTATCCATCAACATT
RsLOG4 F	GTGGAAGCAGCCAAGGCAAGA
RsLOG4 R	TATCCATCAACATTGAGCAAACCCAC
RsLOG5 F	TTCAAGAGGGTTTGTGTGTTCTGT
RsLOG5 R	CATCATCAACGGCTTTATCAATG
RsLOG6 F	GGTTGGTTTATTAAATGTTGATGGATAT
RsLOG6 R	CCTCTAATTTCTCAAACAACTCTTATGC
RsYUC1 F	ATCCTCGTACACGGTCCCATCATC
RsYUC1 R	CCATCCATAAACTTTGCTCCATTTTTTGT
RsYUC3 F	GACATTTTCTCCCGTCGCTGC

RsYUC3 R	GCACCAAACAATCCTTTCCTCGT
RsYUC4 F	CGTAGCGGCTTGTTTATCAAACC
RsYUC4 R	CTTTCACTGCTTGCGTCACTCTAATTT
RsYUC5 F	ACGGTCCAGTTATCATCGGAGCC
RsYUC5 R	TCCTTTCCTCGTGAATCCAGCC
RsYUC7 F	TTACCCAATCTACCCTTCCCCGA
RsYUC7 R	AAGATACTCAAACTCGCAAGAACCG
RsYUC9 F	TGCCAATTACCAAAAATGCCCTTC
RsYUC9 R	GCAAATATACTCCATCTCCTCTCCG
RsGAPDH F	TCTCTTCGGTGAGAAGCCAGTCA
RsGAPDH R	TCAAGTGAGCAGCAGCCTTGTC
RsUBQ F	ACTTGGTCCTCAGGCTTCGTGGT
RsUBQ R	AAAGATCAACCTCTGCTGGTCCG
RsCYCU2-2 F	TTCTGTTCTCGGTGGGGTTC
RsCYCU2-2 R	GCTGGTGGTGGTGATGATGTT
RsXero1 F	GGTGGTGGTGGTTTGAGTGG
RsXero1 R	TGATGCCTTTCTTCTCGTGGT
RsDC-8 F	CGAGGCAGGAGAAGAAGCA
RsDC-8 R	TCCCTAACGCACCCCTTACC
RERF11 F	GAGACCCGACCAAGAAAACC
RsERF11 R	CGAATCAGCAGAGGAGAAACATC
RsOLE F	TGGCTATGGTGGTGGTTATGG
RsOLE R	CGGGCTGAAGATCAGGAAAA
RsWRKY40 F	ACGACAACTGCTTTGGTGGA
RsWRKY40 R	TTTCTTGGGAGGACTGACTTGG
RsBGR F	ACCACGGGAAACAACACGAA
RsBGR R	TCCTCCATAACTCGGCTCTCAC
RsP8B6 F	AGGTGGGACTGGAGGCAAA
RsP8B6 R	CTCGGCGTCTTCCTTGTCAG
RsMYC2 F	CAAAGGAAAGCCCAAACAGAGA
RsMYC2 R	TACCCGCCAACCCAGCAC
RsDEF206 F	GCTTCACTATTCTCGTGCTTGTCC
RsDEF206 R	GGAGGAGTTGGGTATTTGGCTTT
RsDOT1 F	CTGGCATCGGCATAGGTCTC
RsDOT1 R	CACCACCACCTCCTCTTCCA
RsCCT1 F	AATGGCGTGGACAAGTAGGG
RsCCT1 R	CGTAGGCAAAGGGCAAGAGA

RsPLP2 F	GCTGGTGATGTTCAGTGGATG
RsPLP2 R	CCGTTTTCTTCTGCTTTTGGT
RsRDL5 F	CGTTGGTTATCGGCTTGGTT
RsRDL5 R	GTCACTGCTCCTTCCTTCCTC
RsPROF1 F	ATCTCCTGTGCGATGTGGAA
RsPROF1 R	TCCCTTTTTGCCTCTGATGAC
RsSRC2 F	CAACGGCAAGACCAAAGGA
RsSRC2 R	CCTGGAAGAGGCGGGTATG
RsC4H F	CGTCTCTTCACCCAATCTCACC
RsC4H R	ACTCCCACCCTTCCCTGTTC

Appendix 2. Genetics constructs obtained in the paper.

DNA to be inserted into the vector	Entry vector	Destination vector	Resulted construction	Use of vector construction
RsWOX4_cds	pDONR221	pB7WG2D	pB7WG2D-RsWOX4_cds	Investigation of the effect of *RsWOX4* overexpression on root development
RsWOX4_part_of_cds		pH7GWIWG2	pH7GWIWG2-RsWOX4_part_of_cds	Study of the effect of *RsWOX4* RNA interference on root development
RsWOX14_cds		pB7WG2D	pB7WG2D-RsWOX4_cds	Study of the effect of *RsWOX14* overexpression on root development
HD_WOX4	pDONR207	pDEST22	HD_WOX4-pDEST22	Study of TF-DNA interactions using a yeast one-hybrid system
pCLE41		pHisLeu	pCLE41-pHisLeu	
pCLE42			pCLE42-pHisLeu	
pLOG3			pLOG3-pHisLeu	
pRR18			pRR18-pHisLeu	
pYUC7			pYUC7-pHisLeu	