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SPL7 locally regulates copper-homeostasis-related genes in Arabidopsis

Ryoichi Araki[a,b,2], Mélanie Mermod[a,2], Hiroaki Yamasaki[a,1], Takehiro Kamiya[c], Toru Fujiwara[c], Toshiharu Shikanai[a,*]

[a] Department of Botany, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan
[b] Faculty of Education, Wakayama University, Wakayama 640-8510, Japan
[c] Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan

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ABSTRACT

In Arabidopsis, a central regulator of copper (Cu) homeostasis is the transcription factor SQUAMOSA promoter binding protein-like7 (SPL7). Under Cu deficiency, SPL7 induces the expression of *miR398*, which suppresses the expression of the genes *CSD1* and *CSD2*, which encode cytosolic and chloroplastic isoforms of Cu/Zn superoxide dismutase, respectively. Consequently, the limited Cu is preferentially assigned to plastocyanin, which is essential for photosynthetic electron transport. Consistent with this function of *miR398* related to photosynthesis, its expression is strongly induced in leaves. In this study, however, we showed that *SPL7* was transcribed mainly around the vasculature in roots, where Cu levels were likely sensed. To test the possible long-distance signaling of Cu availability from roots to shoots, we conducted a series of grafting experiments using *spl7* mutant and wild-type (WT) plants. Expression of Cu-responsive microRNAs and the resulting suppression of *CSD1* and *CSD2* mRNAs were observed in leaves only when the aerial part was from WT plants, in which a low level of *SPL7* was transcribed also in the vascular tissues. Although local sensing of Cu was disturbed in the *spl7* mutant, the Cu level was not affected in the shoots. *SPL7* is expressed in specific cell layers in both roots and shoots and locally senses Cu availability, transmitting the information to surrounding cells.

1. Introduction

In Arabidopsis, roughly one-third of the total copper (Cu) in shoots is concentrated in chloroplasts (Shikanai et al., 2003), where Cu acts as a cofactor of plastocyanin, a photosynthetic electron carrier localized to the thylakoid lumen. Cu also functions as a cofactor of Cu/zinc (Zn) superoxide dismutase (SOD), which scavenges reactive oxygen species (ROS). CSD1 and CSD2 are cytosolic and plastidic isoforms, respectively, of SOD. Additional roles of Cu can be found in respiratory electron transport, lignin biosynthesis, and ethylene sensing (Ferguson-Miller and Babcock, 1996; Nersissian et al., 1998; Hirayama et al., 1999).

A central player in the regulation of Cu homeostasis in Arabidopsis is SQUAMOSA promoter binding protein-like7 (SPL7) (Yamasaki et al., 2009). Upon sensing Cu deficiency, this transcription factor modifies the expression of genes involved in Cu uptake from the rhizosphere. SPL7 also reorganizes the intracellular distribution of Cu by inducing the expression of microRNA (miRNA) *miR398*, which suppresses the expression of *CSD1* and *CSD2* genes, so that the limited Cu is preferentially assigned to plastocyanin, which is essential for photosynthetic electron transport (Wintz et al., 2003; Abdel-Ghany, 2009). The function of CSD2 is substituted by that of an iron (Fe)-containing superoxide dismutase (FSD1), the transcription of which is directly upregulated by SPL7 (Yamasaki et al., 2007). SPL7 binds to GTAC motifs in the promoter region of target genes (including miRNAs) and induces their expression under Cu deficiency (Yamasaki et al., 2007).

miRNA398 forms a small family consisting of three members, *miR398a*, *miR398b*, and *miR398c*. The sequences of mature *miR398b* and *miR398c* are identical, whereas that of *miR398a* differs at its 32 nucleotide (Sunkar and Zhu, 2004). A low level of *miR398a* is constantly expressed independently of Cu availability. However, *miR398b* and *miR398c* show a clear Cu-dependent expression pattern (Yamasaki et al., 2009). In addition to the *miR398* family, several Cu-responsive miRNAs (Cu-miRNAs) downregulate the production of a series of Cu-

Abbreviations: ACT2, ACTIN2; CSD1, cytosolic copper/zinc superoxide dismutase; CSD2, chloroplastic copper/zinc superoxide dismutase; Cu, copper; Fe, iron; FSD1, iron superoxide dismutase; GFP, green fluorescent protein; GUS, β-glucuronidase; ICP-MS, inductively coupled plasma mass spectrometry; miRNA, microRNA; PYE, POPEYE; qRT-PCR, quantitative reverse transcription PCR; ROS, reactive oxygen species; SPL7, SQUAMOSA promoter binding protein-like7; WT, wild-type; Zn, zinc

* Corresponding author at: Department of Botany, Graduate School of Science, Kyoto University, Oiwake−cho, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan.
E-mail address: shikanai@pmg.bot.kyoto-u.ac.jp (T. Shikanai).
[1] Present address: Central Research Laboratories, DIC corporation, Sakado, Sakura, Chiba 285-8668, Japan.
[2] These authors contributed equally to this work.

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containing proteins (Burkhead et al., 2009). *miR397*, *miR408*, and *miR857* suppress the expression of genes encoding laccases and other blue copper oxidases such as plantacyanin (Zhang and Li, 2013; Wang et al., 2014; Zhao et al., 2015). Whereas *miR398* and *miR408* are ubiquitously expressed (Sunkar et al., 2006; Zhang and Li 2013), in Arabidopsis *miR397* and *miR857* are expressed mainly in the vasculature, where they can regulate the expression of laccases involved in lignification of secondary cell walls (Wang et al., 2014; Zhao et al., 2015).

Increasing evidence indicates that the classical Cu-miRNAs are upregulated not only by low Cu but also by a variety of stress responses. Zhang et al. (2014) showed that the light-responsive transcription factor ELONGATED HYPOCOTYL 5 (HY5) regulates *miR408* by direct interaction with its promoter. In Arabidopsis, expression of Cu-miRNAs is also induced by sucrose (*miR398*), heat shock (*miR398*), and cold treatment (*miR397*) (Sunkar and Zhu, 2004; Dugas and Bartel, 2008; Guan et al., 2013). These treatments, however, may influence the Cu pool, which is sensed by SPL7, rather than activating the expression of the miRNAs directly. A more in-depth discussion of alternative Cu-miRNA induction can be found in a review (Pilon, 2017).

SPL7 mRNA accumulates mainly in the roots but also in stems and flowers. Only a trace amount of RNA has been detected in rosette and cauline leaves (Yamasaki et al., 2009). Despite this fact, *miR398b* and *miR398c*, the expression of which is directly induced by SPL7, are expressed mostly in aerial parts (Sunkar and Zhu, 2004; Sunkar et al., 2006; Yamasaki et al., 2009). Consistent with the idea that Cu levels are sensed in roots, *SPL7* is expressed mainly in the roots (Yamasaki et al., 2009). Similarly, the expression of *miR398b* and *miR398c* in leaves makes sense, as the target genes, *CSD1* and *CSD2*, are expressed in photosynthetic tissues. However, it remains unclear how SPL7 regulates downstream genes that are expressed in different parts of the plant.

In this study, we use a promoter β-glucuronidase (GUS) reporter system to demonstrate that *SPL7* is transcribed mainly in root pericycle cells. Furthermore, we conducted grafting experiments using the *spl7* mutant and the wild type (WT) to shed light on the spatial regulation between SPL7 and its target miRNAs. We found that the expression of *miR398b/c* and their target genes, *CSD1* and *CSD2*, is strictly dependent on the presence of SPL7 in the same part of the plant. Therefore, to regulate the expression of *miR398b/c* in leaves, *SPL7* has to be expressed there.

2. Results

2.1. SPL7 is transcribed mainly in pericycle cells in Arabidopsis roots

Expression of the GUS reporter gene driven by the *SPL7* promoter (*SPL7p:GUS*) revealed that *SPL7* was transcribed mainly in the roots and that gene expression was not affected by the Cu concentration in the medium (Fig. 1A). This result is consistent with our previous result monitoring the accumulation of endogenous transcripts (Yamasaki et al., 2009). In aerial parts, *SPL7* expression was only marginally detectable in the vascular tissue of the petiole and in the leaf veins (Fig. 1B). The GUS signal in roots was observed around the vasculature and at the base of the lateral roots (Fig. 1C). Notably, the GUS expression pattern during lateral root formation resembled that of LRB10, an Arabidopsis marker line that expresses a lateral-root-base-specific construct during the early stage of lateral root primordium development (Malamy and Benfey, 1997). As the lateral root primordium develops from pericycle cells of the primary root, expression of *SPL7* seems to be in the pericycle rather than the endodermis.

2.2. Locally expressed SPL7 is necessary and sufficient for the response to Cu deficiency in shoots

To clarify which tissues of SPL7 are required for the regulation of downstream genes in leaves, we conducted reciprocal grafting using the *spl7* mutant and WT: WT/*spl7* and *spl7*/WT (scion/rootstock). For the control, grafting was performed between the same genotypes, namely WT/WT and *spl7/spl7*. Some grafted plants grew healthily under our experimental conditions and generally showed no symptoms related to grafting (Supplementary Fig. S1), although other plants died within a couple of days after grafting. We carefully checked the seedlings under an optical microscope to ensure that plants with adventitious roots were not used in the following experiments. Calli-like cells were occasionally observed around the junction, but they did not seem to influence plant growth. The success rate of grafting was around 30% or less. To assess possible movement of *SPL7* mRNA from roots to shoots, the shoots of all grafted plants were tested by quantitative reverse-transcription (qRT) PCR for the *SPL7* transcript. Much higher levels of *SPL7* mRNA were detected in the shoots of the WT background than in the shoots of the *spl7* background (Fig. 2A). Although the trace level of the RT-PCR product was amplified in the shoots of the *spl7*/WT plants, a similar level of DNA was amplified in the shoots of the *spl7/spl7* plants. We could not obtain evidence implying the movement of *SPL7* mRNA from roots to shoots.

Grafted seedlings were cultured on medium including high (5 μM) and low (0.1 μM) levels of Cu. Consistent with our previous report (Yamasaki et al., 2009), the *SPL7* transcript level was not affected by Cu concentrations in the medium, and mRNA was detected only when the sample was from the WT plant (Fig. 2). The same level of *SPL7* mRNA was detected in shoots of WT/WT and WT/*spl7* grafted plants under both Cu conditions (Fig. 2).

FSD1 encodes chloroplast Fe SOD, and its transcription is induced directly by SPL7 under Cu deficiency (Yamasaki et al., 2009). Consistently, *FSD1* transcripts were detected in shoots only when the scion was from the WT plant under low Cu conditions (Fig. 2B). Even though the rootstocks were from *spl7* plants, the WT/*spl7* seedlings induced the expression of *FSD1* in shoots. The *SPL7* gene in the roots was not required for the expression of *FSD1* in shoots. In the WT/*spl7* shoots, expression of *FSD1* was higher in low Cu conditions than that in the WT/WT plants, and the *FSD1* transcript was detected also in the presence of high Cu (Fig. 2B). *SPL7* expressed in the shoots is required and sufficient for inducing the expression of *FSD1* in shoots. *SPL7* expressed in the roots is not required for *FSD* expression in shoots but may secondarily modulate SPL7-dependent Cu sensing quantitatively in shoots.

miR398b and *miR398c* genes are direct targets of SPL7 and regulate the stability of *CSD1* and *CSD2* mRNAs (Yamasaki et al., 2009). Their levels were analyzed in mature forms (Fig. 3A). The mature forms of *miR398b* and *miR398c* are identical and were detected together as *miR398b/c*. In WT/WT and WT/*spl7* shoots, both mature forms of *miR398b/c* and *miR408* were induced in response to low Cu (Fig. 3A). Higher expression was observed in the WT/*spl7* shoots than in the WT/WT shoots, as was observed in the case of *FSD1* expression (Fig. 2B), although the difference was not statistically significant. Induction of *miR398b/c* was not detected when the scion was from the *spl7* mutant (Fig. 3A).

miR408 targets *lac3* and plantacyanin mRNAs (Abdel-Ghany and Pilon, 2008), and its expression is also induced by Cu deficiency via the function of SPL7 (Yamasaki et al., 2009). The accumulation of mature miRNA in shoots showed a pattern almost identical to that of *miR398bc* (Fig. 3B).

The expression patterns of *CSD1* and *CSD2*, both of which are downregulated under low Cu via the function of the *miR398* family, were very similar to each other. Consistent with our observation in intact WT seedlings (Yamasaki et al., 2009), Cu-dependent expression of both *CSD1* and *CSD2* was detected in the shoots of WT/WT plants (Fig. 2C and D). The same trend was observed in the shoots of WT/*spl7* plants, but to a lesser extent. The shoots of *spl7/spl7* and *spl7*/WT plants did not respond to a change in Cu concentration.

Taken together, these results clearly demonstrate that Cu-dependent upregulation of *FSD1* and Cu-miRNAs and the subsequent downregulation of *CSD1* and *CSD2* took place normally in the plant part carrying the *SPL7* gene, regardless of whether the other part lacked the