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Abstract

The *FW2.2* gene is associated with the major Quantitative Trait Locus (QTL) governing fruit size in tomato, and acts by negatively controlling cell division during fruit development. *FW2.2* belongs to a multigene family named the *CELL NUMBER REGULATOR* (*CNR*) family. The CNR proteins harbour the uncharacterized PLAC8 motif made of two conserved cysteine-rich domains separated by a variable region that are predicted to be transmembrane segments, and indeed FW2.2 localizes to the plasma membrane. Although *FW2.2* was cloned more than two decades ago, the molecular mechanisms of FW2.2 action remain unknown. Especially, how FW2.2 functions to regulate cell cycle and fruit growth, and thus fruit size, is yet not understood. We here review the current knowledge on PLAC8-containing CNR/FWL proteins in plants, which are described to participate in plant organogenesis and the regulation of organ size, especially in fruits, and in Cadmium resistance, ion homeostasis and/or Ca2+ signalling. Within the plasma membrane, FW2.2 and some CNR/FWL are localized in microdomains, which is supported by recent data from interactomics studies. Hence FW2.2 and CNR/FWL could be involved in a transport function of signalling molecules across membranes, thus influencing organ growth via a cell-to-cell trafficking mechanism.

Keywords

Cell cycle, CELL NUMBER REGULATOR, fruit, FW2.2, growth, microdomains, PLAC8 domain, plasma membrane, tomato

Introduction

Plant growth and development rely on fundamental cellular processes such as cell division, cell expansion and cell differentiation which impact on plant yield and consequently on the quality of plant products. While cell divisions set the number of cells inside a developing organ, cell expansion determines its final size. However, how are cell division and cell expansion spatially and temporally coordinated to control organ growth is still an intensive matter of investigation in plants. Compared to the leaf or root model (Nelissen *et al*., 2016; Motte *et al*., 2019), fruit organogenesis received much less attention, despite an important inference in human nutrition and economic issues. Developmental studies were mostly devoted to ovary formation, fruit set and fruit maturation, while fruit growth fell behind (Gillaspy *et al*., 1993).

The fruit is a specialized organ specific to Angiosperms flowering plants, which results from the development of the ovary after successful flower pollination and fertilization, and provides a suitable environment to fulfill ovule and seed protection during embryo development, and ultimately seed dispersal after maturation (Seymour *et al.*, 2013). This fundamental developmental function contributed to the evolutive success of Angiosperms, and allowed the emergence of a wide diversity of fruit size, form and composition, and of seed and fruit dispersion mechanisms, in response to selective and adaptive pressure (Seymour *et al.*, 2013). In addition, many important traits such as plant and inflorescence architecture, fruit size, fruit weight and shape were modified dramatically following human domestication. This is particularly remarkable within the Solanaceae family, which encompasses nearly 10,000 species, and especially within the same species such as tomato (Knapp, 2002; Périlleux *et al*., 2014; Van der Knaap and Østergaard, 2017).

Tomato (*Solanum lycopersicum* Mill.) was domesticated in central and south America and presents a large diversity in fruit size and shape (Blanca *et al.*, 2015). This large morphological diversity encountered among domesticated tomato varieties and the genetic and genomic tools developed the last 20 years made of tomato a model plant to study the development of fleshy fruits, and especially to unveil the genetic basis of fruit size. Fruit growth in tomato first proceeds from a period of intense mitotic activity according to a spatially- and temporally organized pattern of cell division that sets the number of cells within the whole organ (Cheniclet *et al.*, 2005; Renaudin *et al.*, 2017). Concomitant to cell division, the volume of cells expands as to reach a 30,000-fold increase from initial cell volume, and this cell expansion period lasts for the entire period of fruit growth until ripening, accounting for the final size of the fruit. Remarkably, this spectacular cell hypertrophy is closely correlated to an increase in nuclear DNA levels due to endoreduplication where DNA synthesis occurs independently from mitosis (Edgar and Orr-Weaver, 2001; Chevalier *et al.*, 2014).

Among the approaches undertaken to study the genetic basis of fruit size, marker-assisted mapping studies have allowed the identification of several quantitative trait loci (QTL)/genes encoding regulators of fruit size. Nearly 30 QTLs related to fruit size were identified from the analysis of crosses between small and round wild tomatoes with domesticated varieties of various sizes and shapes (Grandillo *et al.*, 1999; Causse *et al.*, 2007; Rodriguez *et al.*, 2011). Among these 30 QTLs, several exert a major influence on fruit size such as *fw1.1* (fw for fruit weight), *fw2.2*, *fw3.1*, *fw3.2*, *fw4.1*, *fw9.1* and *fw11.3* (Grandillo *et al.*, 1999). Up to now, only three genes underlying such QTLs were cloned in tomato: *fw2.2*, *fw3.2* and *fw11.3*, with yet undisclosed clear function for the respective encoded protein (Frary *et al.*, 2000; Chakrabarti *et al.*, 2013; Mu *et al.*, 2017).

fw2.2 is by far the major QTL governing fruit size in tomato (Grandillo *et al.*, 1999). The *FW2.2* gene associated to the *fw2.2* QTL encodes a 22 KDa protein, which acts as a negative regulator of cell divisions (Alpert and Tanksley, 1996; Frary *et al.*, 2000). Because this function seems to be conserved not only in fruits, but also in leguminous and cereal species, orthologous proteins of FW2.2 have been subsequently named CELL NUMBER REGULATOR (CNR) (Guo *et al.*, 2010) (also referred to as FW2.2-Like or FWL in the literature). We will hereafter refer to CNR/FWL in this review. Despite numerous studies, the true function of FW2.2 and CNR/FWL stays elusive, and the precise biological function and mechanism of action in controlling cell divisions remains totally unknown.

The objective of the present review is to explore the knowledge acquired on FW2.2 and its homologs in tomato and other species, and to discuss recent advances in the characterization of these proteins, in order to help at elucidating their cellular and molecular function.

FW2.2, the major gene controlling fruit weight, encodes a negative regulator of cell division

fw2.2, as the major QTL involved in determining fruit weight, appeared to be responsible for up to 30% of the fruit size variation that resulted from the evolution of tomato between the wild ancestors bearing small fruits and the domesticated tomato (*Solanum lycopersicum*) (Alpert *et al.*, 1995; Alpert and Tanksley, 1996; Frary *et al.*, 2000). Indeed all wild tomato species, such as *Solanum pennellii* or *Solanum pimpinellifolium*, possess a “small fruit allele” at the *fw2.2* locus, while modern tomato species, characterized by enlarged fruits, contain a “large fruit allele” (Alpert *et al.*, 1995). Interestingly, the analysis of the genome sequences of 360 accessions (both wild and cultivated ones) demonstrated that *FW2.2* is rather related to improvement than to domestication (Lin *et al.*, 2014). However, in another study using 1000 accessions, Blanca *et al*. (2015) showed that the frequency of *fw2.2* derived alleles is very low in the tomato ancestor species *S. pimpinellifolium.* The

fw2.2 derived alleles start to spread in the "domesticated" *S. lycopersicum* cerasiforme accessions, and then are fixed in improved large-fruited *S. lycopersicum* accessions. This difference was not noticed in Lin *et al*. (2014). Thus, whether *FW2.2* is related to improvement or domestication of tomato is still a matter of debate. *FW2.2* is definitely not the only gene that contributed to domestication, but it did participate in it with other QTLs, especially because of its strong effect on fruit weight when compared to other QTLs.

A map-based approach allowed the molecular cloning of the associated gene at the *fw2.2* locus (Alpert *et al.*, 1995; Alpert and Tanksley, 1996; Frary *et al.*, 2000). The *FW2.2* gene was found primarily to be expressed in all pre-anthesis floral organs, with higher levels in carpels (Frary *et al.*, 2000). Using nearly isogenic lines (NILs) containing either the large- or small-fruit allele at the *fw2.2* locus, Cong *et al.* (2002) showed that the allelic effects on fruit size were due to different dynamics of transcript accumulation, according to a heterochronic allelic variation in expression and the overall quantity of transcripts. Transcripts from the large-fruit allele accumulate rapidly to reach a peak of expression around 5 days-post-anthesis (DPA), whereas those from the small-fruit allele accumulate more slowly and reach their maximum level of expression nearly a week later (12 DPA) (**Figure 1**). Interestingly, the difference in timing of *FW2.2* expression is inversely correlated to the mitotic activity in the developing fruit (Cong *et al.*, 2002). In addition, no change in cell size within the pericarp and the placenta at any developmental stages can be observed (**Figure 1**) (Cong and Tanksley, 2006), thus suggesting that the amount of cell divisions, rather than the extent of cell expansion, causes the fruit size variation. To test the effect of the overall quantity of transcripts, Liu *et al.* (2003) modified the range of steady-state transcript levels of *FW2.2*, via a transgenic experiment aimed at increasing the number of copies of the "small fruit" allele driven by its own promoter (from zero to four copies) inside the "large fruit allele" background. The increased level of transcripts according to the number of "small fruit" allele copies was indeed negatively correlated with fruit weight. However, since the number of cell layers across the fruit pericarp and therefore the pericarp thickness were not affected, it was concluded that FW2.2 influences fruit growth in a two-dimensional manner through the regulation of both transversely and longitudinally anticlinal cell divisions. Recently, tissue-specific transcriptomic analyses showed that *FW2.2* is transcribed specifically in the epidermis and sub-epidermis of tomato fruit (Shinozaki *et al.*, 2018) where cell divisions occur to generate new cells through periclinal and mostly anticlinal divisions which account for fruit growth (Renaudin *et al.*, 2017).

The allelic effect of *FW2.2* on cell divisions is thus due to a variation in gene expression, and not to a difference in the protein structure. The sequence comparison of the "small fruit" and "large fruit" alleles of *FW2.2* did not reveal any significant difference: four silent Single-Nucleotide

Polymorphisms (SNP) were found in the coding region, and three SNPs cause amino acid changes in the very N-terminal part of the protein sequence. However these SNPs are supposed to be non-synonymous mutations, because two of them were found only in *Solanum pennellii* and the last one is shared among all *Solanum* species, even in the "small fruit" species such as *Solanum pimpinellifolium* (Frary *et al.*, 2000, Nesbitt and Tanksley, 2002). The *fw2.2* phenotype is thus most likely due to one or more SNPs upstream the coding sequence, in the promoter region of the allelic sequences.

The FW2.2 protein thus acts as a negative regulator of cell division activity in pre-anthesis ovary and young developing fruit in tomato, thus modulating the number of cells inside the ovary, and consequently the final fruit size (Frary *et al.*, 2000; Cong *et al.*, 2002; Liu *et al.*, 2003; Nesbitt and Tanksley, 2001). As proposed by Blanca *et al.* (2015), the mutation at the *fw2.2* locus could represent one of the earliest known mutation responsible for a key transition during domestication and improvement of tomato.

FW2.2 and CNR/FWL are transmembrane proteins containing a PLAC8 domain

The function of FW2.2 in the control of fruit size/weight and its inferred impact on fruit quality, relates directly to plant yield and biomass. Therefore, orthologs of FW2.2 have been identified with the objective to search for potential targets to increase agronomical traits in various crop plants. In addition, the analysis of the protein structure of CNR/FWL may help decipher their still unravelled biochemical and physiological function.

FW2.2 and its *CNR/FWL* homologs belong to a complex multigenic gene family. The principal feature of the FW2.2 and CNR/FWL protein sequence is the presence of a PLAC8 (Placenta-specific gene 8 protein) domain originally identified in mammalian placenta proteins (Galaviz-Hernandez *et al.*, 2003). The PLAC8 motif is present in hundreds of proteins in plants, animals and fungi, ranging in size from 108 to 557 amino acids (Guo *et al.*, 2010; Song *et al.*, 2011). In a recent survey, Thibivilliers *et al.* (2020) restricted the plant CNR/FWL family to 134 members across 13 different species belonging to various phyla of the plant kingdom. The PLAC8 domain is composed of one or two hydrophobic segments, predicted to form transmembrane (TM) helices (Song *et al.*, 2004). These hydrophobic segments are composed of cysteine-rich motifs of the type CLXXXXCPC, CCXXXXCPC, CLXXXXFPC or CCXXXXCGPC, separated by a variable region and residing at the N-terminus part of a first transmembrane domain (Cabreira-Cagliari *et al.*, 2018; Guo *et al.*, 2010; Song *et al.*, 2004). In addition, the PLAC8 domain of plant proteins is characterized by the presence of a second conserved motif, the "QXXRELK" motif, at the C-terminal part of the cytosolic domain (Song *et al.*, 2004). As far as tomato is concerned, eleven paralogous proteins to FW2.2 have been identified (Thibivilliers *et*

al., 2020) (**Table I**; **Figure 2**). These 11 SlFW2.2-like (SlFWL) proteins display at least 35% of amino acid sequence identity with FW2.2. The size of the proteins ranges from 144 to 314 amino acids, with the exception of SlFWL4, which is much longer (414 amino acids) due to the presence of an extended N-terminal part compared to other SlFWLs (**Figure 2A**). A more detailed analysis of the primary structure of SlFWL proteins shows that the CLXXXXCPC motif within the PLAC8 domain somehow diverges among the SlFWLs: instead of CC or CL at the beginning of this motif, the amino acids AV, AL or AA are found in SlFWL4, -5 and -6 (**Figure 2B**). Such a diversity is also observed for the second QXXRELK motif of the PLAC8 domain, especially in SlFWL4, -5, -6, -7 and -8 (**Figure 2B**).

Originally, two peptidic regions with high hydrophobicity were predicted to be membrane-spanning α-helices enriched in Cys and Pro residues, at positions 43-64 and 75-94 of the FW2.2 amino acid sequence (Cong and Tanksley, 2006). Using a dedicated software to predict TM helices (https://prabi.ibcp.fr/htm/site/web/services/multipleAlignment), the presence of TM domains was confirmed in SlFWLs (**Figure 2B**), as obtained for other CNR/FWL in various species (Guo *et al.*, 2010; Song *et al.*, 2004; Xu *et al.*, 2013). The presence of these TM domains does confer a localization at the plasma membrane for FW2.2 tagged with GREEN FLUORESCENT PROTEIN (GFP) when transiently expressed in tomato young leaf epidermal cells (Cong and Tanksley, 2006). The plasma membrane localization was observed as well for FW2.2 orthologs from soybean in tobacco epidermal cells (GmFWL1; Libault *et al.*, 2010), from *Physalis floridana* in tobacco protoplasts (PfCNR1; Li and He, 2015) or OsFWL1, -2, -3, -6 in rice protoplasts (Xu *et al.*, 2013). The Arabidopsis homologs of FW2.2 containing a PLAC8-domain, called AtPCRs (see below), also display the presence of two membrane-spanning helices and localize as well to the membrane of mesophyll cells (Song *et al.*, 2004).

A central question then arises: how does a membrane-imbedded protein, like FW2.2, function in controlling fruit size via cell proliferation regulation? To address this question, genetic, molecular and functional analyses of CNR/FWL from different plant species may provide some insights.

FW2.2 and its homologs play a part in plant organogenesis and the regulation of organ size.

As identified by Thibivilliers *et al.* (2020), the CNR/FWL family in tomato is made up of twelve members, including FW2.2. Unfortunately, there is no functional analysis yet available for *FW2.2* and the *SlFWLs.* So far, the sole reported example in the literature comes from a recent study aimed at combining agronomically desirable traits in the wild tomato *Solanum pimpinellifolium* via the use of CRISPR-Cas9 genome editing strategy (Zsögön *et al.*, 2018). Deletion mutant lines of *FW2.2* were generated, but unfortunately these lines were not affected for fruit size. The absence of noticeable phenotype could be due to the conservation of the PLAC8 domain in the truncated proteins,

suggesting that FW2.2 may well be still functional. However, the absence of phenotype could also originate from the specific *S. pimpinellifolium* genetic background.

As a first approach to characterize the *FW2.2* paralogous genes in tomato, the genomic location of *SlFWL* genes was compared to the location of identified QTLs for fruit weight that were described in the literature. The co-localization of *SlFWL* genes within the domestication and improvement selective sweeps defined by Lin *et al*. (2014) was thus searched (**Table I**). Among the eleven *SlFWL* genes, three are located in domestication sweeps and three in improvement sweeps. It is noteworthy that the location of three *SlFWLs* corresponds to two known QTLs : *fw3.1* for *SlFWL4* and *-6* ; *fw12.1* for *SlFWL10*. Whether these co-localizations do correspond to any quantitative effect, and whether these *SlFWL* genes contribute also to the regulation of fruit size, still need to be experimentally demonstrated.

So far, only the comparison of the *SlFWL* gene expression pattern with that of *FW2.2* may provide an information relative to their role during fruit development in tomato. Exploiting the available transcriptomic data in tomato revealed that *FW2.2* and *SlFWLs* are expressed both in reproductive and vegetative organs (**Figure 3**). *FW2.2* is not only expressed early during fruit development, but its transcripts also accumulate in roots even at a higher level than that in fruit. The majority of *SlFWLs* are expressed in roots, and transcripts for *SlFWL2*, *-3*, *-8*, *-9* and *-10* also accumulate significantly in leaf tissues. In reproductive organs, *SlFWL2, -3*, *-7* and *-8* are more specifically expressed in flowers. When compared to *FW2.2* (Cong *et al.*, 2002), most of *SlFWL* genes do not display the same specific timing of expression in fruits. Most of the *SlFWLs* are expressed at high levels throughout fruit development, such as *SlFWL7* and *-10* (**Figure 3**). *SlFWL3, -4* and *-5* are expressed as early as at anthesis; after anthesis and up to 6 DPA, their level of transcripts decreases unlike *FW2.2*, and then resumes to accumulate later on during the cell expansion phase of fruit development. Amongst all *SlFWLs*, *SlFWL2* displays a unique profile of expression with a high transcript level at anthesis, a low level during the cell division phase and the onset of the cell expansion, and a strong increase later on before ripening. The significance of these differential patterns of expression is still unknown.

The function in controlling cell proliferation of many CNR/FWL is conserved in both dicotyledon and monocotyledon plants. Indeed, genetics analyses in several fruit species have shown that CNR/FWL proteins co-localize with strong QTLs governing fruit weight, such as in papaya (Blas *et al.*, 2012), grapevine (Doligez *et al.*, 2013), cherry (De Franceschi *et al.*, 2013), peach (Cao *et al.*, 2016), cucumber (Colle *et al.*, 2017), and in the Solanaceae tomato-related species eggplant (Doganlar *et al.*, 2002). However, a CNR/FWL homolog in pepper, another Solanaceae species highly related to tomato, does not display any major effect in controlling fruit size variation (Zygier *et al.*,

2005), most probably because the pepper fruit is devoid of a developed placenta, while *FW2.2* in tomato is highly expressed in this tissue (Cong *et al.*, 2002) (**Figure 3**). The isolation of *CNR/FWL* orthologs in avocado (*Persea americana*; Dahan *et al.*, 2010), pear (*Pyrus spp*; Tian *et al.*, 2016) and *Physalis floridana* (Li and He, 2015), highlighted that the heterochronic gene expression and transcript accumulation during fruit development are negatively correlated to fruit size, indicating a conserved role in the negative control of cell proliferation as observed in tomato. Moreover, a functional analysis using transgenic plants aimed at over-expressing or down-regulating a *CNR/FWL* ortholog in *Physalis floridana* (*PfCNR1*; Li and He, 2016) showed evidence that multiple organ sizes as well as seed production could be negatively controlled by PfCNR1, by altering significantly the cell number.

The CNR/FWL function is not only conserved in fleshy fruits, but also in cereal and leguminous species, suggesting the existence of a common genetic control of organ size via the regulation of cell number in plants. The overexpression of the maize (*Zea mays*) *ZmCNR1* gene leads to a reduction in organ size and the overall plant stature, while the down-regulation produces opposite effects (Guo *et al.*, 2010). In rice (*Oryza sativa*), the phenotype of T-DNA insertion mutants of the *CNR/FWL OsFWL3 and OsFWL5* is characterized by an increase in grain width and plant height, respectively (Xu *et al.*, 2013). In a recent study aimed at silencing rice *CNR/FWL* by CRISPR/Cas9 targeted mutagenesis, *OsFWL4* was shown to regulate negatively the number of tillers, grain length and plant yield (Gao *et al*. 2020). In both maize and rice, these modifications in organ and plant size originate from the alteration in cell number. Recently, Ruan *et al*. (2020) identified a new semi-dominant quantitative trait locus for grain width and weight, named qTGW2. Using a MAP-based cloning strategy, the corresponding gene *TGW2* was identified as being the rice *CNR/FWL* ortholog *CELL NUMBER REGULATOR 1* (*OsCNR1*). OsCNR1 localizes to the plasma membrane, and is responsible for the observed alteration in grain width and weight by restraining cell proliferation and expansion in glumes (Ruan *et al*., 2020). In soybean (*Glycine max*), the homolog of the tomato *FW2.2* gene, named *GmFWL1*, is highly expressed in root hair cells and nodules in response to inoculation with the nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* (Libault *et al.*, 2010). In addition, the silencing of *GmFWL1* using a RNAi strategy resulted in a significant reduction in the number of nodules, indicating that GmFWL1 is essential for nodule organogenesis, with a putative conserved function in regulating negatively cell division during this process.

Altogether, these results suggest that CNR/FWL proteins behave as cell division regulators across diverse plant species and in multiple organs. However, the true function of FW2.2 and CNR/FWLs stays elusive, and their precise biological function and mechanism of action in controlling cell divisions remains undeciphered.

The PLAC8 domain may confer a function in Cadmium resistance, ion homeostasis and/or Ca^{2+} signalling

The closest homologs to *FW2.2* in Arabidopsis have been named *AtPCR* for *PLANT CADMIUM RESISTANCE* genes, as they were isolated from a complementation screen of a yeast cadmium-sensitive mutant with an Arabidopsis cDNA library (Song *et al.*, 2004). Within this gene family, AtPCR1 improves plant cadmium tolerance by exporting cadmium throughout the plasma membrane (Song *et al.*, 2004). Interestingly, the CCXXXXCPC motif of the PLAC8 domain is essential to confer cadmium resistance, since when deleted or modified at the CC and CPC residues, the cadmium resistance is lost (Song *et al.*, 2004). In accordance with this functional role of the CCXXXXCPC motif, AtPCR8, which displays an AVXXXXVPC motif, does not confer cadmium resistance to yeast cells. A second member of the PCR family, AtPCR2, also confers cadmium tolerance when expressed in yeast cells. However, AtPCR2 was shown to participate in the detoxification of zinc in the presence of high concentrations of zinc and the transfer of zinc from the root to the shoot (Song *et al.*, 2010). As AtPCR2 is expressed in the xylem vascular tissue and in the root epidermis, its functions as a zinc ion efflux transporter was found essential for the plant optimal growth. Interestingly, this transporter function may require the oligomerization of the protein across the membrane, since AtPCR2 can form homo-oligomers when expressed in yeast cells. Whether this property of CNR/FWL proteins and FW2.2 in particular, also occurs in plant cells, was barely investigated, and only reports dealing with rice CNR/FWLs seem to indicate this may be the case (see below).

This metal ion transporter function was also reported in other plant species. The expression in yeast of several rice *CNR/FWL* genes, namely *OsFWL3-7,* confers resistance to Cd (Song *et al*., 2015; Xiong *et al*., 2018), with the strongest effect observed in cells expressing *OsFWL4*. Xiong *et al*. (2018) demonstrated that OsFWL4 is involved in Cd translocation from roots to shoots *in planta*, and that this function as a transporter may require the homo-oligomerization of OsFWL4s. As mentioned before, T-DNA insertion mutants of *OsFWL3* and *OsFWL5* are characterized by increased grain size and plant height, respectively (Xu *et al*. 2013), thus providing examples of CNR/FWL proteins affecting both organ size and metal ion homeostasis. However, this relationship does not appear as clear-cut, since the overexpression of *OsFWL5* (also called *OsPCR1***;** Song *et al*. 2015) increases grain weight, and conversely knockout and knockdown lines produced lighter grains than the wild type. Hence, this phenotype appears the opposite of one might expect from orthologs of the tomato FW2.2 or maize and other rice CNR/FWLs, *i.e*. a reduction in organ size due to an alteration in the number of cells. Moreover, while OsFWL5 enhances Cd resistance and reduces Cd concentration

when heterologously expressed in yeast cells, it confers an inverse sensitivity to zinc, suggesting it may also function as a Zn^{2+} influx transporter (Song *et al*., 2015). In wheat (*Triticum aestivum*), the TaCNR2 protein enhances stress tolerance to cadmium, zinc and manganese when overexpressed in Arabidopsis, and improves the translocation of these cations from roots to shoots when overexpressed in rice (Qiao *et al.*, 2019).

The MID COMPLEMENTING ACTIVITY or MCA proteins are plasma membrane proteins that correlate Ca^{2+} influx with mechanosensing in *Arabidopsis*, and play a part in Ca^{2+} uptake in roots (Nakagawa *et al.*, 2007). These proteins contain a C-terminal PLAC8 domain harbouring two membrane-spanning helices, and only a motif of the LCFXXXXFPC or LCLXXXXFPC type. In maize, *ZmCNR13* was identified as the underlying mutated gene in the *narrow odd dwarf* (*nod*) mutant (Rosa *et al.*, 2017). *ZmCNR13* belongs to the *MCA* family and as such, it was shown to rescue partially the lethal phenotype of the *mid1* mutant from yeast, which lacks a component of a Calcium-permeable channel inducing a calcium uptake deficiency. Similar to the *Osfwl5* mutant, the prominent phenotype in the *nod* mutant induced by the *ZmCNR13* knock-out is an overall reduction in both vegetative and reproduction organ size, thus adding some more confusion in the discovery of the CNR/FWL function.

The above-mentioned examples suggest that the presence of a PLAC8 domain relates with a putative function in the regulation of metal ion homeostasis. However, any relationship with the control of organ size via cell cycle/cell proliferation control is far from being obvious as opposite effects can be recorded. Could it be that the effect on organ size is an indirect consequence of metal ion accumulation or deficiency, or of deregulating Ca^{2+} signalling such as in the case of ZmCNR13? More investigations and functional analyses are required to answer this question. It is noteworthy that *SUN*, a gene involved in the control of elongated-fruit shape in tomato through changes in cell number, may provide such an example. Indeed, Clevenger *et al*. (2015) reported that the main node in the *SUN* gene regulatory network represents genes involved in calcium-regulated processes. Since SUN is characterized by the presence of a calmodulin-binding domain, it is thought to impact fruit growth, and in this case fruit shape, through a calcium signalling cascade.

Can interactomics studies provide advances in the understanding of the mode of action of FW2.2 and its orthologs?

Investigating what is the protein environment of CNR/FWLs is a key element in elucidating their function. This is especially relevant to answer the central question of how transmembrane-localized proteins like FW2.2 and CNR/FWLs regulate the cell cycle and thus cell proliferation, to control fruit/organ size ultimately.

Cong and Tanksley (2006) provided the first results of protein-protein interaction using yeast two-hybrid assays, indicating that FW2.2 can interact with CKIIβ1, a Casein Kinase putatively implicated in cell cycle regulation. This particular interaction was found also for the *Physalis floridana* PfCNR1 protein in yeast cells, but failed to be reproduced in plant cells using Bimolecular Fluorescent Complementation (BiFC) assays (Li and He, 2015). Similarly, these authors showed that PfCNR1 can interact in yeast cells with PfAG2, an AGAMOUS-like MADS-domain regulatory protein which regulates the expression of PfCYCD2;1, a key component involved in the G1/S checkpoint control of the cell cycle. Again, the interaction between PfCNR1 and PfAG2 was not confirmed in plant cells. Arguing that the failure to detect a YFP signal may originate from the non-overlapping subcellular localization of PfCNR1 and its putative interacting proteins, Li and He (2015) used truncated versions of PfCNR1, and demonstrated that the intracellular portion of PfCNR1 (referred to as *PfCNR1*62–78) was able to interact either with PfAG2 or PfCKIIβ1 in plant cells. However, the relevance of these interactions can be questioned, since the full-length protein was not used as a prey, and therefore the proper conformation and structure of the protein in its membranous environment were not respected. So far, the interaction of FW2.2 or its orthologs with CKIIβ1, and a putative CKIIβ1-mediated mechanism of action to explain the effects on cell proliferation still remain unconfirmed to date.

GmFWL1, which is involved in soybean nodule organogenesis, was localised at the plasma membrane (Libault *et al.*, 2010) and more specifically at membrane microdomains according to a punctate localization pattern (Qiao *et al.*, 2017). As a plasma membrane microdomain-associated protein, GmFWL1 was found to interact with specific-protein markers of microdomains, such as remorin, prohibitins and flotillins, using co-immunoprecipitation assays. More precisely, the interaction between GmFWL1 and GmFLOT2/4 was reported as an important feature during legume nodulation in response to rhizobia infection: GmFWL1 and the microdomain-associated proteins may likely participate in the endocytosis of bacteroids into the infected cells (Qiao *et al.*, 2017).

The association of FW2.2 and CNR/FWL proteins to the plasma membrane, and specifically to membrane microdomains as shown in soybean, may thus reflect important biological functions in the process of plant organogenesis. The plasma membrane is a very dynamic structure, which is involved in the perception and transduction of environmental signals, in response to biotic or abiotic stress, in actin cytoskeleton organization, in exocytosis and endocytosis, in membrane transport, and in cell-to-cell communication. All these mechanisms thus influence plant growth and acclimation to a changing environment. Membrane protein effectors associated to these biological functions have been shown to localize to membrane microdomains, and the concentration of specific membrane

components in microdomains accounts for their functional importance (Gronnier *et al*., 2018; Malinsky *et al*., 2013).

Using a split-ubiquitin yeast two-hybrid screen fit to identify membrane protein interactions, Jones *et al.* (2014) established a network of 12,102 interactions between 1523 membrane/signalling proteins from Arabidopsis. Among these interactions, the Arabidopsis closest homolog of FW2.2, AtPCR2, was found to interact with a leucine-rich repeat (LRR)-receptor-like kinase (RLK). This type of LRR-RLK belongs to the large RLK/Pelle kinase family of transmembrane receptors in plants, and is likely to participate in a mechanism of cell-to-cell communication across membranes (Gish and Clark, 2011).

As mentioned above, Ruan *et al*. (2020) identified *OsCNR1* as the gene associated to an important QTL for grain width and weight in rice. Remarkably, it was demonstrated that OsCNR1 interacts with OsKRP1, a specific Cyclin-Dependent Kinase inhibitor, which belongs to the Kip-Related Protein (KRP) family, known to play an important role in the exit from the mitotic cell cycle during rice grain formation (Barroco *et al*., 2006). This interaction revealed by BiFC was consistent with a distribution pattern of OsCNR1 and KRP1 in the cell membrane in *N. benthamiana* leaves and rice protoplasts (Ruan *et al*., 2020). OsCNR1 regulates grain size by restraining cell division and cell expansion, and similar results were obtained when KRP1 was overexpressed in rice (Barroco *et al*., 2006).

Therefore, the work from Ruan *et al*. (2020) provides the first evidence of a direct link between a CNR/FWL protein controlling organ size and a well-established cell cycle regulator inhibiting cell proliferation. It also raises the question whether this interaction is related to a transportation function across membranes. Interestingly, KRPs can act non-cell-autonomously and link decisions on a cellular level with the supracellular division and growth pattern as shown in Arabidopsis leaf epidermis (Weinl *et al.*, 2005). Thus the cell-to-cell movement of KRPs is thought to help arrest of cell proliferation, and one may wonder whether a CNR/FWL-mediated transport across membranes could be also involved in the mechanisms controlling organ growth via cell-to-cell trafficking, alike signalling molecules such as transcription factors (Kitagawa and Jackson, 2017).

Putative functions of PLAC8-domain-containing proteins in plants.

To summarize the available data from the literature, PLAC8 domain-containing proteins in plants fall into three major functional categories, from the least to the most characterized: (i) proteins involved in calcium uptake and signalling, (ii) metal ion homeostasis, and (iii) organ size regulation (**Figure 4**).

The function in calcium uptake seems restricted to a limited number of proteins, namely the MCA proteins showing a particular PLAC8 domain of the LCFXXXXFPC type, which is not present in the FW2.2 and CNR/FWL proteins. Hence this function may not be involved in the organ growth control according to the same mechanism exerted by FW2.2 and its orthologs.

Metal- and more generally speaking ion homeostasis is obviously of fundamental importance for the regulation of cellular processes controlling plant growth, and definitely organ- and fruit growth. This function was first reported for the PCR proteins from Arabidopsis, and then confirmed for some rice and wheat CNR/FWL proteins. For at least three rice proteins, *e.g.* OsFWL3, -4 and -5, the function in ion metal homeostasis was also associated to an effect on organ growth control. However how can metal ion transport control cell division remains unclear.

The vast majority of orthologous proteins to FW2.2 can be assigned a function in regulating organ growth, through the negative regulation of cell division. Comparative quantitative data from genetics analyses confirmed the co-localization of CNR/FWL genes with strong QTLs governing fruit weight in several fruit species, with grain size and plant height in rice and maize. Still, functional analyses of CNR/FWL genes remain scarce in the literature, but in some instances they allowed to demonstrate the effect on organ size determination in maize (ZmCNR1 and ZmCNR2) and rice (OsFWL4), and revealed an important role in a precise development process such as nodule organogenesis in response to rhizobium infection in the case of the soybean GmFWL1 gene.

Conclusion

The elucidation of FW2.2 and CNR/FWL biological function is thus still a challenging matter, but recent discoveries shed light on an exciting hypothesis to conciliate their specific localization at the site of the plasma membrane and microdomains. Whether cell-to-cell communication and/or the putative cell-to-cell movement of key cell cycle regulators such as KRPs, is involved in spatially enabling the control of cell division and ultimately influencing organ size and more precisely fruit size in tomato, is an exciting new subject of investigations.

Acknowledgements

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Author contributions

All authors conceptualized the review and contributed to the writing of the manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest

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Figure legends

Figure 1: Schematic representation of FW2.2 function as a negative regulator of cell division during tomato fruit development (adapted from Cong *et al*., 2002 and Liu *et al*., 2003)**.**

At the *fw2.2* locus, the large- or small-fruit allele effects on fruit size are due to a heterochronic allelic variation in expression and the overall quantity of transcripts. The allelic difference in timing of *FW2.2* expression affects the cell division rate in an inversely correlated manner, but does not induce any change in cell size within the fruit tissues during fruit development. Across the pericarp, the expression level of *FW2.2* does not affect the number of cell layers, and therefore the pericarp thickness. On the contrary the small-fruit allele expression regulates negatively the transversely and longitudinally anticlinal cell divisions in a two-dimensional manner thereby influencing the overall fruit growth (indicated by the double-head arrow). DPA: days post-anthesis.

Figure 2: Protein structure of FW2.2 and SlFWLs. (**A**) Schematic representation of FW2.2 and SlFWLs protein structure deduced from computational analysis of protein primary sequences, and designed by using the Mydomain prosite (Expasy) (https://prosite.expasy.org/mydomains). Transmembrane domains were predicted using the Prabi website (https://prabi.ibcp.fr/htm/site/web/services/multipleAlignment). The scale refers to the protein sizes (in amino acid residues). (**B**) Amino acid sequence comparison of the PLAC8 domain in FW2.2 and SlFWLs, using the BAR clustalW multiple alignment software (http://bar.utoronto.ca/ntools/cgi-bin/ntools_multiplealign_w_mview.cgi). TM: Transmembrane domain.

Figure 3: Fruit tissue- and developmental stage specific gene expression for *FW2.2* and *SlFWLs* in tomato. Transcriptomic data for *FW2.2* and *SlFWLs* in whole fruit and vegetative organs were collected from the BAR database (http://bar.utoronto.ca/efp_tomato/cgi-bin/efpWeb.cgi). Transcriptomic data for *FW2.2* and *SlFWLs* in fruit tissues were collected from the Tomato Expression Atlas (http://tea.solgenomics.net/) (Shinozaki *et al*., 2018). The gene expression values were transformed using log10 base as to provide a Heatmap representation using the ggplot package in R software.

Figure 4: Putative functions of PLAC8-domain-containing proteins in plants. Three distinct functions have been assigned in the literature to PLAC8-domain-containing proteins including the different CNR/FWL proteins: a function in the regulation of organ size, metal ion homeostasis and calcium uptake. The different proteins are grouped according to their assigned function(s) and the amino acid sequence of the first PLAC8 motif.

Table I. Genomic localization of *FW2.2* and the 11 *SlFWL* genes, and putative association to domestication or improvement trend according to Lin *et al*. (2014).

Gene name[a]	Gene ID.	Chr. nb	Within domestication sweep[b]	Within improvement sweep[b]	Sweep nb[b]	QTL overlapping[b]
FW2.2	Solyc02g090730	2	no	yes	IS033	*fw2.2*
SlFWL2	Solyc01g005470	1	no	no		
SlFWL4	Solyc03g119660	3	no	yes	IS056	*fw3.1*
SlFWL6	Solyc03g120600	3	no	yes	IS056	*fw3.1*
SlFWL3	Solyc04g007900	4	yes	no	DS050	
SlFWL1	Solyc05g009620	5	yes	no	DS056	
SlFWL5	Solyc06g066590	6	no	no		
SlFWL7	Solyc08g013910	8	no	no		
SlFWL8	Solyc08g013920	8	no	no		
SlFWL9	Solyc10g081410	10	no	no		
SlFWL10	Solyc12g013570	12	yes	no	DS178	*fw12.1*
SlFWL11	Solyc12g037950	12	no	no		

[a] Gene names are following the nomenclature proposed by Thibivilliers *et al*. (2020).

[b] The location of *SlFWL* genes in domestication sweeps and improvement sweeps defined in Lin *et al*. (2014) is reported, as well as its correspondence to already identified QTLs.

Figure 1

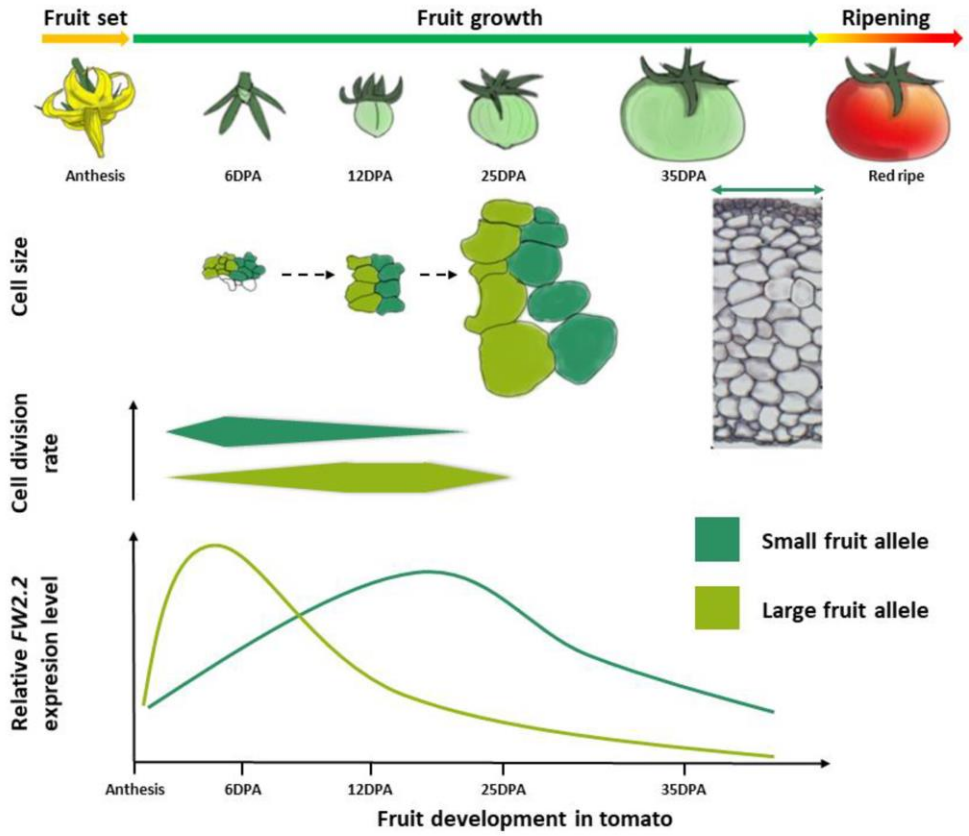

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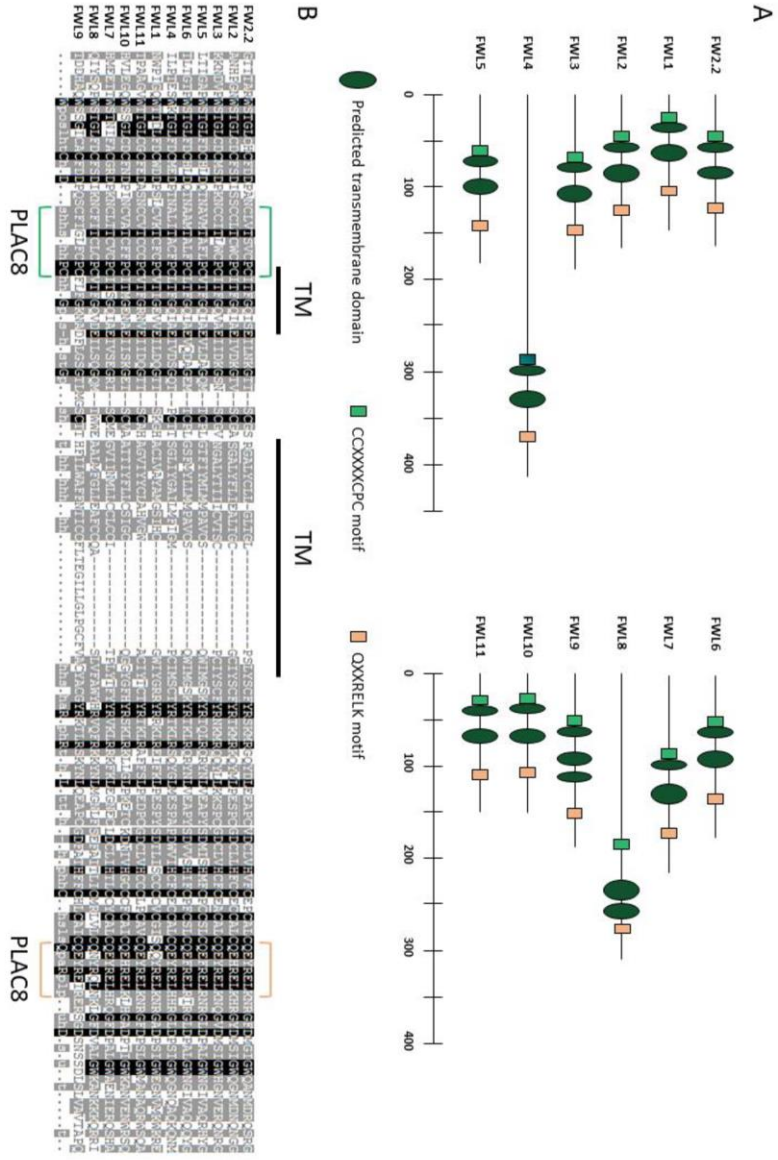
Figure 2



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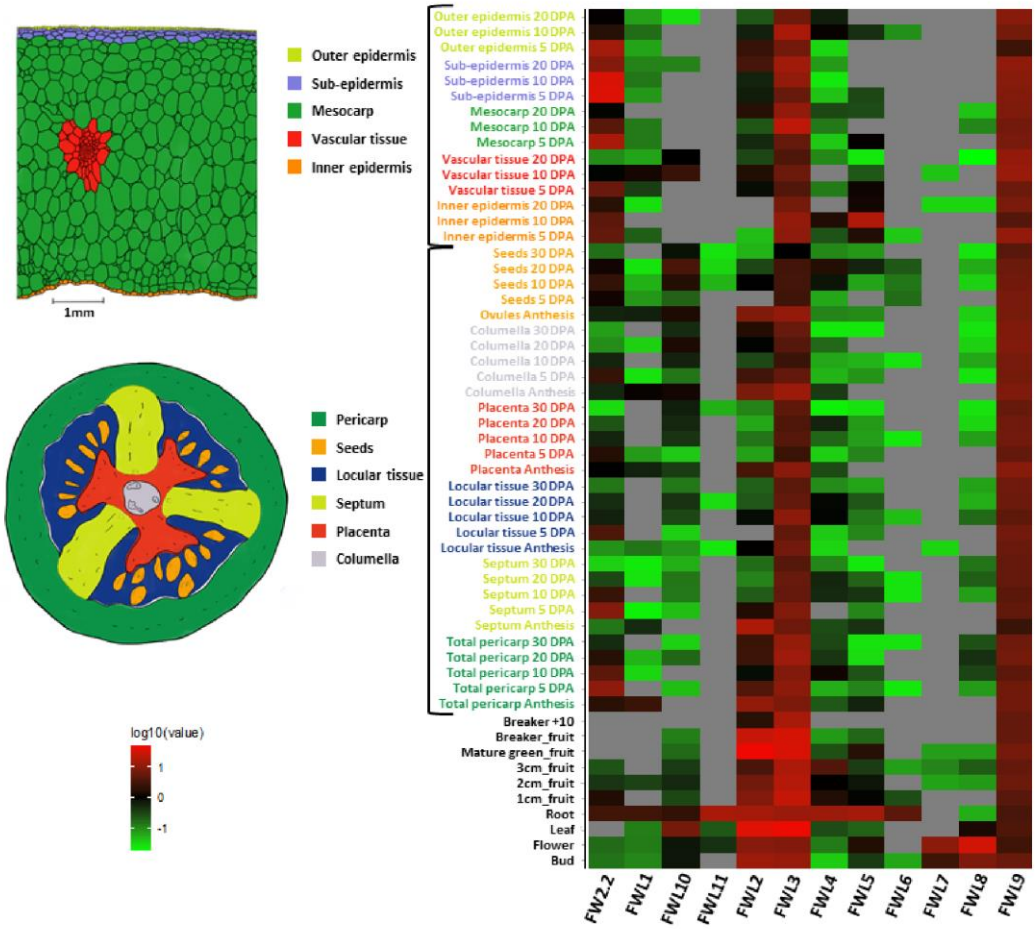

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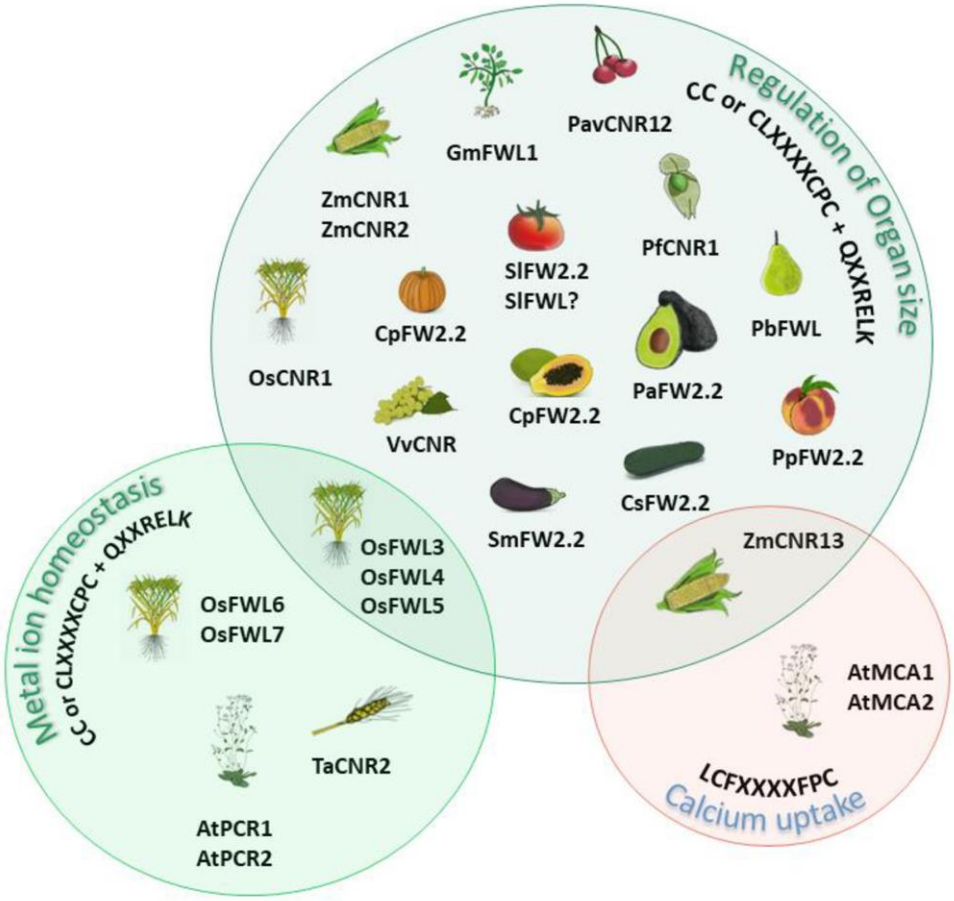
Figure 4



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