Review

Identification of the Causative Genes of Calcium Deficiency Disorders in Horticulture Crops: A Systematic Review

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Abstract: The occurrence of calcium (Ca^{2+}) deficiency disorders is a severe problem in the production of horticulture crops. Recently, several studies have investigated the role of gene expression in Ca^{2+} deficiency disorders and/or Ca^{2+} accumulation, providing an indication of the mechanism of Ca^{2+} deficiency disorders at the genetic level. To determine the relation between gene expression and the occurrence of Ca^{2+} deficiency disorders, we conducted a systematic review of the literature using the Preferred Reporting Items for Systematic Reviews and Meta-analyses protocol. In our initial search, we extracted studies investigating the relationships between Ca^{2+} deficiency disorders (tipburn and blossom-end rot) and gene expression. In our second search, we extracted studies involving functional analyses of the genes associated with Ca^{2+} deficiency and/or Ca^{2+} accumulation in plant organs. Thirty-seven articles were extracted from both searches. Studies on Ca^{2+} movement-related genes (Ca^{2+} antiporters, calreticulin, Ca^{2+} pumps, Ca^{2+} channels, and pectin methylesterases) accounted for the majority of these articles. Particularly, the effects of the expression of CAXs (Ca^{2+} / H^{+} antiporters) and CRT (calreticulin) on the occurrence of Ca^{2+} deficiency disorders were demonstrated in studies extracted from both searches. Further research focusing on these genes may reveal the causative genes for Ca deficiency disorders in different horticulture crops. We hope that the knowledge synthesized in this systematic review will contribute to the accumulation of further knowledge and elucidation of the causes of Ca^{2+} deficiency disorders.

Keywords: Ca^{2+} deficiency; gene expression; transcriptome; QTL; Ca^{2+} accumulation; Ca^{2+} antiporter; Ca^{2+} channel; Ca^{2+} pump; Ca^{2+} binding protein

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1. Introduction

Calcium (Ca^{2+}) is an essential plant macronutrient, mainly transported via the xylem in the transpiration stream [1]. However, its mobility from older plant organs to newly developed tissues is poor. Thus, Ca^{2+} deficiency disorders often occur in new leaves and fruits even when sufficient Ca^{2+} is supplied in the field [2,3]. The occurrence of Ca^{2+} deficiency disorders is a serious concern in horticulture crop production because it causes significant economic losses [4–6].

Two Ca^{2+} deficiency disorders, tipburn and blossom-end rot (BER), have been studied since the 1800s, and it is becoming clearer how environmental conditions (e.g., humidity [7,8], light [5,9], and air flow [10,11]) can mitigate the damage caused by these disorders. However, no environmental control techniques to eliminate the occurrence of Ca^{2+} deficiency disorders have been found, and farmers instead often force the selection and production of cultivars with high resistance to Ca^{2+} deficiency disorders.

In *Arabidopsis*, many Ca^{2+} transporter and binding protein-encoding genes have been identified [12–14], and their functions have been extensively demonstrated by functional analysis [15,16]. These findings have contributed to increased clarity in the understanding of plant Ca^{2+} signaling systems [17–19]. Moreover, an increasing number of studies have utilized transcriptome or QTL (quantitative trait locus) analysis to identify the causative

genes of Ca^{2+} deficiency disorders. Some studies have also performed functional analyses of the genes associated with Ca^{2+} deficiency [20,21].

To synthesize the current knowledge regarding the role of gene expression in the occurrence of Ca^{2+} deficiency disorders, we conducted a systematic literature review using Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) [22]. In this review, we focused on plants exhibiting symptoms of two Ca^{2+} deficiency disorders, tipburn and BER. We firstly extracted studies that investigated the relationship between gene expression and these disorders, and secondly studies in which a functional analysis of the genes associated with Ca^{2+} deficiency and/or Ca^{2+} accumulation in plant organs was conducted. The information synthesized in this review will contribute to future research on the causative genes of Ca^{2+} deficiency.

2. Methods

This systematic review was conducted following the PRISMA principles [22], and we referred to several other systematic reviews [23–26]. Web of Science and PubMed were selected as the scholarly online databases. The publication date of the articles was set from 1 January 1983 to 7 June 2021 (the date on which the last search was conducted). Firstly, to capture all empirical studies that used genetic analysis to identify the causative genes for Ca deficiency disorders, we used the following search terms with multiple combinations: “Tipburn” OR “blossom end rot” AND “gene expression” OR “transcriptome” OR “QTL.” We defined this search as Search 1. For our second search (defined as Search 2), to capture all studies involving the functional analysis of genes associated with Ca^{2+} deficiency and/or Ca^{2+} accumulation at the plant organ level, we used the following search terms: “transgenic plant” and “calcium deficiency”.

After document retrieval, we read the titles and abstracts of all the articles and removed duplicate articles. The full text of an article was reviewed if the title and abstract met the purpose of each literature search (see above for details). Only articles written in English and published as original articles were extracted. When we searched for studies that investigated the causative genes for Ca^{2+} deficiency disorders by conducting genetic analysis (Search 1), we focused only on two Ca^{2+} deficiency disorders, namely, tipburn and blossom-end rot (BER). Additionally, when extracting studies involving the functional analysis of genes associated with Ca^{2+} deficiency disorders and/or Ca^{2+} accumulation (Search 2), we considered only those that demonstrated clear results at the plant organ level (i.e., articles that demonstrated gene function associated with Ca accumulation at the cellular level were excluded).

3. Results and Discussion

3.1. Search 1: Studies Using Genetic Analysis to Identify the Causative Genes for Ca^{2+} Deficiency Disorders (Tipburn and BER)

A total of 64 articles were obtained through document retrieval (Figure 1). After removal of duplicate articles ($n = 23$) and review articles ($n = 4$), 37 articles were selected for full-text evaluation. Following the eligibility evaluation, 15 articles were determined as eligible for this systematic review. The 15 extracted articles were organized by plant materials and Ca^{2+} deficiency disorders (tipburn or BER), as shown in Table 1. Given below is a review of the experimental results for each deficiency disorder and plant type.

3.1.1. Tipburn in Lettuce

Four studies investigated the causative genes of tipburn in lettuce by conducting QTL and transcriptome analyses (Table 1). In 2013, Jenni et al. [27] conducted QTL analysis to determine the genetic basis of heat stress-induced physiological disorders (including tipburn incidence and severity) in lettuce. This study was the first to analyze the relevance of tipburn and its genetic basis in lettuce. A single QTL, *qTPB5.2* on LG (linkage group) 5b, was detected in multiple trials, and described 38–70% of the variation in tipburn incidence. Macias-González et al. [28] conducted multiple large-scale QTL analyses in different years

and growing environments. They reported two major QTLs for tipburn (including *qTPB5.2* on LG 5b), which determined up to 45% and 65% of the phenotypic variance, respectively. The latest research by the authors [29] identified 12 genes in *qTPB5.2* as candidate genes for tipburn resistance. The authors identified these candidate genes by dissecting the QTL (*qTPB5.2*) using multiple RIL (recombinant inbred line) populations. In addition, two of the 12 genes were found to encode proteins with a sequence similarity to Ca transporters.

In contrast, Seki et al. [30] conducted a QTL analysis to investigate the relationship between leaf shape and tipburn incidence. They used Empire-type (serrated leaf) cultivars and Salinas-type (wavy leaf) cultivars in the field cultivation test. The Empire-type cultivars showed late bolting and high tipburn susceptibility compared with the Salinas-type cultivars. Their research revealed that leaf shape and bolting time were controlled by a single locus in LG5. In addition, using further fine mapping and transcriptome analysis, the authors identified *LsTCP4* as a candidate gene for both traits (leaf shape and bolting time). Therefore, the authors suggested that the trait of tipburn incidence may also be controlled by *LsTCP4*.

Figure 1. Systematic review flow chart for Search 1.

Table 1. Overview of the studies using genetic analysis to identify the causative genes of Ca²⁺ deficiency disorders (tipburn and BER) obtained via Search 1.

Year	Author	Plant Materials	Ca ²⁺ Deficiency Disorder	Main Methods	Main Comparison	Causal Candidate Genes	Ref No.
2013	Jenni et al.	Lettuce (<i>Lactuca sativa</i> L.) leaves	Tipburn	QTL mapping	Empire type vs. Salinas type (Genetic mapping was conducted using 152 F ₇ RIL (recombinant inbred line) populations developed from an intra-specific cross of the Empire type × Salinas type)	<i>qTPB5.2</i> on LG 5b (largest tipburn QTL)	[27]
2019	Macias-González et al.	Lettuce (<i>Lactuca sativa</i> L.) leaves	Tipburn	QTL mapping	Seven RIL populations were analyzed in multiple environments and years.	Two major QTLs for tipburn incidence were identified in LG1 (<i>qTBIN_1_QC11</i>) and LG5 (<i>qTPB5.2</i>).	[28]
2021	Macias-González et al.	Lettuce (<i>Lactuca sativa</i> L.) leaves	Tipburn	QTL mapping RNA-seq	Eleven RIL population were analyzed in multiple environments and years.	12 candidate genes including Ca transporter genes in <i>qTPB5.2</i> .	[29]
2020	Seki et al.	Lettuce (<i>Lactuca sativa</i> L.) leaves	Tipburn	ddRAD-seq QTL mapping RNA-seq Real-time PCR	Empire type (serrated leaf) vs. Salinas type (wavy leaf) (Genetic mapping was conducted using F ₂ population derived from a cross between the Empire-type cultivar and Salinas-type cultivar.)	<i>LsTCP4</i> (Lsat_1_v5_gn_5_127021 locus)	[30]
2016	Su et al.	Chinese cabbage (<i>Brassica rapa</i> L. ssp. <i>pekinensis</i>) leaves	Tipburn	Real-time PCR	Tipburn-susceptible inbred line vs. tipburn-resistant inbred line	Ca ²⁺ transporter genes (e.g., <i>CAX1</i> orthologs (<i>Bra005131</i> and <i>Bra017134</i>))	[31]
2019	Su et al.	Chinese cabbage (<i>Brassica rapa</i> L. ssp. <i>pekinensis</i>) leaves	Tipburn	GWA mapping QTL mapping Functional analysis	Tipburn-susceptible plants vs. Tipburn-resistant plants (Genetic mapping was conducted using 194 divers inbred lines, a doubled haploid (DH100) population, and an F ₂ population)	<i>BraCRT2</i> (<i>Bra018575</i>)	[32]

Table 1. Cont.

Year	Author	Plant Materials	Ca ²⁺ Deficiency Disorder	Main Methods	Main Comparison	Causal Candidate Genes	Ref No.
2019	Wang et al.	Chinese cabbage (<i>Brassica rapa</i> L. ssp. <i>pekinensis</i>) leaves	Tipburn	circRNA sequencing qRT-PCR	Ca ₂₊ deficiency treatments (0, 3, and 6 day after treatment)	<i>ABF2</i> (Bra040260) et al. (23 and 22 circRNAs differentially expressed)	[33]
2013	Lee et al.	Cabbage (<i>Brassica oleracea</i>) leaves (apex, middle, and base)	Tipburn	Real-time PCR	Tipburn-susceptible line vs. tipburn-resistant line	Ca ²⁺ transporter genes (e.g., <i>BoCAX2</i> and <i>BoCAX5</i>)	[34]
2016	Lee et al.	Cabbage (<i>Brassica oleracea</i> var. <i>capitata</i>) leaves (apex, middle, and base)	Tipburn	RNA-seq	Tipburn-susceptible cabbage vs. tipburn-resistant cabbage vs. Kale	1844 loci were identified as tipburn related phenotype genes	[35]
2020	Gonzalo et al.	Tomato (<i>Solanum lycopersicum</i> cv. ‘Moneymaker’ × <i>S. pimpinellifolium</i> accession TO-937)	Tipburn	QTL mapping	Temperature treatments (T1: 25 °C day/20 °C night; T2: 30 °C day/25 °C night; T3: 35 °C day/30 °C night) (Genetic mapping was conducted using 168 RILs and 52 Introgression Line (IL))	a QTL located in chromosome 7	[36]
2021	Kuronuma and Watanabe	Lisianthus (<i>Eustoma grandiflorum</i> (Raf.) Shinn.) leaves and roots	Tipburn	RNA-seq	Tipburn-susceptible cultivar, which accumulated excessive Ca in roots, vs. tipburn-resistant cultivar	Ca ²⁺ transporter genes (e.g., <i>CAX5</i> and <i>ACA12</i>) and <i>PMEs</i>	[37]
2012	Freitas et al.	Tomato <i>Solanum lycopersicum</i> L. pericarp	BER	Real-time PCR Functional analysis	Gibberellin (GA) treatments (Control (water), GA ₄₊₇ , and prohexadione-calcium (GA biosynthesis inhibitor))	<i>CAX</i> and <i>Ca²⁺-ATPase</i> genes (e.g., <i>CAX3</i> and <i>Ca²⁺-ATPase1</i>)	[38]

Table 1. Cont.

Year	Author	Plant Materials	Ca ²⁺ Deficiency Disorder	Main Methods	Main Comparison	Causal Candidate Genes	Ref No.
2018	Freitas et al.	Tomato <i>Solanum lycopersicum</i> L. pericarp	BER	Microarray Real-time PCR	Growth regulator treatments (Control (water), GA ₄₊₇ , prohexadione-calcium (GA biosynthesis inhibitor), and ABA)	35 candidate genes inhibiting BER and 7 candidate genes triggering BER	[39]
2012	Uozumi et al.	Tomato <i>Solanum lycopersicum</i> L. pericarp	BER	Growth assay using introgression lines	IL8-3 vs. the <i>S. lycopersicum</i> parent (M82d)	the IL8-3 region of the <i>S. pennellii</i> genome	[40]
2016	Ikeda et al.	Tomato <i>Solanum pennellii</i> pericarp	BER	Real-time PCR	BER-resistant introgression line (IL8-3) vs. the parent ('M82'), which is a BER-susceptible cultivar.	Ca ²⁺ -movement-related genes (e.g., <i>CAX4</i> , <i>Ca²⁺-ATPase3</i> , and <i>Solyc07g062700 (NCX)</i>)	[41]

3.1.2. Tipburn in Chinese Cabbage

Three studies investigating the causative genes for tipburn in Chinese cabbage were extracted (Table 1). Su et al. [31] examined the expression of 12 Ca^{2+} transporter genes in tipburn-susceptible and tipburn-resistant plants under different Ca^{2+} concentrations. Most of the Ca^{2+} transporter genes were expressed at lower levels in the inner leaves of tipburn-susceptible plants than those in the inner leaves of tipburn-resistant plants. Additionally, salicylic acid (SA) levels were higher in tipburn-susceptible plants than in tipburn-resistant plants. These results suggest that cytoplasmic Ca^{2+} fluctuation-induced downstream signaling events (including SA biosynthesis) are related to tipburn resistance and response to Ca^{2+} deficiency conditions in Chinese cabbage. Furthermore, in 2019, the same authors conducted GWA (genome-wide association) mapping and QTL mapping using 194 diverse inbred lines, a doubled haploid (DH100) population, and an F_2 population [32]. This research identified a novel tipburn-associated locus (*gqbTRA06*) and found that *BrCRT2* (*Bra018575*, CRT: calreticulin) exhibited higher expression levels during the disease development. Subsequent functional analysis of *BrCRT2* suggested that *BrCRT2* is a candidate gene for controlling tipburn in Chinese cabbage.

One study investigated the relevance of circular RNA (circRNA) expression and response to tipburn in Chinese cabbage [33]. Circular RNAs are non-coding RNAs that play significant roles in the function and transcriptional regulation of microRNA. Wang et al. [33] conducted circRNA sequencing and showed that circRNAs were differentially expressed in response to different stages of tipburn damage. The authors also suggested that *ABF2*, which is a host gene of differentially expressed circRNA and a positive regulator of the abiotic stress response and abscisic acid (ABA)-dependent pathway, is a key gene in the tipburn response process, since they found that ABA also accumulated during Ca^{2+} deficiency stress.

3.1.3. Tipburn in Cabbage

Lee et al. conducted two studies investigating causative genes of tipburn in cabbage [34,35]. In 2013, the authors investigated the expression of Ca^{2+} transporter genes in tipburn-susceptible and tipburn-resistant lines [34]. Interestingly, the authors found that *BoCAX2* (CAX: $\text{Ca}^{2+}/\text{H}^{+}$ antiporter) and *BoCAX5* were differentially expressed in the tipburn-susceptible and tipburn-resistant lines. However, clear relevance of the expression of these genes and Ca^{2+} concentration in vacuoles was not observed. In 2016, the same authors conducted RNA-seq (RNA sequencing) using tipburn-susceptible cabbage, tipburn-resistant cabbage, and kale samples, from which they identified 1844 loci as tipburn-related phenotype-specific genes [35]. Of these, 16 were specific to tipburn-susceptible cabbage, while 747 were specific to tipburn-resistant cabbage.

3.1.4. Tipburn in the Other Horticulture Crops

To obtain insight into the genetic control of heat tolerance in tomato plants, Gonzalo et al. [36] investigated QTLs involved in reproductive traits and tipburn incidence under different temperature treatments. A QTL located on chromosome 7 was identified as associated with a low incidence of tipburn.

In *lisianthus*, Kuronuma and Watanabe [37] conducted RNA-seq using both tipburn-susceptible cultivars, which accumulated excessive Ca^{2+} in roots [42,43], and tipburn-resistant cultivars. Of the Ca^{2+} transporter genes, *CAX5* was upregulated and *ACA12* (ACA: Ca^{2+} -ATPase) was downregulated in the roots of the tipburn-susceptible cultivar. Thus, the authors suggested that *CAX5* and *ACA12* are candidate genes associated with excessive Ca^{2+} accumulation in the roots of the tipburn-susceptible cultivar, which inhibits Ca^{2+} distribution to the leaves.

3.1.5. Blossom-End Rot in Tomato

Four studies have been conducted on the causative genes of BER in tomatoes. In 2012, Freitas et al. [38] investigated the expression of the Ca^{2+} transporter genes under different gibberellin (GA) treatments. The authors found that GA treatment enhanced the expression of putative CAX and Ca^{2+} -ATPase genes. Consequently, the water-soluble apoplastic Ca^{2+} concentration was reduced and the BER incidence increased in plants treated with GA. In 2018, the authors attempted to identify the candidate genes and gene networks that inhibit or trigger BER [39]. Candidate genes that inhibit or trigger BER were identified through a correlation analysis between the gene expression levels and BER incidence. The authors suggested that the GA synthesis inhibitor and ABA reduced BER because of increased tissue resistance to reactive oxygen species (ROS) and other toxic compounds (i.e., GA triggered BER by increasing the levels of fruit oxidative stress).

To explore agriculturally useful traits, including salt tolerance and resistance to BER, Uozumi et al. [40] used an introgression line (IL), IL8-3, in which a *Solanum pennellii* chromosomal segment was introgressed into *Solanum lycopersicum* chromosome 8. The IL8-3 line exhibited a significantly lower incidence of BER and higher Ca^{2+} concentration in fruits than the 'M82d' parent line did. Thus, a gene that enhances tolerance to BER is likely to be present in the IL8-3 region of the *S. pennellii* genome. Using similar plants, Ikeda et al. [41] compared the Ca^{2+} concentrations and expression levels of the Ca^{2+} -movement-related genes in IL8-3 (BER resistant introgression line) and the parent line ('M82'). Ca concentrations were higher in the fruit and leaves of IL8-3 than in those of 'M82'. Additionally, the expression level of CAX4 was significantly higher in IL8-3 than in M82. Accordingly, it was suggested that the expression levels of the Ca^{2+} -movement-related genes are heavily involved in the development of BER by promoting Ca^{2+} transfer.

3.2. Search 2: Studies Involving Functional Analysis of Genes Associated with Ca^{2+} Deficiency Disorders and/or Ca^{2+} Accumulation in Plant Organs

A total of 103 articles were obtained through document retrieval (Figure 2). After removal of duplicate articles ($n = 16$), review articles ($n = 9$), and articles written in languages other than English ($n = 1$), 77 articles were selected for full-text evaluation. Fourteen of these were extracted following eligibility evaluation. Additionally, five full-text articles were added through references and three full-text articles were added from Search 1. The 22 articles selected for this systematic review are shown in Table 2. Given below is a review of the experimental results for each gene.

3.2.1. Effects of Genes Encoding Ca^{2+} Antiporters

Nine of the extracted studies involved functional analysis of the genes encoding tonoplast localized $\text{Ca}^{2+}/\text{H}^{+}$ antiporters (CAXs), which are associated with Ca^{2+} deficiency disorders and/or Ca^{2+} accumulation in plant organs (Table 2). In 1999, Hirschi [20] created transgenic tobacco plants expressing *Arabidopsis* CAX1. The CAX1-expressing tobacco showed significantly higher Ca^{2+} accumulation in roots and leaves than vector control plants did. However, CAX1-expressing plants also displayed symptoms of Ca^{2+} deficiency (leaf necrosis). To our knowledge, this study was the first to conduct a functional analysis of the genes associated with Ca^{2+} deficiency disorders and Ca^{2+} accumulation at the plant organ level.

In tomatoes, Park et al. [21] created transgenic plants expressing sCAX1 and CAX4 and found that the transgenic plants contained significantly more Ca^{2+} than vector controls did. In contrast, only the sCAX1-expressing tomatoes showed a significant increase in the occurrence of BER. Cheng et al. [44] reported that sCAX2A-expressing tomato exhibited a significantly higher Ca^{2+} concentration in fruit compared with the wild-type, despite there being no observed differences in morphological traits between the sCAX2A-expressing tomato and the wild-type, and no increase in BER incidence in the sCAX2A-expressing tomatoes. Freitas et al. [45] identified the reason that the sCAX1-expressing tomato exhibited increased the BER incidence despite accumulating more Ca^{2+} than that in the

wild-type. To do this, the authors investigated gene expression and localization of Ca^{2+} in the cells. The *sCAX1*-expressing tomato was found to have accumulated excessive Ca^{2+} in the vacuole, and thus contained lower apoplastic and cytosolic Ca^{2+} concentrations than the wild-type did. These results clearly indicated that high expression of *sCAX1* reduces the mobility of Ca^{2+} , which leads to the development of BER in fruits.

Park et al. [46] conducted the first attempts to use biotechnology to increase the Ca^{2+} content in potato tubers. In both leaves and tubers, Ca^{2+} accumulation was significantly higher in transgenic *sCAX1*-expressing potatoes than in the wild-type control. Similarly, Kim et al. [47] demonstrated that *CAX2B*-expressing potato accumulated 50–60% more Ca^{2+} in tubers than the wild-type did. In these two reports, no deleterious alternations in plant growth and morphological traits between transgenic plants and wild types were observed. In contrast, Zorrilla et al. [48] and Gao et al. [49] reported that *sCAX*-expressing potatoes showed shoot rip necrosis and hollow heart, while the total and tuber Ca^{2+} concentrations were higher than those of the wild-type. The authors demonstrated that this result was due to *sCAX*-expressing potatoes sequestering Ca^{2+} in vacuoles as calcium oxalate crystals, reducing the water-soluble Ca^{2+} concentration.

Figure 2. Systematic review flow chart for Search 2.

Table 2. Overview of the studies involving the functional analysis of genes associated with Ca²⁺ deficiency disorders and Ca²⁺ accumulation in plant organs obtained via Search 2.

Year	Author	Subject Genes		Plant Materials	Ca ²⁺ Deficiency Disorder	Main Comparison	Mani Methods	Ref No.
1999	Hirschi	<i>CAX1</i>	Ca ²⁺ antiporter	Tobacco (<i>Nicotiana tabacum</i> ‘KY160’)	Leaf necrosis	Vector controls vs. <i>CAX1</i> -expressing plants	Functional analysis	[20]
2005	Park et al.	<i>sCAX1</i> and <i>CAX4</i>	Ca ²⁺ antiporter	Tomato (<i>Solanum lycopersicum</i>)	Blossom-end rot	Wild-type vs. <i>sCAX1</i> -expressing line vs. <i>CAX4</i> -expressing line	Functional analysis	[21]
2010	Chung et al.	<i>sCAX2A</i>	Ca ²⁺ antiporter	Tomato (<i>Solanum lycopersicum</i> ‘Ailsa Craig’)	Tipburn Blossom-end rot	Wild type vs. <i>sCAX2A</i> -expressing plants	Functional analysis	[44]
2011	Freitas et al.	<i>sCAX1</i>	Ca ²⁺ antiporter	Tomato (<i>Solanum lycopersicum</i> ‘FM9’)	Blossom-end rot	Wild-type vs. <i>sCAX1</i> -expressing plants	Functional analysis Microarray Real-time PCR	[45]
2005	Park et al.	<i>sCAX1</i>	Ca ²⁺ antiporter	Potato (<i>Solanum tuberosum</i> ‘Russt Norkotah’)	—	Wild-type vs. <i>sCAX1</i> -expressing lines	Functional analysis	[46]
2006	Kim et al.	<i>CAX2</i>	Ca ²⁺ antiporter	Potato (<i>Solanum tuberosum</i> ‘Daejiree’)	—	Wild-type vs. <i>CAX2B</i> -expressing lines	Functional analysis	[47]
2019	Zorrilla et al.	<i>sCAX1</i>	Ca ²⁺ antiporter	Potato (<i>Solanum tuberosum</i> L. ‘Atlantic’)	Shoot tips necrosis Hollow heart	Wild type vs. <i>sCAX1</i> overexpression line	Functional analysis Real-time PCR	[48]
2020	Gao et al.	<i>sCAX1</i>	Ca ²⁺ antiporter	Potato (<i>Solanum tuberosum</i> L. ‘Atlantic’)	Shoot tips necrosis Hollow heart	Wild type vs. <i>sCAX1</i> overexpression line	Functional analysis Real-time PCR	[49]

Table 2. Cont.

Year	Author	Subject Genes		Plant Materials	Ca ²⁺ Deficiency Disorder	Main Comparison	Mani Methods	Ref No.
2004	Park et al.	<i>CAX1</i>	Ca ²⁺ antiporter	Carrot (<i>Daucus carota</i> ‘Danver 60’)	—	Vector control vs. <i>CAX1</i> -expressing line	Functional analysis	[50]
2001	Persson et al.	<i>CRT</i> (<i>Zea mays</i>)	Calreticulin	Tobacco (<i>Nicotiana tabacum</i> ‘NT1’)	—	Wild-type vs. <i>CRT</i> -overproducing line	Functional analysis	[51]
2002	Wyatt et al.	<i>CRT</i>	Calreticulin	<i>Arabidopsis thaliana</i>	—	Wild type vs. plants induced the mGFP5- <i>CRT</i> C-domain fusion protein	Functional analysis	[52]
2012	Wu et al.	<i>CRT</i> and <i>sCAX1</i>	Calreticulin and Ca ²⁺ antiporter	Tobacco (<i>Nicotiana tabacum</i> ‘KY160’)	Necrosis on the tips of the new leaves	<i>sCAX1</i> -expressing plants vs. <i>sCAX1</i> and <i>CRT</i> -co-expressing plants	Functional analysis	[53]
				Tomato (<i>Solanum lycopersicum</i> ‘Rubion’)	Necrosis in leaf tips BER			
2007	Mills et al.	<i>ECA3</i>	Ca ²⁺ -ATPase	<i>Arabidopsis thaliana</i>	—	Wild type vs. <i>eca2</i> mutant vs. <i>eca3</i> mutant	Functional analysis	[54]
2011	Barabasz et al.	<i>ECA3</i>	Ca ²⁺ -ATPase	Tobacco (<i>Nicotiana tabacum</i> ‘Xanthi’)	Chlorotic leaf	Wild type vs. <i>AtECA3</i> overexpression line	Functional analysis	[55]
2001	Kim et al.	<i>AtGluR2</i>	Ca ²⁺ channel	<i>Arabidopsis thaliana</i>	Browning and death of the shoot apex Necrosis of leaf tip Deformation of leaves	Vector control vs. <i>AtGluR2</i> overexpression line	Functional analysis	[56]
2012	Aouini et al.	<i>SLGLR1.1</i> and <i>SLGLR3.5</i> (<i>Solanum lycopersicum</i>)	Ca ²⁺ channel	<i>Arabidopsis thaliana</i>	Necrosis of the tips of young leaves	Wild type vs. <i>SLGLR1.1</i> overexpressing plant vs. <i>SLGLR3.5</i> overexpressing plant	Functional analysis	[57]

Table 2. Cont.

Year	Author	Subject Genes		Plant Materials	Ca ²⁺ Deficiency Disorder	Main Comparison	Mani Methods	Ref No.
2007	Nakagawa et al.	<i>MCA1</i>	Ca ²⁺ channel	<i>Arabidopsis thaliana</i>	—	Wild-type vs. <i>mca1</i> -null mutant vs. <i>MCA1</i> -overexpression line	Functional analysis	[58]
2010	Yamanaka et al.	<i>MCA1</i> and <i>MCA2</i>	Ca ²⁺ channel	<i>Arabidopsis thaliana</i>	—	Wild-type vs. <i>mca2</i> -null single mutant vs. <i>mca1</i> -null and <i>mca2</i> -null double mutant	Functional analysis Real-time PCR	[59]
2012	Freitas et al.	<i>PME</i>	The others	Tomato (<i>Solanum lycopersicum</i>)	Blossom-end rot	Wild-type vs. <i>PME</i> -silenced plant	Functional analysis	[60]
2013	Tanaka et al.	<i>PCaP2</i>	The others	<i>Arabidopsis thaliana</i>	—	Wild type vs. NR23 (Root hair-less line: overexpression of a 23 amino acid peptide in the N-terminal region of <i>PCaP2</i>)	Functional analysis Real-time PCR	[61]
2002	Świądrych et al.	<i>Solanum tuberosum</i> mRNA for 14-3-3 protein, clone 20R. (EMBL/GenBank database account no. X87370)	The others	Potato (<i>Solanum tuberosum</i> L. 'Desiree')	—	Wild type vs. J4 (underexpressing the potato 14-3-3 isoform cDNA 20R)	Functional analysis	[62]
2019	Fasani et al.	<i>MYB59</i>	The others	<i>Arabidopsis thaliana</i>	—	Wild type vs. <i>myb59</i> knockout mutant vs. <i>MYB59</i> overexpressing mutant	Functional analysis Microarray Real-time PCR	[63]

In carrot, Park et al. [50] created a *CAX1*-expressing transgenic line. Compared to the vector controls, this mutation did not alter the morphology and growth characteristics, including those of the edible portion. In contrast, *CAX1*-expressing carrots accumulated significantly more Ca^{2+} (1.4-to 1.6-fold) than the vector controls did.

3.2.2. Effects of Genes Encoding Calreticulin

Three studies have undertaken functional analysis of the genes encoding calreticulin (*CRT*) (Table 2). *CRT* is a Ca^{2+} -binding protein localized in the endoplasmic reticulum lumen, which is a main Ca^{2+} -retaining pool. Persson et al. [51] created transgenic tobacco plants expressing maize (*Zea mays*) *CRT*. The *CRT*-overexpressing tobacco exhibited a twofold increase in ATP-dependent Ca^{2+} uptake compared to the wild-type line. In addition, they revealed that the ER from the antisense line showed a 40% decrease in Ca^{2+} uptake, suggesting that there is a positive correlation between *CRT* production and ATP-dependent Ca^{2+} uptake. Wyatt et al. [52] also reported that the total Ca^{2+} concentration in plants, which induced the mGFP5-*CRT* C-domain fusion protein (GFP: green fluorescent protein), increased by 9–35.7% compared to the wild type. These results indicate that the expression of the calreticulin C-domain increases the Ca^{2+} stores.

Wu et al. [53] investigated the occurrence of Ca^{2+} deficiency disorders and Ca^{2+} accumulation in both *sCAX1*-expressing plants and *sCAX1* and *CRT*-co-expressing plants. The authors found that *sCAX1* + *CRT*-expressing tobacco and tomato exhibited lower Ca^{2+} deficiency symptoms (leaf tip necrosis and BER) than the lines expressing *sCAX1* did alone. In contrast, there were no significant differences in Ca^{2+} concentration between the *sCAX* + *CRT*-expressing and the *sCAX*-expressing lines. These results suggest that co-expression of *CRT* and *sCAX1* enhances the Ca^{2+} content of plants without any apparent detrimental effects potentially caused by *sCAX1* expression.

3.2.3. Effects of Genes Encoding Ca^{2+} -ATPasess (Pumps)

Two studies have been conducted on the functional analysis of the genes encoding $\text{P}_{2\text{A}}$ -type ATPases (*ECA*) (Table 2). Ca^{2+} -ATPasess play a role in controlling the Ca^{2+} efflux from the cytosol to organelles and/or to the apoplast. Under $0.5 \times$ Murashige and Skoog with Mangan (Mn^{2+}) omitted (0 Mn^{2+}), Mills et al. [54] investigated the Ca^{2+} concentration in roots of *eca3* mutants in which *ECA* was functionally disrupted. The authors found that, as a result of this disruption, the Ca^{2+} concentration in the roots of the mutant line was significantly higher than that of the wild type. However, the *eca3* mutants showed a marked reduction in growth compared to the wild type. The authors thus suggested that *ECA3* is important for Mn^{2+} and Ca^{2+} homeostasis. Four years later, Barabasz et al. [55] created *AtECA*-expressing tobacco. Under Ca^{2+} deficiency conditions, wild-type plants had chlorotic older leaves, whereas no symptoms were observed in *AtECA*-expressing tobacco. In contrast, there were no significant differences in the biomass and Ca^{2+} concentrations of shoots and roots between wild-type and *AtECA*-expressing plants. These results suggest that *ECA* expression contributes to increased tolerance to Ca^{2+} deficiency.

3.2.4. Effects of Genes Encoding Ca^{2+} Channels

Four studies involved functional analysis of the genes encoding Ca^{2+} channels (Table 2). For the ionotropic glutamate receptor (GLR), which acts as a non-selective Ca^{2+} channel, Kim et al. [56] reported that an *AtGluR2* overexpression line showed Ca^{2+} deficiency disorders (necrosis and browning of shoot tips and curling of leaves), while no symptoms were observed in the vector control line. In contrast, there was no significant difference in the Ca^{2+} concentration in the shoots between the control and transgenic plants. The authors suggested that Ca^{2+} deficiency in transgenic plants is mainly due to reduced utilization of the transported Ca^{2+} . Aouini et al. [57] created transgenic *Arabidopsis* plants expressing *S. lycopersicum* *GLR1.1*, *GLR3.5* (*SiGLR1.1* and *SiGLR3.5*). Although there were no significant differences in the Ca^{2+} concentration in the aerial parts of the wild-type and transgenic

plants, symptoms of Ca^{2+} deficiency (necrosis of the tips and margins of young leaves, dwarf stature, and curled and deformed leaves) were observed in transgenic plants.

For MCA, which is a Ca^{2+} -permeable stretch-activated channel component, Nakagawa et al. [58] demonstrated that an *MCA1*-overexpression line accumulated significantly higher Ca than did the wild-type and *mca1*-null lines. However, there was no significant difference in Ca^{2+} uptake between the wild-type and *mca1*-null lines, meaning that the function of *MCA1* remains uncertain. Additionally, Yamanaka et al. [59] created an *MCA2*-expressing plant. The Ca^{2+} uptake activity of the *MCA2*-expressing cells was significantly higher than that of the wild-type. This result suggested that *MCA2* functions as a Ca^{2+} uptake protein in the root.

3.2.5. Effects of Genes Encoding the Other Proteins

Freitas et al. [60] created *PME*-silenced tomato plants (Table 2). Pectin methylesterase (*PME*) is an enzyme that esterifies the glycan regions within the HG (homogalacturonan) domain of pectin. This enables Ca^{2+} crosslinking in the cell wall. *PME*-silenced fruit had a higher total and apoplastic water-soluble Ca^{2+} concentrations in the pericarp tissue than the wild-type fruit did. Moreover, *PME*-silenced fruit had a lower incidence of BER than that of the wild-type fruit.

Plasma membrane-associated cation-binding protein 2 (*PCaP2*) is involved in root hair growth. Tanaka et al. [61] demonstrated that NR23 (root hairless line: overexpression of a 23 amino acid peptide in the N-terminal region of *PCaP2*) did not produce root hairs, and the concentrations of individual minerals, including Ca^{2+} , were relatively low (Table 2). This result indicated that morphological traits, including root hairs, also play significant roles in Ca^{2+} acquisition.

Świądrych et al. [62] investigated the growth and ion content of 14-3-3-repressed transgenic potato (Table 2). The 14-3-3 protein acts directly with nitrate reductase (NR) and sucrose phosphate synthase (SPS), regulating the process of nitrogen fixation and carbohydrate metabolism. There was no significant difference in dry weight between the wild-type and 14-3-3-repressed transgenic potatoes. However, there was a significant increase in the Ca^{2+} content of the transgenic potato tubers compared to the wild-type plants, while there were few differences in the concentrations of other minerals (e.g., phosphorus, magnesium, and iron) between the wild-type and transgenic potatoes.

Several studies have also reported effects of transcription factors on Ca^{2+} accumulation. Fasani et al. [63] investigated the function of *MYB59* in *Arabidopsis* (Table 2). *MYB59* is known to be a transcription factor that regulates cell cycle progression and root elongation. The root tips of the *myb59* knockout mutant used in this study accumulated significantly higher Ca^{2+} than the wild type did under 25 μM CdSO_4 . In addition, transcriptome analysis revealed upregulation of calmodulin-like proteins and Ca^{2+} transporter genes in the *myb59* mutant. These results indicate that *MYB59* negatively regulates Ca^{2+} homeostasis and signaling during periods of Ca^{2+} deficiency.

3.3. Synthesizing Current Knowledge Regarding the Role of Gene Expression in the Occurrence of Ca^{2+} Deficiency Disorders

To determine the role of gene expression in the occurrence of Ca deficiency disorders and/or Ca accumulation, 37 articles were reviewed in detail (Tables 1 and 2 and Figures 1 and 2). Twenty-six of the thirty-seven reports showed an association between Ca deficiency and/or Ca accumulation and the expression of Ca^{2+} -movement-related genes (Ca^{2+} antiporters [27,29,32,35,36,39,42–50,53], calreticulin [30,51–53], Ca^{2+} -ATPase [35,36,39,54,55], Ca^{2+} channels [55–58], and *PME* [35,59]). Of the remaining studies, five studies identified QTLs that described variation in tipburn incidence [25,26,33,34,38]. Two studies investigated the causative genes of Ca deficiency disorders and Ca accumulation by focusing on morphological traits (leaf shape [28] and root hair production [61]). In addition, other studies identified the relevance of the expression of genes encoding circRNA [31], plant hormones [37], 14-3-3 protein [62], and transcription factors [63] to Ca

deficiency disorders and/or Ca accumulation in plant organs. Therefore, many genes are likely to be involved in the occurrence of Ca deficiency disorders, but Ca^{2+} -movement-related genes seem to be the most influential.

Only *CAXs* ($\text{Ca}^{2+}/\text{H}^{+}$ antiporter) and *CRT* (calreticulin) were identified as genes associated with the occurrence of Ca deficiency disorders in studies obtained from both document retrievals (Searches 1 and 2). In particular, the relevance of *CAXs* expression to Ca deficiency disorders and/or Ca accumulation has been well-documented [29,32,35,36,39,42–45,48,49,53]. Thus, it is plausible that *CAXs* act as a causative gene for Ca deficiency disorders in horticultural crops. However, it should be noted that two diametrically opposed hypotheses as to the mechanism by which expression of *CAXs* is associated with Ca deficiency disorders are advanced by these studies. In Search 2 (Table 2), many studies [42,43,45,48,49] indicated that *CAX1*-expressing transgenic plants exhibited Ca deficiency disorders because excessive Ca accumulation in vacuoles inhibited the distribution of Ca to new leaves and fruits. Based on this phenomenon, two studies suggested *CAXs* as a causative gene for Ca deficiency disorders using transcriptome analysis [35,36]. In contrast, five studies demonstrated that transgenic plants overexpressing *CAXs* (*CAX1* [46,50], *CAX2* [44,47], and *CAX4* [43]) did not differ from wild-type plants in morphological traits, and thus that overexpression of *CAXs* did not cause Ca deficiency disorders in these plants, despite their Ca contents being significantly higher than those of the wild-type. Two studies also concluded that the occurrence of tipburn and BER in Ca deficiency disorder-susceptible lines is due to reduced expression of *CAXs* compared to resistant lines [29,39]. Although it is clear that *CAXs* contribute to an increase in Ca acquisition, the effect of *CAXs* on the occurrence of Ca deficiency disorders needs to be investigated in more detail. It is particularly important that future studies demonstrate localized Ca accumulation and gene expression and carefully evaluate their role in Ca deficiency disorders.

Compared with *CAXs*, the function of *CRT* in Ca deficiency disorders and/or Ca accumulation is not well-documented. However, this systematic review indicated that expression of *CRT* likely enhances Ca accumulation and decreases the occurrence of Ca deficiency disorders [51–53]. In addition, a single study by Su et al. [30], which involved both QTL analysis and functional analysis, indicated that *BrCRT2* is a candidate gene for controlling tipburn in Chinese cabbage. It is important that future studies conduct both exhaustive gene expression and functional analyses to identify the causative genes of Ca deficiency disorders, as in the report by Su et al. [30]. This systematic review also suggests that the causative genes of Ca deficiency disorders differ between different horticultural crops and cultivars.

Additionally, this systematic review suggests that expression of *ECA3* plays a contributive role in mitigating the occurrence of Ca deficiency disorders [55,60], and that overexpression of *GLRs* and *PME* may cause Ca deficiency disorders [56,57]. However, the effects of genes for which few studies on Ca deficiency disorders exist need to be further investigated. In addition, it is known that plant hormones affect the occurrence of Ca deficiency disorders [64–66]. In this systematic review, some reports indicated that plant hormones and the expression of related genes affected the occurrence of Ca deficiency disorders [31,36,37]. Quantification of not only Ca distribution, but also plant hormone localization, may provide further understanding of the mechanism of Ca deficiency disorders in plants.

4. Perspectives

We do not yet have fully systematized knowledge of the causative genes for Ca deficiency disorders in different horticulture crops. However, this systematic review showed that many studies, in various plant systems and from various perspectives, have been conducted to identify the causative genes for Ca deficiency disorders. It is likely that the relationship between gene expression and Ca distribution will be systematically understood, and the mechanism of occurrence of Ca deficiency disorders in horticulture crops will be explained genetically in the near future. We hope that the knowledge synthesized

in this systematic review will contribute to the accumulation of further knowledge and elucidation of the causes of Ca deficiency disorders.

5. Conclusions

To synthesize current knowledge regarding the role of gene expression in the occurrence of Ca²⁺ deficiency disorders, we conducted a systematic literature review using PRISMA. In total, 37 articles were extracted in this review. In particular, the effects of the expression of CAXs and CRT on the occurrence of Ca²⁺ deficiency disorders were well demonstrated. However, the causative genes for Ca²⁺ deficiency disorders in each horticulture crop are not clear. We hope that the knowledge synthesized in this systematic review will contribute to the accumulation of further knowledge and elucidation of the causes of Ca²⁺ deficiency disorders.

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