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Invited Review

# Functions and structure of roots and their contributions to salinity tolerance in plants

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Soil salinity is an increasing threat to the productivity of glycophytic crops worldwide. The root plays vital roles under various stress conditions, including salinity, as well as has diverse functions in non-stress soil environments. In this review, we focus on the essential functions of roots such as in ion homeostasis mediated by several different membrane transporters and signaling molecules under salinity stress and describe recent advances in the impacts of quantitative trait loci (QTLs) or genetic loci (and their causal genes, if applicable) on salinity tolerance. Furthermore, we introduce important literature for the development of barriers against the apoplastic flow of ions, including $Na^+$, as well as for understanding the functions and components of the barrier structure under salinity stress.

**Key Words:** salinity stress, osmotic stress, roots, quantitative trait loci, $Na^+$ exclusion, Casparian strip, apoplastic transport barrier.

## Introduction

Salinity stress induced by soil salinization is one of the major constraints that limit crop production worldwide as most of the major crops are glycophytes that have evolved by adaptation to soils with low sodium levels (Cheeseman 2015). Soils that accumulate excessive soluble salts in the root zone are often labelled as salt-affected soils and generally divided into two major categories, saline and sodic soils (**Table 1**; Horie *et al.* 2012, Tuyen *et al.* 2010). Saline soils occur in estuaries and coastal fringes as well as arid regions and are defined to have an electrical conductivity (EC) of more than 4 dS/m, which is approximately 40 mM NaCl (Horie *et al.* 2012, Munns and Tester 2008). Sodic soils are found in arid and semi-arid regions and are dominated by $Na^+$ at the exchangeable site of clay particles, with high concentrations of carbonate or bicarbonate anions, which leads to high pH of >8.5 (Horie *et al.* 2012, Tuyen *et al.* 2010). Saline-sodic soils, which show features of both soils, occasionally occur in salt-affected lands (CISEAU, IPTRID and AGLL, FAO 2005). The percentage of salt-affected soils is remarkably high in the region of Asia and the pacific and Australia (**Table 1**), where important crops, including rice, wheat, barley, soybean, corn, and so on are widely cultivated.

Breeding of salt-tolerant crop cultivars is thought to be one of the means for addressing the problem of soil salinization in arable lands (CISEAU, IPTRID and AGLL, FAO 2005). In the past few decades, numerous studies have attempted to elucidate the underlying mechanisms of plant salt tolerance at the molecular, physiological, and genetic levels focusing on not only the model plant *Arabidopsis thaliana* but also crop plants including halophytes (Blumwald 2000, Deinlein *et al.* 2014, Ismail and Horie 2017, Munns *et al.* 2020a, 2020b, Munns and Tester 2008, Shabala 2013, Teakle and Tyerman 2010, Zhu 2002).

The root, which generally develop underneath the surface of the soil, is an indispensable organ of vascular plants. Roots play vital roles in the growth and reproduction of plants, such as uptake of water and nutrients; perception of changes in soil environments, including soil salinity; and even interaction with microorganisms. Under salinity stress, roots encounter two major difficulties; (i) insufficient water absorption (or loss of water depending on the extent of the stress) due to hyperosmosis-induced reduction in soil water potential, and (ii) massive influx of toxic ions such as $Na^+$ and $Cl^-$, which eventually over-accumulate in leaves and trigger ion toxicity (Horie *et al.* 2012, Munns and Tester 2008). In the past few decades, many root-based quantitative trait loci (QTLs), which have a remarkable impact on salt tolerance, have been identified with the development of genome sciences and bioinformatics. This has led to several remarkable discoveries in the

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**Table 1.** The extent of salt-affected soil in arable and non-arable lands of the world. Each area is shown in million km$^2$. Note that this table was prepared referring FAO SOILS PORTAL (http://www.fao.org/soils-portal/soil-management/management-of-some-problem-soils/salt-affected-soils/more-information-on-salt-affected-soils/en/) with some minor modifications

| Regions | Total area | Saline soils | % | Sodic soils | % |
|---|---|---|---|---|---|
| Africa | 18.99 | 0.39 | 2.04 | 0.34 | 1.76 |
| Asia and the Pacific and Australia | 31.07 | 1.95 | 6.28 | 2.49 | 8.00 |
| Europe | 20.11 | 0.07 | 0.33 | 0.73 | 3.62 |
| Latin America | 20.39 | 0.61 | 2.97 | 0.51 | 2.50 |
| Near East | 18.02 | 0.92 | 5.08 | 0.14 | 0.78 |
| North America | 19.24 | 0.05 | 0.24 | 0.15 | 0.75 |
| Total | 127.81 | 3.97 | 3.11 | 4.34 | 3.40 |

molecular physiological mechanisms of $Na^+/K^+$ transport and plant salt tolerance (Bassil and Blumwald 2014, Horie *et al.* 2009, Ismail and Horie 2017, Zhu 2002, and references therein). Furthermore, the impacts of the functions of plant membrane transporters on stress tolerance mechanisms, including salinity, have been identified and discussed (Schroeder *et al.* 2013). However, genes or loci governing other key mechanisms such as as-yet-uncovered ion homeostasis, osmotic stress tolerance, management of reactive oxygen species (ROS), and essential sensor or signaling molecules under salinity stress remain to be identified.

In this review, we discuss the recent advances in the study of plant salt tolerance; the paper is divided into four major sections. In the first two sections, QTLs or genetic loci and their responsible genes, if applicable, which govern ion homeostasis and osmotic adjustment under salinity stress, are referred, and the third section summarizes important questions regarding $Na^+$ homeostasis. In the last section, we introduce recent findings on the structure of roots and its relevance to salinity tolerance.

## 1. QTLs or loci associated with the functions and biomass of roots under salinity stress

### *1.1. Overview: The basis of salinity stress-related QTLs or genetic loci*

In general, important agronomic traits are controlled by polygenes, that is, quantitative trait loci (QTLs) (Ashikari and Matsuoka 2006). The development of DNA sequencing technologies has dramatically expanded genomic sequence information and molecular markers, which has facilitated QTL analysis in the field of plant science. Salinity tolerance is based on multifactorial inheritance, and such a polygenic nature has led to the identification of numerous salinity stress-related QTLs. Narrowing down the QTLs identified recently by using root-based indices such as ion contents and biomass, many QTLs still can be found in various plant species including Arabidopsis (Kobayashi *et al.* 2016, Roy *et al.* 2013), barley (Gill *et al.* 2017, 2019, Lonergan *et al.* 2009, Long *et al.* 2013, Nguyen *et al.* 2013, Rivandi *et al.* 2011, Shavrukov *et al.* 2010, Xue *et al.* 2009, 2017), *Brassica rapa* (Basnet *et al.* 2015), Cotton (Oluoch *et al.* 2016), grapevine (Henderson *et al.* 2018), maize (Cao *et al.* 2019, Zhang *et al.* 2018, 2019a), rice (Bonilla *et al.* 2002, De Leon *et al.* 2017, Gimhani *et al.* 2016, Haq *et al.* 2010, Li *et al.* 2020, Lin *et al.* 2004, Sabouri *et al.* 2009, Thomson *et al.* 2010, Tian *et al.* 2011, Wang *et al.* 2012a, 2012b), tomato (Villalta *et al.* 2008), wheat (Lindsay *et al.* 2004), *Medicago truncatula* (Arraouadi *et al.* 2012), soybean (Zhang *et al.* 2014), and white clover (Wang *et al.* 2010). Major QTLs/loci that have been identified by using indices relevant to the function and biomass of roots under salinity stress are summarized in **Table 2**.

$Na^+$ is a major toxic element in both saline and sodic soils, and glycophytic plants should preferably not over-accumulate $Na^+$ in leaves under salinity stress. Therefore, restricting $Na^+$ transfer from the roots to shoots can be an essential mechanism for the salinity tolerance of glycophytes (Ismail and Horie 2017). Conversely, $K^+$ is a positive element, which functions in maintaining a favorable cytosolic environment for cellular metabolism, including the regulation of membrane potential and playing a role in signaling against salinity stress (Hauser and Horie 2010, Rubio *et al.* 2020, Shabala 2017). Considering that $K^+/Na^+$ selectivity can be competitive at the level of membrane transport in plant cells (Blumwald 2000), the consequence tends to appear in tissue $K^+/Na^+$ contents. Therefore, $Na^+$ and $K^+$ contents and their ratios ($K^+/Na^+$ or $Na^+/K^+$) in tissues often became target-indices for seeking loci that have a large impact on salinity stress tolerance (**Table 2**). In some cases, significant correlation between such indices and salinity tolerance or actual impacts of the alteration of $Na^+/K^+$ homeostasis on salinity tolerance have been proven (ex. Cao *et al.* 2019, Do *et al.* 2016, Lin *et al.* 2004, Liu *et al.* 2016, Long *et al.* 2013, Munns *et al.* 2012, Xue *et al.* 2009, Zhang *et al.* 2018, 2019a). Consequently, the responsible genes for salinity stress-related QTLs or loci encode either a membrane transporter or a signaling protein that has been shown to play a key role in salinity tolerance in a $Ca^{2+}$-dependent manner except in the case of a recent study of alkali stress tolerance in rice (**Table 2**; Li *et al.* 2020).

Like $Na^+$, $Cl^-$ is also a dominant toxic element in saline soil, although it is an essential micronutrient for plants under non-stress conditions (Teakle and Tyerman 2010). Since most studies on salinity tolerance QTLs have focused

**Table 2.** Major salnity stress-related QTLs or genetic loci, which were identified with root-based phenotype-indices. Note that QTLs or loci identified by $Na^+$, $K^+$ contents and their ratios in leaves or shoots are included as indices for the function of roots as $K^+/Na^+$ selectivity of roots can be highly relevant to the accumulation of those ions in aerial parts. In addition, QTLs under hypoxia (in barley) are also included due to significant relevance of ROS production to salinity stress (see the text)

| Plant species used | Source | Index | Identified chromosome | Name of QTL or locus | Percentage of phenotypic variance explained | Candidate responsible gene (*) or identifined responsible gene (†), and its encoded protein | References |
|---|---|---|---|---|---|---|---|
| Arabidopsis (*Arabidopsis thaliana*) | RILs of Bay-0 × Shahdara | shoot Na/K ratio | 2 | n/a | 10.0% | *AtCIPK16* (†); CBL-interacting protein kinase family | Roy *et al.* 2013 |
| | RILs of Ler × Cvi | Relative root length under salt stress/ control conditions | 2, 5 | *QTL2*, *QTL5-2* | 10.0–11.0% | | Kobayashi *et al.* 2016 |
| Barley (*Hordeum vulgare*) | DHLs of salt tolerant CM72 × Gairdner | shoot Na/K ratio | 6 | *qNAK6s* | 29.8% | | Xue *et al.* 2009 |
| | DHLs of landrace Sahara 3771 × Clipper3 | shoot Na contents | 1HL | *HvNax4* | 35–84% | *HvCBL4* (*); Calcineurin B-like protein family | Lonergan *et al.* 2009, Rivandi *et al.* 2011 |
| | DHLs of wild barley CPI-71284-48 × cv. Barque | shoot Na contents | 7H | *HvNax3* | 51.0% | | Shavrukov *et al.* 2010 |
| | 192 barley accessions | shoot Na contents | 4H | n/a | 22.4% | | Long *et al.* 2013 |
| | | shoot K contents | 4H | n/a | 39.0% | | |
| | | shoot Na/K ratio | 4H | n/a | 39.0% | | |
| | | shoot Mg contents | 6H | n/a | 26.0% | | |
| | | shoot Cl contents | 4H | n/a | 39.0% | | |
| | DHLs of salt tolerant Steptoe × Morex | root dry weight & ion contents in roots and shoots | 2H | n/a | 17.9–23.8% | | Nguyen *et al.* 2013 |
| | DHLs of salt tolerant Nure × Tremois | root elongation | 7HS | *QTL5* | 38.6–45.3% | | Xue *et al.* 2017 |
| | DHLs of landrace TX9425 × cv. Naso Nijo | plasma membrane potential of root epidermal cells under hypoxia | 2H | *QMP.TxNn.2H* | 22.0% | | Gill *et al.* 2017 |
| | DHLs of landrace TX9425 × cv. Naso Nijo | root ROS contents under hypoxia | 2H | *QSO.TxNn.2H*, *QHP.TxNn.2H* | 23.0–24.0% | | Gill *et al.* 2019 |
| Cotton (*Gossypium hirsutum*, *G. tomentosum*) | an interspecific cross population of CRI-12 × wild species $AD_3$-00 | Longest root length | 16 | *qRL-Chr16-1* | 11.9, 18.4% | | Oluoch *et al.* 2016 |
| Grapevine (*Vitus champinii*, *V. riparia*, *V. berlandieri*, *V. rupestris*) | a hybrid rootstocks population: K51-40 × 140 Ruggeri | shoot Na contents | 11 | *NaE* | 72.0% | *VisHKT1;1* (*); High affinity $K^+$ transporter family subgroup 1 ($Na^+$ selective transport) | Henderson *et al.* 2018 |
| Maize (*Zea mays*) | RILs of salt tolerant Zheng58 × Chang7-2 | leaf Na contents | 3 | *ZmNC1* | n/a | *ZmHKT1* (†); High affinity $K^+$ transporter family subgroup 1 ($Na^+$ selective transport) | Zhang *et al.* 2018 |
| | RILs of maize W22 × teosinte accession 8759 | shoot K contents | 1, 4, 5 | *qKC1*, *qKC2*, *qKC3* | 7.9, 22.9, 6.7%, | *ZmHKT2* (*qKC3*) (†); High affinity $K^+$ transporter family subgroup 2 ($Na^+$-$K^+$ co-transport) | Cao *et al.* 2019 |
| | | shoot Na contents | 1, 4, 3, 10 | *qNaC1*, *qNaC2*, *qNaC3*, *qNaC4* | 7.8, 25.9, 4.4, 5.8% | | |
| | 513 maize inbred lines; RILs of HuangC × X178 | shoot Na contents | 4 | *ZmNC2* (*qNaC4-1*) | 11.0% | *ZmHAK4* (†); KT/HAK/KUP-type high affinity $K^+$ transporter family ($Na^+$-selective transport) | Zhang *et al.* 2019a |
| Rice (*Oryza sativa*, *O. rufipogon*) | RILs of IR29 × salt tolerant Pokkali | Shoot Na/K ratio | 1 | *Saltol* | 43.0% | | Bonilla *et al.* 2002, Thomson *et al.* 2010 |
| | RILs of salt tolerant Nona Bokra × Koshihikari | shoot Na contents | 7 | *SNC7* | 48.5% | | Lin *et al.* 2004 |
| | | shoot K contents | 1 | *SKC1* | 40.1% | *OsHKT1;5* (*); High affinity $K^+$ transporter family subgroup 1 ($Na^+$ selective transport) | Lin *et al.* 2004, Ren *et al.* 2005 |
| | RILs of *O. rufipogon* Griff. × Teqing | Relative root dry weight under salt stress/control conditions | 6, 7, 10 | *qRRW6*, *qRRW7*, *qRRW10* | 33.0, 22.3, 22.7% | | Tian *et al.* 2011 |

**Table 2.** (continued)

| Plant species used | Source | Index | Identified chromosome | Name of QTL or locus | Percentage of phenotypic variance explained | Candidate responsible gene (*) or identifined responsible gene (†), and its encoded protein | References |
|---|---|---|---|---|---|---|---|
| Rice (*Oryza sativa*, *O. rufipogon*) | RILs of salt tolerant Jiucaiqing × IR26 | shoot Na contents | 11 | *qSNC11* | 14.9–16.1% | | Wang *et al.* 2012a |
| | RILs of salt-tolerant At354 × Bg352 | shoot Na/K ratio | 1, 2, 3, 4, 10 | *qSNK1*, *qSNK2*, *qSNK3*, *qSNK4.1*, *qSNK4.2*, *qSNK10* | 21.8, 24.6, 23.5, 14.2, 13.9% | | Gimhani *et al.* 2016 |
| | | shoot Na contents | 1, 2, 3, 5 | *qSNC1*, *qSNC2*, *qSNC3*, *qSNC5* | 17.9, 19.9, 21.5, 14.2% | | |
| | | shoot K contents | 1, 2, 4 | *qSKC1*, *qSKC2*, *qSNC4* | 19.0, 19.4, 12.4% | | |
| | | Root length | 6 | *qRL6* | 14.6% | | |
| | | Root fresh weights | 2, 3, 8 | *qRFW2*, *qRFW3*, *qRFW8* | 29.1, 19.6, 21.8% | | |
| | | Root dry weights | 2, 4, 5 | *qRDW2*, *qRDW4.2*, *qRDW5* | 24.5, 18.4, 20.1% | | |
| | RILs of salt tolerant Pokkali × Bengal | shoot K contents | 1 | *qK1.3863* | 10.7% | | De Leon *et al.* 2017 |
| | | shoot Na/K ratio | 3 | *qNaK3.32* | 11.3% | | |
| | 390 rice accessions | root Na contents | 4 | *RNC4* | 15.0% | *OsHKT1;1* (†); High affinity $K^+$ transporter family subgroup 1 ($Na^+$ selective transport) | Campbell *et al.* 2017 |
| | RILs of alkali tolerant Xiaobaijingzi × Kongyu131 | Root length under alkali stress | 11 | *qAT11* | 11.4% | *LOC_Os11g37390* (*); F-box domain containing protein | Li *et al.* 2020 |
| Tomato (*Solanum lycopersicum*, *S. cheesmaniae*) | RILs of salt toletant Fosberg × var. cerasiforme | Leaf K contents | 7 | *lkc7.1* | 25.0–27.0% | | Villalta *et al.* 2008 |
| | | Leaf Na contents | 1 | *lnc1.1* | 10.0% | *LeNHX3* (*); $Na^+$ (Cation)-$H^+$ exchanger family | |
| | | | 7 | *lnc7.1* | 43.0% | | |
| | | | 8 | *lnc8.1* | 12.0% | | |
| | | Leaf Na/K ratio | 7 | *lkn7.1* | 30.0% | | |
| | | Total Na contents in shoots | 7 | *tn7* | 23.0–24.0% | | |
| Wheat (*Triticum aestivum*; *T. turgidum*; *T. monococcum*) | Disomic substitution lines: bread wheat × durum wheat cv. Langdon | Leaf K/Na ratio | 4D | *Kna1* | 60.0% | *TaHKT1;5-D* (†); High affinity $K^+$ transporter family subgroup 1 ($Na^+$ selective transport) | Gorham *et al.* 1990, Dubcovsky *et al.* 1996, Byrt *et al.* 2014 |
| | Durum wheat Line 149; T. monococcum (C68-101) × cv. Marrocos | Leaf Na contents | 2A, 5A | *Nax1*, *Nax2* | 38.0%, n/a | *TmHKT1;4-A2* (*Nax1*) (*); *TmHKT1;5-A* (*Nax2*) (*); High affinity $K^+$ transporter family subgroup 1 ($Na^+$ selective transport) | Lindsay *et al.* 2004, James *et al.* 2006, Huang *et al.* 2006, Byrt *et al.* 2007 |

on $Na^+$ but not $Cl^-$, the information regarding QTLs linked to $Cl^-$ is considerably less than that for $Na^+$. Correlation of $Cl^-$ exclusion (i.e., low $Cl^-$ contents in leaves or shoots) with salinity tolerance was reported in several plant species including soybean, *Hordeum marinum*, and *M. truncatula* (Teakle and Tyerman 2010, and references therein). In addition, increased sensitivity to excessive amount of $Cl^-$ was observed in some barley cultivars (Tavakkoli *et al.* 2011). Analyses of barley accessions revealed a significant negative correlation of $Cl^-$ content in the shoots and roots to salinity tolerance and led to the identification of a QTL controlling the shoot $Cl^-$ content (**Table 2**; Long *et al.* 2013). Thus, focusing on $Cl^-$-related indices in the analysis of salinity QTLs might unravel novel mechanisms for $Cl^-$ homeostasis during salinity stress.

The number of QTLs based on root biomass under salinity stress are comparatively less and their responsible genes remain largely unknown (**Table 2**). A genome-wide association study (GWAS) on the average relative root length of more than 200 natural *A. thaliana* accessions highlighted the 10 most significant single nucleotide polymorphisms (SNPs) in two different NaCl conditions, which included genes of ATP-binding cassette B10 and vacuolar $H^+$-ATPase subunit A isoform 2, implying the importance of auxin efflux and regulation of vacuolar $H^+$-ATPase on the maintenance of root growth under salinity stress (Kobayashi *et al.* 2016). Furthermore, a recent QTL mapping on the root length of rice under sodic alkali stress

identified a strong QTL *qAT11* on chromosome 11 (**Table 2**; Li *et al.* 2020). Subsequent linkage mapping and GWAS led to the narrowing down of the candidates to three genes and, among which the most significant candidate was *LOC_Os11g37390*, which encodes an F-box domain containing protein (**Table 2**; Li *et al.* 2020). Unlike the QTLs governing ion accumulation in tissues, one can expect more to isolate key genes that contribute to hyperosmotic stress tolerance from root biomass-based salinity QTLs. In fact, as salinity-induced osmotic stress impairs water uptake and distribution (Horie *et al.* 2012, Munns and Tester 2008), the coping mechanisms can be partly expected to overlap with those for drought stress tolerance. Therefore, to gain insight into the coping mechanism of salinity-triggered osmotic stress, investigating the effect of QTLs for drought stress tolerance under salinity stress might be an effective strategy. Indeed, some QTLs for drought stress tolerance in pearl millet were shown to confer salinity stress tolerance as well (Sharma *et al.* 2011, 2014). To promote detection of hidden QTLs for osmotic adjustments under salinity, developing novel approaches such as a high-throughput imaging technology might be a key (Al-Tamimi *et al.* 2016). Yichie *et al.* (2018) successfully assessed the growth and water-use efficiency of wild rice accessions under salinity stress by using high-throughput imaging and phenotyping, which, for example, could have a potential to be extended to the screening of osmotic QTLs.

### *1.2. Interaction of soil waterlogging-related QTLs and salinity tolerance*

Recent studies indicated the detection of QTLs that are highly correlated with hypoxia tolerance (**Table 2**; Gill *et al.* 2017, 2019). Hypoxia is often caused by soil waterlogged conditions and triggers a reduction in ATP production through respiration in root cells. Therefore, waterlogging stress damages roots and eventually impairs the supply of water and nutrients from the roots to shoots. A significant reduction in ATP production has a large impact on broad cell metabolism, including membrane potential and pH homeostasis, by decreasing the activity of $H^+$-ATPase at the plasma membrane (Gill *et al.* 2017). Oxygen-deprived conditions facilitate the generation of ROS, which further reduce the viability of plants by damaging biological molecules and reducing enzymatic activity (Bailey-Serres and Chang 2005). Salinity stress is known to induce ROS, and the detoxification of ROS is one of the critical mechanisms for salinity tolerance (Bose *et al.* 2014). Waterlogging stress concurrently occurs with salinity stress and salinity-induced damages tend to be exacerbated in combination with waterlogging (Ma *et al.* 2015). Indeed, a combination of salinity and waterlogging caused greater damage on barley plants than with salinity stress alone (Ma *et al.* 2015). By using a double haploid (DH) population of salt-tolerant and salt-sensitive cultivars, Ma *et al.* (2015) identified salinity tolerance QTLs at chromosomes 2H and 5H under salinity stress with or without waterlogging. The same research group later applied distinct methods to identify QTLs related to salinity and waterlogging stress in barley. In one method, a positive relationship was found between the maintenance of highly negative membrane potential (MP) and tolerance of the plant to salinity and waterlogging stress, based on which a major QTL maintaining higher MP in root epidermal cells under hypoxia has been detected using a barley DH population (**Table 2**; Gill *et al.* 2017). Subsequently, in another method, the same QTL identified in the aforementioned MP-based screening was detected by measuring ROS content in the roots of the same barley DH population (**Table 2**; Gill *et al.* 2019). Interestingly, these QTLs were found to be located at chromosome 2H, which was deduced to be the same locus as that for the QTLs at chromosome 2H reported by Ma *et al.* (2015). These findings reinstate the link between salinity and waterlogging stress. Wang *et al.* (2019) recently proposed novel root-based high-throughput phenotyping methods to screen germplasm for oxidative stress tolerance, which can be expected to be utilized for identifying salinity tolerance QTLs in different germplasm as well as different plant species.

## 2. Essential root-based mechanisms contributing to ion homeostasis and salinity tolerance

### *2.1. Maintenance of $Na^+$ exclusion and $K^+$ accumulation in shoots, mediated by HKT and HAK genes*

Genes that belong to the High affinity $K^+$ Transporter (*HKT*) family (Schachtman and Schroeder 1994, Rubio *et al.* 1995) have been shown to be responsible for important salinity QTLs in monocot crops and woody grapevine (**Table 2**). *HKT* genes can be divided into at least two subgroups, HKT1s and HKT2s, which exhibit $Na^+$ selective transport and $Na^+$-$K^+$ co-transport, respectively (Horie *et al.* 2009). Lin *et al.* (2004) reported two major QTLs in rice for $K^+$ and $Na^+$ contents of shoots under salinity (**Table 2**). The candidate responsible gene for one of the QTLs, named *SKC1*, has been found to be the *OsHKT1;5* gene encoding an $Na^+$ selective plasma membrane transporter (Ren *et al.* 2005). *SKC1*-dependent phenotypes of near isogenic lines (NILs) that maintain the locus from the salt tolerant Nona Bokra were suggested to be attributed to OsHKT1;5-dependent $Na^+$-selective transport in the root vasculature, in particular, xylem (Ren *et al.* 2005). The physiological role of OsHKT1;5 in rice under salinity was deduced to be similar to the proposed model of a $Na^+$ selective transporter AtHKT1;1 in Arabidopsis (Sunarpi *et al.* 2005), in which HKT1-mediated $Na^+$ absorption at the xylem parenchyma cells prevents $Na^+$ loading (and thus $Na^+$ exclusion from leaves), which in turn could accelerate $K^+$ loading (and thus $K^+$ accumulation in leaves), stimulated by $Na^+$ influx-induced membrane depolarization (**Fig. 1**; Horie *et al.* 2009 and references therein). More detailed physiological roles of OsHKT1;5 in salinity tolerance of rice have been proposed by Kobayashi *et al.* (2017). Based on phenotyping of

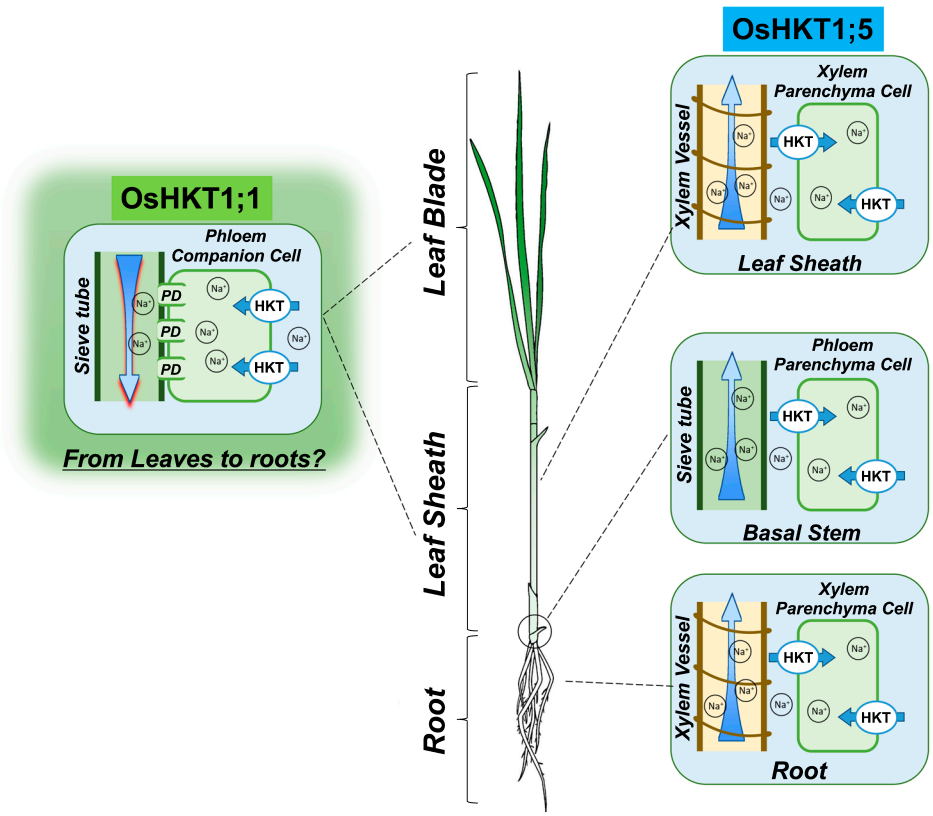


**Fig. 1.** Schematic drawings of OsHKT1-mediated $Na^+$ exclusion in rice (The rice model). OsHKT1;5 was proposed to function in $Na^+$ unloading from the xylem in roots and leaf sheaths and from the phloem in basal nodes at the vegetative growth stage under salinity stress. Note that the phloem of the tissue in basal nodes where OsHKT1;5 functions appear to have an unusual structure: phloem companion cells next to sieve tubes are lacking, but phloem parenchyma cells are retained (Kobayashi *et al.* 2017). A suggested function of OsHKT1;1-mediated $Na^+$ recirculation needs to be elucidated.

stable *OsHKT1;5* mutants and immuno-staining of rice tissues, OsHKT1;5 was proposed to function in $Na^+$ exclusion from leaves by mediating $Na^+$ unloading from xylem vessels in not only roots but also leaf sheaths, with a novel unexpected function in $Na^+$ unloading from the phloem in basal nodes (**Fig. 1**; Kobayashi *et al.* 2017).

Comparing the proposed functions of *OsHKT1;5* in rice, it is interesting to note that the $Na^+$ unloading function of OsHKT1;5 in roots and sheaths well overlaps with that of *TmHKT1;4-A2*, the candidate responsible gene of the *Nax1* QTL in durum wheat (**Table 2**; Huang *et al.* 2006, James *et al.* 2006, Lindsay *et al.* 2004). Interestingly, another important QTL, *Nax2*, was also identified in durum wheat and found to encode *TmHKT1;5-A*, which functions in $Na^+$ unloading from the xylem only in roots (**Table 2**; Byrt *et al.* 2007, James *et al.* 2006). The *Nax2* locus was suggested to be homoeologous to the *Kna1* locus that has long been known to harbor a key gene controlling leaf $Na^+$ exclusion and a high $K^+/Na^+$ ratio in the leaves of bread wheat under salinity stress (**Table 2**; Byrt *et al.* 2007). Byrt *et al.* (2014) showed that *TaHKT1;5-D*, the product of which shows $Na^+$-selective transport as *TmHKT1;5-A*, is most likely to be the causal gene of *Kna1* (**Table 2**). In fact, the *Nax2*-mediated leaf $Na^+$ exclusion trait was shown to confer salinity tolerance with a significant increase in yield to a durum wheat cultivar at the level of salt-affected field assessment (Munns *et al.* 2012).

Rice also has the *OsHKT1;4* gene that encodes a plasma membrane-localized $Na^+$ selective transporter (Suzuki *et al.* 2016). The expression of *OsHKT1;4* in roots was relatively weak, and reduction in *OsHKT1;4* expression via RNAi did not disturb both $Na^+$ and $K^+$ accumulation in any tissues of a japonica rice cultivar in the vegetative growth stage (Campbell *et al.* 2017, Suzuki *et al.* 2016). However, a recent study on transgenic rice lines expressing artificial microRNA indicated the involvement of OsHKT1;4 in $Na^+$ unloading from the xylem in roots under a wide range of external $Na^+$ concentrations (Khan *et al.* 2020). Further studies are required to completely understand the function and role of OsHKT1;4 in rice.

The mechanism of HKT1-mediated $Na^+$ exclusion under salinity stress in monocot plants shows a more complicated view. A genetic study using diverse rice accessions led to the discovery of a novel locus named *RCN4* that governs root $Na^+$ contents and root $Na^+/K^+$ ratios under salinity stress (**Table 2**; Campbell *et al.* 2017). The causal gene for the *RCN4* locus was concluded to be *OsHKT1;1*, which encodes an inward-rectified $Na^+$ selective transporter

(Campbell *et al.* 2017). In fact, the involvement of OsHKT1;1 in $Na^+$ exclusion of rice under salinity has also been reported by independent groups (Takagi *et al.* 2015, Wang *et al.* 2015). According to phenotype assessment of the *oshkt1;1* mutant plant, OsHKT1;1 was suggested to mediate "$Na^+$ recirculation from shoots to roots" via the phloem to exclude $Na^+$ from leaves (**Fig. 1**; Wang *et al.* 2015). Notably, HKT1-mediated $Na^+$ recirculation has been proposed in the AtHKT1;1-mediated salt tolerance mechanism in Arabidopsis (Hauser and Horie 2010 and references therein). In barley, a molecular physiological study showed that HvHKT1;1-mediated $Na^+$ selective transport contributes to the lowering of $Na^+$ accumulation in both roots and leaves of a Tibetan wild barley accession (Han *et al.* 2018). In contrast, the role of the HKT1;5 transporter in salinity tolerance of barley plants is yet to be determined as contrasting results have been reported (Huang *et al.* 2020, van Bezouw *et al.* 2019).

Studies of salinity tolerance in maize (*Zea mays*) also led to the identification of important genes. A QTL named *ZmNC1* was discovered by leaf $Na^+$ content analysis of a recombinant inbred line (RIL; **Table 2**; Zhang *et al.* 2018). The *ZmHKT1* gene was found to be localized within the QTL (Zhang *et al.* 2018). Phenotyping of CRISPR-Cas9-derived transgenic maize (*ZmHKT1*$^{Crispr}$) mutants revealed that ZmHKT1 mediates $Na^+$ unloading from the xylem in root stelar cells as other HKT1 transporters such as AtHKT1;1 and OsHKT1;5 (**Fig. 1A**; Horie *et al.* 2009). Moreover, an independent salinity QTL study using RILs of teosinte and maize identified several QTLs related to $Na^+$ and $K^+$ contents in shoots (**Table 2**; Cao *et al.* 2019). One of the QTLs, *qKC3*, was determined to be the *ZmHKT2* gene that encoded a $Na^+$ and $K^+$ co-transporter based on the assessments of *ZmHKT2*$^{Crispr}$ mutants (Cao *et al.* 2019). In this case, the proposed mechanism is unique such that the maize-derived ZmHKT2, which exhibits significant reduction in the $K^+$-transport activity compared with that from teosinte, unloads less $K^+$ from xylem under salinity stress, which accounts for an increase in the $K^+$ content in the xylem sap and thus in leaves (Cao *et al.* 2019). More recently, the same research group identified the *ZmNC2* locus by conducting a GWAS for $Na^+$ content using 513 maize inbred lines (Zhang *et al.* 2019a). The causal gene for the *ZmNC2* locus was deduced to encode a KT/HAK/KUP-type high affinity $K^+$ transporter, ZmHAK4, which appears to function as a $Na^+$-selective transporter under salinity stress (Zhang *et al.* 2019a). Based on the phenotypes of *ZmHAK4*$^{Crispr}$ mutants and its expression in root stelar cells, the authors proposed a model that ZmHAK4 mediates $Na^+$ unloading to reduce $Na^+$ accumulation in the leaves of maize under salinity stress (Zhang *et al.* 2019a), similar to the proposed function of HKT1 transporters in the root xylem (**Fig. 1**). These findings suggest that $Na^+$ unloading in roots is mediated by multiple transporter families to circumvent the triggering of $Na^+$ toxicity in leaves.

### *2.2. The Saltol QTL-mediated salt tolerance and its interaction with the SKC1 QTL (OsHKT1;5) in rice*

A major QTL associated with salt tolerance of rice seedlings, named *Saltol*, has been identified on chromosome 1 by using RILs derived from salt-tolerant Pokkali and salt-sensitive IR29 (**Table 2**; Bonilla *et al.* 2002, Thomson *et al.* 2010). This QTL had a major influence on $Na^+$ exclusion from rice leaves. One highly salt-tolerant RIL (FL478) containing the *Saltol* QTL contributed to increased salt tolerance of some cultivars as a donor in breeding programs (for more details, see review by Ismail and Horie 2017). On the other hand, the effect of the *Saltol* QTL was found to be "not straightforward" (Thomson *et al.* 2010), and its responsible gene(s) remain to be determined. In fact, by using a different rice RIL population, Haq *et al.* (2010) identified QTLs for $Na^+$ contents and $K^+/Na^+$ ratio in shoots under salinity stress in an overlapping region with the *Saltol* QTL. However, two QTL clusters were detected in the region with one more relatively minor QTL immediately adjacent to the overlapping genomic region. Furthermore, transcriptome analysis based on the Affymetrix rice genome array performed using FL478 and IR29 suggested that the source of the core of the *Saltol* QTL in FL478 appears to be derived from the sensitive IR29 (Walia *et al.* 2005). Note that the *Saltol* QTL includes the above-mentioned *SKC1* (*OsHKT1;5*) locus and this gene is expected to be at least one of the causal genes. However, a recent study on NIL-*SKC1* plants indicated that the NIL plants showed increased sensitivity to moderate salinity of 80 mM NaCl with unexpected overexpression of *OsHKT1;5* in roots (Al Nayef *et al.* 2020). Furthermore, electrophysiological experiments using the microelectrode ion flux estimation (MIFE) technique indicated substantial reduction in the activity of $Na^+$ reabsorption (i.e., $Na^+$ unloading) in the xylem of NIL-*SKC1* (Al Nayef *et al.* 2020). These results altogether imply the involvement and complex interactions of several different genes, including *OsHKT1;5*, in the mechanism of salinity tolerance mediated by the QTLs, and further research is needed to elucidate the underlying molecular mechanisms.

### *2.3. Salinity QTLs in soybean and responsible genes*

HKT1-mediated $Na^+$ exclusion in roots was shown to be an important salinity tolerance mechanism even in woody grapevine based on the analysis of the *NaE* QTL identified using a hybrid rootstock population (**Table 2**; Henderson *et al.* 2018). However, the tolerance mechanism against salinity so far looks distinct in the dicot soybean. Many salinity QTLs, including a major QTL for sodic-alkaline stress, have been identified by assessing the degree of tolerance and measuring the leaf chlorophyll content (Zhang *et al.* 2019b and references therein, Tuyen *et al.* 2010). A major QTL for salinity stress was repeatedly detected on chromosome 3 (Guan *et al.* 2014, and references therein). Guan *et al.* (2014) isolated the causal gene by map-based cloning, and it was found to encode a cation/$H^+$ exchanger family

member protein, *Glyma03g32900* and was named *GmSALT3* (for *Glycine max* salt tolerance-associated gene on chromosome 3). The expression of GmSALT3 in the endoplasmic reticulum membrane of roots was shown to be highly associated with $Na^+$ exclusion from the shoots and greater salt tolerance (Guan *et al.* 2014). Independent studies based on whole-genome *de novo* sequencing and a map-based cloning strategy have also isolated the same gene as the causal gene (*GmCHX1* and *Ncl*, respectively; Qi *et al.* 2014, Do *et al.* 2016). Later GmSALT3 was shown to contribute to $Cl^-$ exclusion from leaves as well as $Na^+$ exclusion (Do *et al.* 2016, Liu *et al.* 2016) and improve soybean yield (higher seed weights) under saline field conditions (Liu *et al.* 2016). Detailed transport and exclusion mechanisms mediated by GmSALT3/GmCHX1/Ncl have not yet been elucidated.

Recently, a new QTL named *qST8* for the sensitivity to salinity stress at the soybean germination stage was mapped on chromosome 8 (Zhang *et al.* 2019b). In combination with a GWAS analysis, the candidate responsible gene was identified (*Glyma08g102000*) and found to encode a cation diffusion facilitator (CDF) family protein (GmCDF1). Transgenic hairy roots and haplotype analyses indicated that the function of GmCDF1 is negatively correlated with salinity tolerance of soybean (Zhang *et al.* 2019b).

### *2.4. Important factors in signaling under salinity stress*

The importance of a CIPK (CBL-interacting protein kinase)-CBL (Calcineurin B-like $Ca^{2+}$ sensor protein) complex in the mechanism of plant salt tolerance has been established by studies of the regulation on SOS1 (plasma membrane $Na^+$-$H^+$ antiporter) by the SOS2 (CIPK24)-SOS3 (CBL4) complex in Arabidopsis (Zhu 2002). Evidence of the involvement of a CIPK-CBL complex in resistant mechanisms to various biotic or abiotic stress has been provided (Ma *et al.* 2020). A CIPK protein and a CBL $Ca^{2+}$ sensor were identified as candidate responsible genes for the QTLs that control $Na^+/K^+$ homeostasis in Arabidopsis and barley, respectively (**Table 2**). An impact of a key signaling molecule on plant salt tolerance and breeding of tolerant cultivars has also been highlighted by Takagi *et al.* (2015). A salt-tolerant japonica rice mutant *hst1*, derived from an ethylmethanesulfonate (EMS)-mutagenized population, harbored a single recessive mutation in the gene *Os06g0183100*, which encodes a B-type response regulator OsRR22 (Takagi *et al.* 2015). *OsRR22* expression was shown to complement a loss-of-function mutant of the Arabidopsis type-B RR family, which suggested the involvement of OsRR22 in cytokinin signaling (Tsai *et al.* 2012). The *OsHKT1;1* gene was identified as one of the highly expressed genes in the *hst1* mutant in comparison with wild-type (Takagi *et al.* 2015). These findings imply an important role of cytokinin in the regulation of $Na^+$ homeostasis and salinity tolerance of rice. Recently, a salt-responsive $Ca^{2+}$ sensor calmodulin in barley, HvCaM1 was shown to be a negative regulator of salt tolerance in barley plants (Shen *et al.* 2020). RNAi-mediated *HvCaM1* knockdown rendered the barley lines more salt tolerant with lower $Na^+$ accumulation in shoots and higher expression of the *HvHKT1;1* gene, suggesting that, at least in part, upregulated *HvHKT1;1* conferred salt tolerance to the transgenic lines (Shen *et al.* 2020).

## 3. Important questions remain to be elucidated for understanding the physiological functions of root under salinity stress

### *3.1. $Na^+$ influx mediated by non-selective cation channels and possible involvement of plasma membrane intrinsic proteins*

Several important salt tolerance mechanisms and key genes involved have been discovered over the past few decades. Nevertheless, some more questions in relation to $Na^+$ homeostasis remain to be addressed. One of them is the plasma membrane protein which can be a major entry for $Na^+$ under salinity stress. Previous electrophysiological studies have implicated that non-selective cation channels (NSCCs) are likely to be the primary candidates that mediate $Na^+$ influx into roots subjected to salinity stress (see Demidchik and Maathuis 2007). However, the molecular identity of NSCCs still remains elusive. Cyclic nucleotide gated channels and glutamate receptors are thought to be potential candidates (Ismail and Horie 2017, and references therein), but crucial evidence for the molecular identity is lacking. Recently, water channels called plasma membrane intrinsic protein (PIP) in Arabidopsis, AtPIP2;1 and AtPIP2;2, were reported to show ion channel activity for $Na^+$ and $K^+$ when the external $Ca^{2+}$ concentration is low (Byrt *et al.* 2017, Kourghi *et al.* 2017). Given the highly abundant nature of the protein and the sensitivity of AtPIP2;1-mediated $Na^+$ channel activity in *Xenopus laevis* oocytes to low pH and external $Ca^{2+}$ concentration, which was found to be similar to the features of $Na^+$ currents mediated by NSCCs in root protoplasts from Arabidopsis, AtPIP2;1 was considered as a candidate for NSCCs in plant roots (Byrt *et al.* 2017, McGaughey *et al.* 2018). More recently, like AtPIP2;1, HvPIP2;8 in barley was shown to exhibit ion channel activity for $Na^+$ and $K^+$ in a $Ca^{2+}$ sensitive manner (Tran *et al.* 2020). In addition, OsPIP1;3 in rice was shown to mediate the transport of nitrate anions in mammalian HEK293 cells in addition to being a water channel (Liu *et al.* 2020). Whether PIP aquaporins from different plant species also show dual ion and water permeability and whether these features are reminiscent of the nature of NSCCs in plant cells need to be elucidated in the future research.

### *3.2. Does futile $Na^+$ cycling or rapid transmembrane $Na^+$ cycling occur across the plasma membrane?*

When subjected to soil salinity, plants need to pay significant energy costs for osmotic and ionic adjustments, which has a large influence on crop production in saline or sodic

soils (Munns and Gilliham 2015). Energy costs associated with plant salinity tolerance mechanisms have recently been highlighted with extensive reviews (ex. See Munns *et al.* 2020a, 2020b, Shabala *et al.* 2020, Tyerman *et al.* 2019).

Under salinity stress, it is thought that a large amount of $Na^+$ rapidly and passively enters the epidermal and cortex cells of plant roots. Cytosolic $Na^+$ concentrations are maintained low by extruding $Na^+$ ions out of the cells across the plasma membrane against the gradient of electrochemical potential and by sequestering $Na^+$ ions into vacuoles. The extrusion of $Na^+$ is exclusively carried out by the plasma membrane transporter SOS1 owing to the electrochemical $H^+$ gradient (Zhu 2002), which is established by the $H^+$-pump ATPase by using the energy from ATP hydrolysis (**Fig. 2A**). Previous studies have shown that $Na^+$ efflux/influx ratios across the plasma membrane increase in accordance with an increase in the external $Na^+$ concentration and eventually get to nearly 1, suggesting that the cycling of $Na^+$ across the plasma membrane is driven using a large amount of energy (for details of the energy cost, see Munns *et al.* 2020a). These processes look at a glance futile and energetically costly (**Fig. 2A**; Britto and Kronzucker 2006, 2015). Such futile $Na^+$ cycling is referred to as "Rapid Transmembrane Sodium Cycling (RTSC)" (Britto and Kronzucker 2015). The $^{24}Na^+$ tracer experiments performed using a salt-tolerant variety of rice Pokkali and a sensitive cultivar IR29 indicated that unidirectional influx and efflux of $Na^+$ in IR29 were more than 4 times higher than those in Pokkali at the external $Na^+$ concentration of 25 mM (Malagoli *et al.* 2008). Furthermore, the estimation of the respiratory oxygen consumption rate at 25 mM $Na^+$ revealed that the amount of $O_2$ consumed in the roots of IR29, but not of Pokkali, is insufficient for driving the active $Na^+$ efflux via the $Na^+/H^+$ antiporters at the plasma membrane (Britto and Kronzucker 2015, Malagoli *et al.* 2008). These phenomena lead to an assumption that an unknown transport mechanism might function in the root $Na^+$ extrusion. This mechanism would be coupled with the passive fluxes of some ion and/or vesicular transport of $Na^+$ (**Fig. 2A**; Flowers *et al.* 2019, Malagoli *et al.* 2008). Revealing the detailed mechanism of $Na^+$ efflux out of the root under salinity stress as well as understanding how plants manage the cost for this process are crucial questions to be addressed.

### *3.3. The problem of energy cost for futile sodium leak in vacuoles*

The $Na^+/H^+$ antiport activity across the tonoplast via transporters such as NHX-type cation/$H^+$ antiporters is crucial for plant salt tolerance since it promotes $Na^+$ sequestration into vacuoles as has been extensively investigated and reviewed (e.g., see Bassil and Blumwald 2014, and references therein, Bassil *et al.* 2019). A salinity QTL analysis performed using tomato detected the *lnc1.1* locus, the causal gene of which was deduced to be *LeNHX3*, which

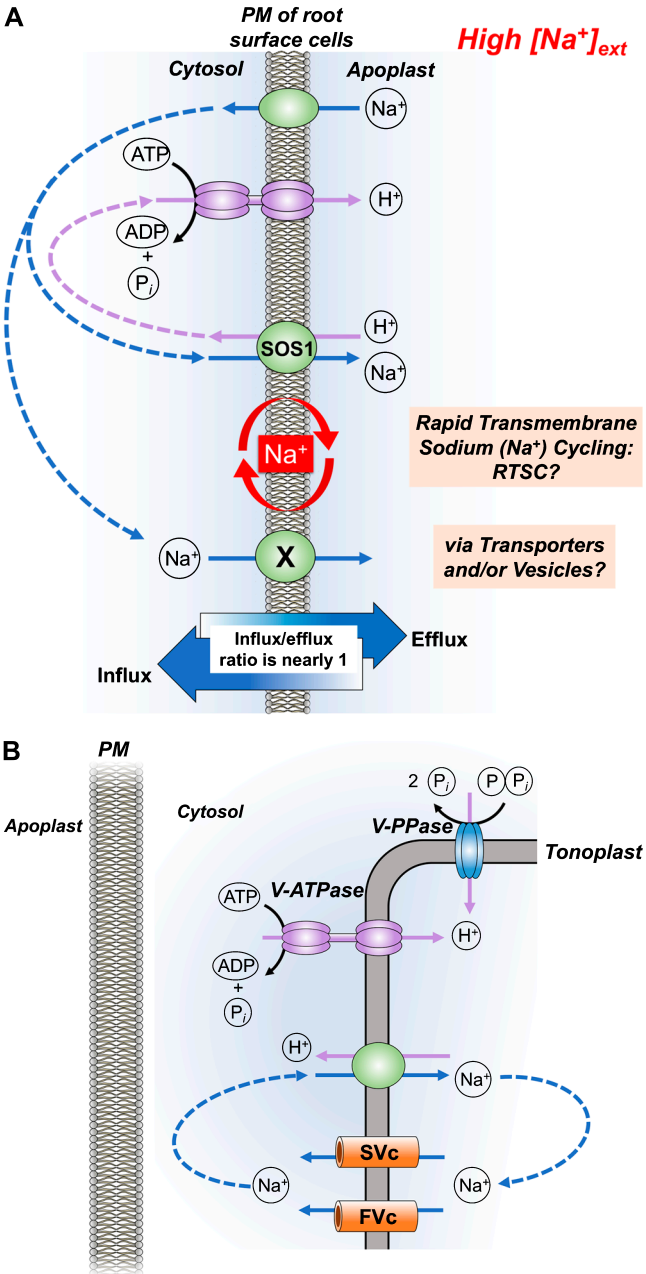


**Fig. 2.** Schematic drawings of the energy-consuming $Na^+$ transport mechanisms in plants subjected to salinity stress, which need to be elucidated. (A) A hypothesis of futile $Na^+$ cycling in the root epidermal cells under salinity stress, which is called Rapid Transmembrane Sodium Cycling. "X" represents an unknown transporter(s) or mechanisms such as vesicular transport, which mediate rapid $Na^+$ efflux. (B) A futile $Na^+$ leak from vacuoles via non-selective vacuolar channels, called slow-activating (SVc) and fast-activating (FVc) channels. PM represents plasma membrane.

controls $Na^+$ contents in leaves (**Table 2**; Villalta *et al.* 2008). This result might imply that $Na^+$ sequestration in root cells could have a robust impact on the overall salt tolerance. Recent findings in barley support this possibility, in which a combination of techniques of electrophysiological MIFE and confocal fluorescence dye imaging on the

assessment of barley accessions revealed a positive correlation of the ability for root vacuolar $Na^+$ sequestration, but not for $Na^+$ extrusion from roots, with the overall salt tolerance (Wu *et al.* 2019). There is, however, another problem of futile $Na^+$ cycling in the mechanism of vacuolar $Na^+$ sequestration and energy costs (see Shabala *et al.* 2020, and references therein). In brief, $Na^+$ retention in vacuoles is not stable as two types of $Na^+$-permeable non-selective vacuolar ion channels, called "slow-activating (SV) channels" and "fast-activating (FV) channels", mediate $Na^+$ extrusion from the inside of vacuoles to the cytosol, that is, "back-leak of $Na^+$ into cytosol" (**Fig. 2B**; Shabala *et al.* 2020). Since the $Na^+/H^+$ antiport activity across the tonoplast largely depends on the activity of V-ATPase that hydrolyzes ATP for $H^+$ pumping (**Fig. 2B**), the futile cycle of "$Na^+$ sequestration-$Na^+$ back-leak" across the tonoplast under salinity conditions can be very costly: According to the model calculations, plants would need to retain only a very small number of SV channels open (0.1%) to avoid the futile cycle (Shabala *et al.* 2020). However, electrophysiological studies on SV and FV channels in halophytes have provided evidence that halophytes retain traits to significantly reduce the number of open SV and FV channels under salinity stress (Bonales-Alatorre *et al.* 2013a, 2013b). The two-pore channel 1 (TPC1) protein appears to function as an SV channel by forming two TPC protein subunits, which mainly mediate $Ca^{2+}$ and $Na^+$ transport (Guo *et al.* 2017). The estimated number of TPCs in halophytes was found to be similar as that in glycophytes (Shabala *et al.* 2020). Therefore, understanding the regulatory mechanism on the gating of TPC1 (SV) and FV channels as well as determining the molecular identity of the FV channel are primary subjects to be elucidated. Such information would form the basis for breeding salt tolerant crops.

## 4. Development of the structural features of roots in response to salinity stress

We mainly focused on advances in this field of research over the last decade since we published a relevant review (Horie *et al.* 2012). Since then, omics and other emerging technologies have been introduced in this field, and remarkable progresses have been made. Herein, topics related to structural or anatomical aspect are mainly focused, and relevant reviews are also introduced. First, we begin with the endodermis that plays an important role as an apoplastic barrier in younger parts of roots both in eudicots and monocots.

### *4.1. Molecular mechanisms of endodermal Casparian strip development*

Among issues related to the endodermis, in the past decade, remarkable progress has been achieved for the Casparian strip research at the molecular level by using Arabidopsis. Attention has been paid by not only basic plant scientists but also cell biologists. Excellent reviews dealing with these achievements have also been published. Nevertheless, we have briefly summarized the recent progress regarding this topic while referring to some previous related, but uncommon studies.

In Arabidopsis, starting from endodermal differentiation, the cells at the cortex or endodermis initially undergo asymmetrical formative division to regenerate themselves and produce daughter cells at the tip of the root having a simple structural organization. The daughter cells divide to produce the progenitors of endodermal cells and of normal cortical cells (Liberman *et al.* 2015). Transcriptional factors regulating the initial daughter cell division of the cortex or endodermis and those regulating endodermal cell-fate specification have been identified. SHORTROOT (SHR) expressed in the stele moves into the daughter cells and the endodermis and induces the expression of SCARECROW (SCR) (Nakajima *et al.* 2001). Next, SCR directly activates the expression of a transcription factor, MYB DOMAIN PROTEIN 36 (MYB36; Liberman *et al.* 2015), which is the master regulator of Casparian strip formation (Kamiya *et al.* 2015).

The formation of Casparian strip is initiated by the localization of CASPARIAN STRIP DOMAIN PROTEINs (CASPs) at the site of the Casparian strip (Casparian strip domain, CSD; Roppolo *et al.* 2011). CASPs then recruit peroxidase 64 (PER64)—a respiratory burst oxidase homolog F—and a dirigent-like protein Enhanced Suberin 1 (ESB1), to polymerize lignin precursors (Kamiya *et al.* 2015). A receptor-like kinase protein SCHENGEN3 (SGN3/GASSHO1) has been shown to be necessary for localizing CASPs into an uninterrupted, ring-like domain (Pfister *et al.* 2014). Peptides named Casparian strip Integrity Factors 1 and 2 (CIF1 and CIF2, respectively), which are expressed in the stele, specifically bind SGN3 (Doblas *et al.* 2017b, Nakayama *et al.* 2017), mediating the surveillance of root apoplastic barrier integrity (Doblas *et al.* 2017a). For more information on the recent Casparian strip research, please refer to more detailed reviews by the authors of the abovementioned publications, that is, by Geldner (2013) and Doblas *et al.* (2017a).

We would like to also present our viewpoint. A unique experiment performed using surgical manipulation of *Pisum sativum* L. (pea) stems, affecting the physical environment of endodermal cells, indicated that some positional information is accumulated in the radial wall of endodermal cells that defines the future site of the formation of the strip and its width, i.e., CSD, at an early stage of endodermal cell development (Yokoyama and Karahara 2001). The Casparian strip is occasionally, but naturally, observed in two neighboring endodermal cells in the radial direction (**Fig. 3**). Considering this case, such positional information has been speculated to be accumulated even before the final periclinal division to form an endodermal cell. The same experimental system performed using surgical manipulation also indicated the involvement of brefeldin A-sensitive secretory transport for not only the modification of cell

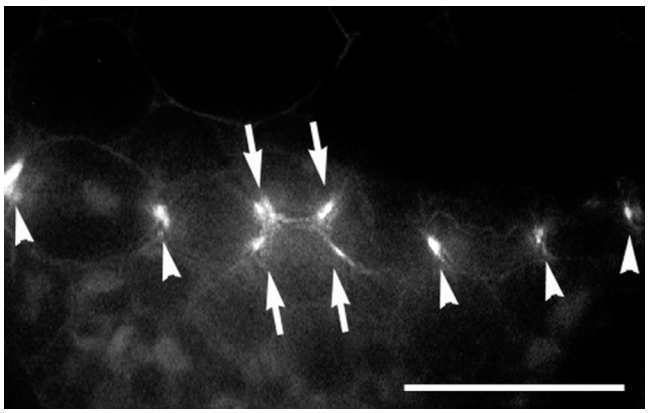

**Fig. 3.** The Casparian strip formed in two neighboring endodermal cells in the radial direction (arrows). A fluorescence cross-sectional image of a pea stem observed under UV light. Arrowheads: the Casparian strips formed in normal endodermal cells. This image is adapted from Karahara (2000).

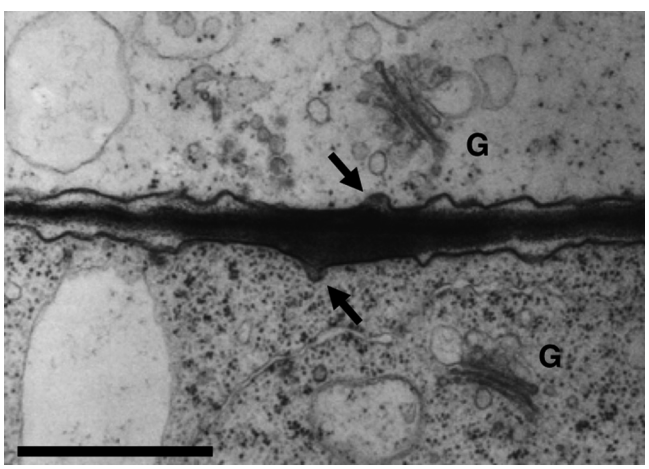


**Fig. 4.** Ultrastructure of endodermal cells developing a Casparian strip in a cross-section of a pea root cut at 15 mm from the root tip. It appears as if the vesicles carrying electron-dense materials fuse with the plasma membrane at CSD (arrows). G: Golgi apparatus. Bar, 1 μm. This image is adapted from Karahara (1995).

wall but also the completion of the tight adhesion of the plasma membrane at the CSD, and that the former precedes the latter (Karahara and Shibaoka 1998, Karahara 2012).

In fact, when the ultrastructure of endodermal cells developing Casparian strip was carefully observed in a pea root, Golgi apparatuses and vesicles were found in the vicinity of the developing Casparian strip, and vesicles carrying electron dense materials were found to fuse with the plasma membrane at the CSD (**Fig. 4**; Karahara 1995). The radial width of the Casparian strip, a morphological parameter that should be related to the effectiveness of the strip as a barrier, increased under salinity stress in *Zea mays* L. roots (Karahara *et al.* 2004).

A comparative study between salinity-sensitive IR24 and Nipponbare and salinity-tolerant cultivars of rice Nona Bokra and Pokkali showed that the Casparian strip matured closer to the root tip after high NaCl treatment in all cultivars than in the control (without NaCl treatment; Ferdose *et al.* 2009). Determining whether salinity accelerated the development of the Casparian strip formation would be interesting (Karahara *et al.* 2008). More interestingly, the development of the Casparian strip was more prominent in the salinity-sensitive cultivars than in the salinity-tolerant cultivars, and the distance between the root tip to the lowermost position of the endodermal Casparian strip was comparatively shorter in the salinity-sensitive cultivars than in the salinity-tolerant ones. This result is consistent with those obtained by the same group that $Na^+$ exclusion across the endodermis is more efficient in a sensitive cultivar than in a resistant one (Tsuchiya *et al.* 1994). These results indicate that the developmental regulation of the Casparian strip responding to salinity stress might be more complicated than was suggested elsewhere (Chen *et al.* 2011, Krishnamurthy *et al.* 2009).

Regarding the issue about the acceleration of the development of the Casparian strip under salinity stress, salinity can be thought to accelerate the formation of Casparian strip when the distance from the root tip to the lowest position of the strip decreases under salinity stress. However, because the distance from the root tip to the lowest position of the Casparian strip depends on the cell division rate, cell elongation rate, and the time required for the formation of the band in individual cells, these factors need to be considered. This issue was tested in maize roots, and the estimated time required for formation of the endodermal Casparian strip in an individual cell was found to not substantially change under salinity stress even when the distance from the root tip to the lowest position of the strip decreased (Karahara *et al.* 2004). Furthermore, a unique integrative method was established to monitor the changes in the developmental processes of a particular cell type in the root, i.e., the rates of cell differentiation, production, and elongation (Karahara *et al.* 2008). A shortcoming of this method is that cell production rate needs to be measured, which is a laborious procedure, even solely to analyze cell differentiation. To overcome this difficulty, the authors developed the unique "sandwich" method (Karahara *et al.* 2012), in which the roots are sandwiched between two different agar media, and are thereby unilaterally exposed to different environmental conditions. By using this method, the authors showed that the formation of the Casparian strip was not promoted under osmotic stress (Karahara *et al.* 2009).

### *4.2. Endodermal lignification*

The Casparian strip is formed in the, so called, state I developmental stage of endodermal cells (Haas and Carothers 1975). One of the most interesting findings of the previous apoplastic barrier research is that lignin polymer plays an important role in the function of the apoplastic barrier of the Casparian strip in roots in state I endodermal cells (Naseer *et al.* 2012). This finding is surprising

because lignin itself has been considered to be more hydrophilic than suberin (Geldner 2013). Nonetheless, considering lignin as an apoplastic barrier component is becoming important. Barbosa *et al.* (2019) even suggested that the Casparian strip can be used in lignin research. Lignin might be present in a specialized form in the Casparian strip (Nawrath *et al.* 2013). Conversely, whether, like in Arabidopsis, lignin solely functions as the apoplastic barrier in other plant species needs to be determined because the roots of *Pisum sativum* contain aliphatic suberin besides lignin in the Casparian strip (Zeier *et al.* 1999). Furthermore, the tight adhesion of the plasma membrane to the CSD also needs to be considered as an important feature of the apoplastic barrier of the Casparian strip (Karahara and Shibaoka 1992).

Recently, an interesting finding was reported that Laccase3, which is involved in lignin monomer polymerization, is indicated to provide possible positional information for directing Casparian strip formation in Arabidopsis by pharmacological experiments (Zhuang *et al.* 2020). This finding is surprising because an enzyme that acts at the final stage of lignin formation provides positional information needed at the initial stage of the Casparian strip formation. However, further careful examination is needed for this issue because peroxidases, rather than laccases, have been shown to be required in the Casparian strip lignification by genetic experiments (Rojas-Murcia *et al.* 2020).

Regarding the relationship between lignin formation and salinity stress, genes responsible for lignin biosynthesis such as *PHENYLALANINE AMMONIA LYASE*, *CAFFEIC ACID-O-METHYLTRANSFERASE*, and *CINNAMYL ALCOHOL DEHYDROGENASE* are upregulated in the roots of a loss-of-function rice mutant of a nuclear factor gene *RICE SALT SENSITIVE3* (Toda *et al.* 2013a, 2013b), which shows severer reduction of root growth under salinity stress than under normal conditions. This fact suggests that a complicated relationship exists between lignin formation and salinity stress when the recently revealed role of lignin in the apoplastic barrier at the Casparian strip is considered, although cell type specificity of the upregulated expression of these genes under salinity stress is still not known.

### *4.3. Endodermal suberization*

After the Casparian strip is developed, endodermal cell walls deposit suberin, which is called state II of the endodermal cells (Haas and Carothers 1975). The permeability of suberin to water and solutes has already been discussed well in a review by Ranathunge *et al.* (2011b). Since suberin lamellae themselves are deposited between the plasma membrane and cell wall as a secondary wall component, their role is to prevent the blocking of the apoplastic diffusion between cells in the cell wall, but to limit transmembrane transport into cells at least in the state II endodermal cells (Hosmani *et al.* 2013).

Although the mechanism regulating the transition from state I to state II endodermal cell is still largely unknown, a transcription factor MYB41, the promoter of which is activated by abscisic acid (ABA) and salinity stress, is suggested to be a candidate (Barberon 2017, Kosma *et al.* 2014). In addition to transcription factors that positively regulate suberin synthesis, such as MYBs, some were recently found to be negative regulators, such as ANAC46, which is expressed in the endodermis (Mahmood *et al.* 2019).

In addition to transcription factors, an enzyme called docosanoic acid synthase, which is involved in the biosynthesis of aliphatic suberin, has been identified in roots (Franke *et al.* 2009, Lee *et al.* 2009). Yadav *et al.* (2014) showed that the G subfamily of the ATP-binding cassette (ABCG) half-transporters are involved in the synthesis of suberin, and their gene expression is positively regulated by ABA for endodermal barrier function in Arabidopsis roots. They also showed that a triple mutant of the genes of these transporters (*abcg2-1*, *abcg6-1*, and *abcg20-1*) still developed a functional Casparian strip. Regarding the regulation of suberization in response to environment changes, the development of endodermal suberin was shown to be induced unilaterally in the root side exposed to the air or in contact with cadmium in maize (Líška *et al.* 2016), suggesting the flexibility of suberization regulation.

The role of suberin in the function of a transport barrier was elucidated by using modern biological tools such as mutants of suberin deposition, chemical analysis of suberin composition, and ionome analysis. A combination of these tools actually revealed that the *enhanced suberin1* (*esb1*) mutant of Arabidopsis showed decreased accumulation of Ca, Mn, and Zn and increased accumulation of Na, S, K, As, Se, and Mo in the shoot (Baxter *et al.* 2009). They suggested that the increase of Na concentration in the shoot of the *esb1* mutant was attributed to a possible decrease in the apoplastic bypass flow for this element at low external Na concentrations in the soil. Therefore, they also speculated that the *esb1* mutant might be salinity tolerant when the external Na concentration is elevated. These suggestions seem to be reasonable when facts that *esb1* mutant showed drought tolerance (Franke *et al.* 2012) and that rice cultivars having elevated suberin showed salinity tolerance (Krishnamurthy *et al.* 2009) are considered. Conversely, given that *esb1* mutants form defective lignin-based Casparian strips (Hosmani *et al.* 2013), another possible cause, i.e., incomplete apoplastic barrier at the Casparian strip, might be considered for the increase of Na concentration in the shoot of the *esb1* mutants.

Recently, a transcriptional factor SUBERMAN (MYB39) was shown to positively regulate suberin lamellae formation in root endodermal cells (Cohen *et al.* 2020). Interestingly, the rosette leaves of the SUBERMAN overexpressor showed significant accumulation of elements such as Mg, P, S, K, Ca, Mn, and Fe, but not of Na (Cohen *et al.* 2020). However, the results of these two studies (Baxter *et al.* 2009, Cohen *et al.* 2020) were not concerning

Na, indicating that a careful evaluation about the difference in the degree, site, and timing of altered suberization in these mutants is needed for the interpretation of these results. In addition, a combination of the manipulation of suberin deposition and ionome analysis raised the fundamental question of why the barrier function of suberin is different between different elements.

### 4.4. Exodermis

When the hypodermis, a cell layer beneath the root epidermis (rhizodermis), forms the Casparian strip, the cell layer is called exodermis (Peterson 1988). The exodermis is observed in many species, including gramineous plants such as rice and maize. Apoplastic transport barriers in roots have been considered to exist even in the exodermis (Perumalla and Peterson 1986), and many studies have been providing evidence of this (Kreszies *et al.* 2018).

In the case of rice roots, a lignified sclerenchymatous cell layer develops beneath the exodermis in the outer part of the root (OPR). Since studies have been focusing on the importance of lignin in the apoplastic barrier function of the endodermal Casparian strip, the contribution of sclerenchyma to the barrier function of the OPR needs to be further considered.

The barrier function of the exodermis is important as a radial oxygen loss (ROL) barrier (Shiono *et al.* 2014). When rice is grown in oxygen-deprived medium, the root exodermis as a ROL barrier is strengthened to reduce oxygen leakage from the root, and, in such roots, solute permeability for NaCl was reduced (Ranathunge *et al.* 2011a), indicating that enhancement of the OPR is also effective as a barrier to NaCl.

In maize, interestingly, high salinity at 100 mM NaCl induced suberization over a large part of the cortex, but did not notably influence the suberization of the endodermal cell wall (Shen *et al.* 2015). In contrast, osmotic stress after treatment with 20% (w/v) polyethylene glycol accelerated suberization both in the endodermis and exodermis, but suberization induced by osmotic stress was limited to several cell layers in the outer cortex (Shen *et al.* 2015). This indicates differences in roles between the endodermis and exodermis to salinity and osmotic stress, although careful interpretation is needed because salinity stress itself imposes osmotic stress.

An inspiring computer simulation modeling of silicon uptake in rice roots suggested that the double-layer structure of the Casparian strips (both in the endodermis and exodermis) is an important factor in the high silicon uptake by rice (Sakurai *et al.* 2015). Such an *in silico* simulation modeling might be applicable to sodium uptake or exclusion as well. Actually, a mathematical approach is being used to evaluate the effectiveness of the endodermal Casparian strip and suberin lamellae in the salinity stress response of plant roots (Foster and Miklavcic 2017).

Previous studies have been focusing on understanding the molecular mechanisms of suberin or lignin formation in the exodermis. Reduced suberin deposition was observed in the root exodermis of RNAi plants of a gene encoding the ABC half-transporter, *ABCG1* (Landgraf *et al.* 2014). Fleck *et al.* (2011) conducted histochemical analysis of rice roots treated with silicon and found enhanced development of endodermal and exodermal Casparian strip. They also showed that the expression of genes of ABC transporters (including *OsABCG25*) was upregulated in the roots of rice. Utilizing this experimental system, i.e., analyzing rice root treated with silicon, in combination with gene overexpression and knockout techniques, Hinrichs *et al.* (2017) revealed that an ABC transporter gene *OsABCG25* contributed to Casparian strip formation in the exodermis of rice roots. Conversely, chemical analyses by Fleck *et al.* (2015) showed that suberin content, but not lignin content, in the outer part of roots decreased owing to a decrease in its aromatic components, and the silicon content increased when silicon treatment promoted exodermal Casparian strip formation in rice and maize roots. They suggested that silicon-induced enhancement of the Casparian strip might be attributed to the chemical interaction of phenolic compounds with silicon.

### 4.5. Bypass flow of solutes

Regardless of the development of the apoplastic barrier in roots, sodium ions leak in roots via the apoplast, which is called "apoplastic bypass flow", and its possible location has been discussed to be either the OPR, root tip, or breaks created by lateral root emergences (Horie *et al.* 2012). Since this issue has been thoroughly reviewed recently (Kreszies *et al.* 2018), we have only mentioned some new interesting aspects regarding this.

Regarding the bypass flow at breaks created by lateral root emergences, a newly characterized gene *LOTR1*, which is essential for Casparian strip formation in Arabidopsis, was shown to be involved in the formation of suberin lamellae as an apoplastic barrier at sites of lateral root emergence where Casparian strips are disrupted (Li *et al.* 2017).

The alleviation of salinity stress by the application of silicon possibly involves the reduction of the bypass flow of ions to the shoot, although the exact mechanism is not yet clear (Thorne *et al.* 2020). The blockage of salt may involve the polymerization of silicic acid within the endodermal apoplast, for example, via complexation with lignin and other phenolics (Thorne *et al.* 2020). This is implied from the results of material science studies showing that $SiO_2$ can bind to lignin (Cabrera *et al.* 2016, Strzemiecka *et al.* 2016).

### 4.6. Cell wall components other than suberin and lignin related to barrier function and salinity stress

Suberin and lignin are not the only components of cell wall related to root function under salinity stress. The effects of abiotic stress on cell wall modification are generally categorized into two aspects: one is on the primary cell

walls and the other is on the secondary cell walls. In the latter, the modification of the secondary cell walls is mainly related to the apoplastic barrier function in the case of roots.

The visible effects of abiotic stresses such as salinity stress on plant organs are growth inhibition. Organ growth inhibition is caused by the inhibition of cell elongation, which is related to the modification of the primary cell walls. Byrt *et al.* (2018) introduced an interesting viewpoint that, under salinity stress, $Na^+$ can physically and directly interact with the cell wall components and change their chemical properties. That is, $Na^+$ could displace $Ca^{2+}$ from its binding sites of pectin, reducing pectin crosslinking (Byrt *et al.* 2018), which possibly leads to changes in cell elongation through pectin dynamics (Proseus and Boyer 2012). Reviews by researchers such as Le Gall *et al.* (2015) and Byrt *et al.* (2018) also address this issue.

### *4.7. Hormone signaling in the endodermis*

As mentioned above, the endodermis plays an important role as an apoplastic barrier, and this function is regulated through hormone signaling. In particular, endodermal suberization is highly plastic in response to many nutritional stress conditions, which exert their effect on suberin through ethylene and ABA hormonal pathways: ABA promotes endodermal suberization, and ethylene induces the disappearance of suberin from state II endodermal cells (Barberon *et al.* 2016).

In addition, Duan *et al.* (2013) indicated the importance of the endodermis in regulating root growth under salinity stress through ABA signaling based on the result that postemergence growth of lateral roots is strongly suppressed during salinity stress through endodermal ABA signaling. Furthermore, many studies have revealed the importance of endodermis in plant hormonal regulation of root growth (Dinneny 2014, Robbins *et al.* 2014). Ubeda-Tomás *et al.* (2008) showed that the endodermis represents the primary gibberellin-responsive tissue that regulates entire root growth. Zhang *et al.* (2011) and Heo *et al.* (2011) showed that the endodermis-expressed *SCARECROW-LIKE 3*, the promoter of which is directly induced by SCR and SHR heterodimer, mediates gibberellin-promoted cell elongation in the root. In Arabidopsis, osmotic stress via ABA signaling in meristematic endodermal cells induces the differentiation of protoxylem (Bloch *et al.* 2019). These facts raise the question whether the endodermis functioning as a communication center during root responses to the external environment as well as an important apoplastic barrier is a coincidence.

## Conclusions

Recent advances in technologies for plant breeding, genomic science, and bioinformatics have accelerated to detect many important QTLs or genetic loci, which function in the mechanism of salinity tolerance of crops. Identifying the responsible genes for crop QTLs is not easy, but information of key genes for salinity tolerance is increasing. In general, since $Na^+$ toxicity has a larger impact on the sensitivity of herbaceous crops to salinity stress than $Cl^-$ toxicity, the primary focus has been toward the coping mechanisms with $Na^+$. However, the mechanism to circumvent $Na^+$ toxicity is yet to be elucidated, and impacts of $Cl^-$ toxicity on crop salinity tolerance need to be determined. In addition to the problem of ion toxicity, elucidation of the tolerance mechanisms to hyperosmotic conditions is essential as salinity-induced osmotic stress causes serious water deficiency and growth inhibition. Applying novel strategies, including bioimaging, for screening genes and using halophytes as donors of genes may open a path toward the identification of unknown genes that are indispensable to salinity tolerance.

Regarding studies on the structural and developmental features of roots in response to salinity stress, omics and other emerging technologies have been introduced in this field of researches, and remarkable progresses have been made over the last decade. In particular, we gained a deeper understanding of the molecular mechanisms of the endodermal Casparian strip formation as well as endodermal lignification and suberization by using Arabidopsis as a model plant. Furthermore, studies have been attempting to understand the developmental features of exodermis, which is not formed in Arabidopsis but in gramineous plants and also plays an important role as an apoplastic transport barrier. If more knowledge on the developmental and structural features of roots is obtained, computer simulation modeling would allow the understanding of solute uptake mechanisms. Emergence of new technologies might provide interesting insights in this field of research.

## Author Contribution Statement

TH and IK developed ideas for the structure of this review. TH prepared the text, figures, and tables for sections 1–3. IK prepared the text and figures for section 4.

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