From Organelle to Organ: ZRIZI MATE-Type Transporter is an Organelle Transporter that Enhances Organ Initiation

Yoge Burko¹, Yosef Geva¹, Aya Refael-Cohen¹, Sharona Shleizer-Burko¹, Eilon Shani¹, Yael Berger¹, Eyal Halon¹, George Chuck², Menachem Moshelion¹,∗ and Naomi Ori¹,∗

¹The Robert H. Smith Institute of Plant Sciences and Genetics in Agriculture and The Otto Warburg Minerva Center for Agricultural Biotechnology, Hebrew University, Rehovot 76100, Israel
²Plant Gene Expression Center, 800 Buchanan St., Albany, CA 94710, USA
*Corresponding authors: Naomi Ori, E-mail, ori@agri.huji.ac.il; Fax, +972-8-948-9322; Menachem Moshelion, E-mail, moshelio@agri.huji.ac.il; Fax, +972-8-968-9781
(Received November 19, 2010; Accepted January 11, 2011)

Plant architecture is a predictable but flexible trait. The timing and position of organ initiation from the shoot apical meristem (SAM) contribute to the final plant form. While much progress has been made recently in understanding how the site of leaf initiation is determined, the mechanism underlying the temporal interval between leaf primordia is still largely unknown. The Arabidopsis ZRIZI (ZRZ) gene belongs to a large gene family encoding multidrug and toxic compound extrusion (MATE) transporters. Unique among plant MATE transporters identified so far, ZRZ is localized to the membrane of a small organelle, possibly the mitochondria. Plants overexpressing ZRZ in initiating leaves are short, produce leaves much faster than wild-type plants and show enhanced growth of axillary buds. These results suggest that ZRZ is involved in communicating a leaf-borne signal that determines the rate of organ initiation.

Keywords: Arabidopsis • Architecture • Enhancer trap • MATE transporters • Organ initiation rate • ZF14.
Abbreviations: GFP, green fluorescent protein; GUS, β-glucuronidase; MATE, multidrug and toxic compound extrusion; OP, operator; qRT–PCR, quantitative reverse transcription0–PCR; SAM, shoot apical meristem.

Introduction

The plant body is formed through a series of morphogenetic events, coordinated by meristems. The shoot apical meristem (SAM) continuously produces lateral organs, axillary meristems and stems, successively building the plant shoot (Bowman and Eshed 2000, Carles and Fletcher 2003, Veit 2006, Barton 2010). While the outcome of this sequence of events is unique in every single plant (Sachs 2004), reflecting the changing internal and external circumstances, many features of plant architecture are highly predictable within a given species. Thus, organ formation and growth at the SAM are tightly regulated in a manner that enables this combination of consistency and flexibility.

The time period between the initiation of two successive leaves is termed the plastochron. The timing of leaf initiation is thought to be determined by non-autonomous signals from the previously initiated leaf (Lee et al. 2009). The timing of organ initiation from the SAM, the extent of stem growth between initiated organs and the fate of the axillary meristems represent central developmental choices affecting plant architecture (Reinhardt and Kuhlemeier 2002, McSteen and Leyser 2005). Mutant analysis suggests that these features, as well as leaf size, may be related, as they are often commonly affected by a single genetic lesion (Schwarz et al. 2008, Wang et al. 2008).

The Arabidopsis altered meristem program1 (amp1) mutant, which is allelic to primordia timing (pt), hauptling (hpt) and constitutive morphogenesis 2 (cop2), shows an increased rate of leaf initiation, shorter internodes, reduced apical dominance, de-etiolation in the dark and earlier flowering time (Chaudhury et al. 1993, Hou et al. 1993, Conway and Poethig 1997, Mordhorst et al. 1998). The AMP1 gene encodes a putative glutamate carboxypeptidase, and has been implicated in the production of a signaling molecule involved in SAM function (Helliwell et al. 2001). amp1 mutants contain increased cytokinin levels, and many aspects of its altered phenotype are also associated with exogenous cytokinin application (Chaudhury et al. 1993). However, some of the amp1 effects cannot be phenocopied by cytokinin application. This, and the nature of the AMP1 gene, suggests that the elevated cytokinin level may not be the primary effect of the mutation, and that additional, cytokinin-independent processes may be impaired in amp1 mutants (Helliwell et al. 2001, Saibo et al. 2006).

Mutations in the SUPERSHOOT (SPS) gene, encoding a cytochrome P450, also cause a dramatic increase in cytokinin levels. sps mutants are characterized by an extreme overgrowth of axillary shoots, which may be mediated by the increased cytokinin levels (Tantikanjana et al. 2001). The phenotypes of sps and amp1 mutants only partially overlap despite the common
feature of elevated cytokinin levels, suggesting that relative spatial and temporal cytokinin levels play a crucial role in these phenotypes.

The miR156-regulated transcription factors SPL9 and SPL15 have been shown to regulate leaf initiation rate and branching redundantly. Reduced activity of SPL9 and SPL15 resulted in an increased leaf initiation rate, suggesting that factors negatively affect leaf initiation (Schwarz et al. 2008, Wang et al. 2008). Double mutants in CYP78AS/KLUH and its close homolog CYP78A7 also cause an increased leaf initiation rate, probably in a pathway parallel to that of SPL (Anastasiou et al. 2007, Wang et al. 2008). An increased rate of leaf initiation has also been observed in the maize terminal ear1 (te1) and the rice plastochnon1 (pla1) and pla2 mutants. te1 and pla2 represent mutations in orthologous genes encoding RNA-binding proteins (Veit et al. 1998, Kawakatsu et al. 2006). While TE1 is expressed in both the maize SAM and leaf primordia, the PLA2 gene is expressed mostly in leaf primordia. The PLA1 gene encodes the cytochrome P450 CYP78A11, and is expressed in specific regions of developing leaves (Miyoshi et al. 2004). Thus, control of the rate of leaf initiation seems to involve diverse biochemical activities and complex communication between the initiating leaf and the SAM. Moreover, it appears that many of the players involved in this delicately balanced process are currently not yet known.

Multidrug and toxic compound extrusion (MATE) is a family of transporter proteins, which is widely distributed in all living kingdoms (Brown et al. 1999, Omote et al. 2006). The plant MATE gene family is particularly large, with 58 members in the Arabidopsis genome (Hvrup et al. 2003, Omote et al. 2006). However, the biological function of most of its family members is still unknown. Plant MATE proteins are reported to be localized to the tonoplast or the plasma membrane, and to transport secondary metabolites and xenobiotics through an \( \Delta^+ \) exchange mechanism. They are implicated in various processes, including anthocyanin accumulation (Debeaujon et al. 2001, Mathews et al. 2003, Marinova et al. 2007, Gomez et al. 2009, Zhao and Dixon 2009), protection of roots from inhibitory compounds (Diener et al. 2001), salicylic acid-dependent disease resistance signaling (Nawrath et al. 2002), iron homeostasis (Rogers and Guerinot 2002, Durrett et al. 2007), aluminum tolerance (Magalhaes et al. 2007, Li et al. 2009, Maron et al. 2010) and detoxification of lipophilic cations and cadmium (Li et al. 2002). To date, MATE proteins have not been implicated in plant development.

Here, we describe the characterization of a member of the Arabidopsis MATE gene family, ZRIZI (ZRZ; ‘ZARIZ’ means agile in Hebrew). ZRZ overexpression in initiating leaves resulted in short, bushy plants with a dramatically increased leaf initiation rate. Intracellular localization analysis revealed that ZRZ is located in the membrane of mitochondria, or an organelle that is biochemically related to mitochondria. The ZRZ transporter is thus proposed to be involved in the determination of the leaf initiation rate through an unknown mechanism.

### Results

**ZRIZI—a MATE gene with enriched expression in shoot apices**

A screen of enhancer trap lines has identified a line, designated 1015, with enriched expression in the boundaries between the developing leaf primordia and the developing stem (Supplementary Fig. S1A–C). In the inflorescence, strong staining was observed in the receptacles, in addition to the inflorescence meristem (Supplementary Fig. S1D).

The insertion site in 1015 was located on chromosome 1, about 5 kb upstream of the annotated gene At1g58340 (ZF14; Kato et al. 1999), and about 7 kb downstream from At1g58330 (ZW2) (Fig. 1A). Examination of ZF14 transcript levels in Arabidopsis shoots using quantitative reverse transcription–PCR (qRT–PCR) revealed that its expression is enriched in shoot apices relative to leaves, in agreement with the 1015 expression pattern (Fig. 1B–E). In contrast, ZW2 is expressed ubiquitously in the plant, as revealed by the Arabidopsis eFP browser (http://babycot.botany.utoronto.ca/efp/cgi-bin/efpWeb.cgi) (Winter et al. 2007), and is transcribed in the opposite direction with respect to the insertion. We chose to characterize further the ZF14 (At1g58340) gene, referred to below as ZRZ. ZRZ encodes a protein from the MATE family of multidrug-transporter proteins.

**Subcellular localization of ZRZ**

To follow ZRZ cellular localization, we transiently expressed a chimeric green fluorescent protein (GFP)–ZRZ fusion protein under the control of two copies of the 35S promoter in protoplasts from Arabidopsis mesophyll. GFP–ZRZ expression was localized to organelles (Fig. 2A, B) with a similar distribution profile and number to those of the mitochondria (detected using the mitochondrial-selective red dye MitoTracker; Fig. 2C). However, GFP–ZRZ and MitoTracker did not co-localize to the same organelle (Fig. 2D; see also Supplementary Fig. S2). Interestingly, in cells strongly expressing GFP–ZRZ the mitochondria showed weaker labeling with MitoTracker (Supplementary Fig. S2). This weaker labeling was specifically related to ZRZ expression, as the control, which expressed free GFP, did not show such an effect (Supplementary Fig. S2). These results indicate the existence of a direct or indirect relationship between ZRZ and mitochondrial activity.

**ZRZ misexpression causes dramatic developmental phenotypes**

To examine ZRZ function, we characterized mutant lines with insertions in the ZRZ gene. zrz-1 mutants, homozygote for an insertion in the first exon of ZRZ, about 100 bp downstream from the start codon (Fig. 1A), did not show any aberrant phenotype compared with the wild type (Supplementary Fig. S3A, B). qRT–PCR analysis showed 83-fold lower ZRZ mRNA levels in zrz-1 seedlings (Supplementary Fig. S3C).
The lack of apparent phenotype in zrz-1 mutants may result from redundant activity of additional MATE genes. Therefore, to understand ZRZ function further, we misexpressed the gene by the use of several different promoters. ZRZ expression driven by the 35S promoter resulted in tiny, bushy and almost infertile plants (Fig. 3A–F).

We used a transactivation system (Moore et al. 1998) to analyze further the specific effect of ZRZ misexpression in different domains. The AINTEGUMENTA (ANT) promoter is expressed in lateral organ primordia from a very early stage (Elliott et al. 1996, Schoof et al. 2000). ZRZ expression driven by the ANT promoter resulted in similar phenotypes to those of 35S:ZRZ plants (Fig. 4A–H, L; compare with Fig. 3A–F). However, the phenotypes were milder and the plants were partially fertile.

ANT>>ZRZ plants produced leaves much faster than wild-type plants (Figs. 4A, B, 5A). While in 14-day-old wild-type plants an average of 6.4 (±0.5) leaves were produced, ANT>>ZRZ plants of comparable age produced 9.6 (±0.8) leaves (Fig. 5A). Similarly, 16-day-old wild-type and ANT>>ZRZ plants had 8 (± 0.67) and 11.81 (±0.74) leaves, respectively. While ANT>>ZRZ plants flowered early with respect to chronological age, they produced 11.81 (±0.74) leaves before flowering, relative to 8.4 (±0.84) leaves in the wild type. Similarly, upon flowering, ANT>>ZRZ plants produced flowers faster than the wild type (Fig. 5B). In addition, they featured reduced stem elongation and altered phylotaxis (Figs. 4E, G, H, K, L, 5C). A role for ZRZ in the control of leaf initiation rate is in agreement with the enriched expression of ZRZ in young apices relative to older leaves (Fig. 1B).

The bushy appearance of ANT>>ZRZ plants resulted from the combination of reduced stem elongation and reduced apical dominance. More rosette leaves developed a visible axillary bud in ANT>>ZRZ than in wild-type plants (Fig. 4C, D). To quantify this phenotype, the proportion of individuals producing a visible axillary bud in the axil of each leaf was recorded. As can be seen in Fig. 5E, 44% of ANT>>ZRZ plants produced axillary buds in the axil of the cotyledon, while in wild-type
ANT> >ZRZ and 3SSZRZ plants produced organs with aberrant shape and smaller size. Leaves were smaller and more serrated than wild-type leaves (Figs. 3A, D, 4A, B). Flowers were smaller than in the wild type (Fig. 4G, H), and siliques were shorter and aberrant (Fig. 4F). Flowers showed reduced fertility.

To confirm that the observed phenotypes were caused by overexpression of the ZRZ gene, levels of ZRZ mRNA were compared between whole seedlings of the wild type and ANT> >ZRZ. ANT> >ZRZ seedlings were found to have a dramatically elevated ZRZ mRNA level relative to the wild type (Fig. 4N).

To test the effect of the location of ZRZ expression on the phenotype, we expressed ZRZ with the STM promoter. The STM promoter drives expression in the SAM and not in leaf primordia, thus representing complementary expression to that of ANT. ZRZ expression driven by the STM promoter did not have an obvious effect during the vegetative phase of plant development, but after the transition to flowering these plants had similar phenotypes to those of ANT> >ZRZ (Fig. 4I–M). Thus, accurate ZRZ expression is critical for properly balanced organ production at the SAM. While ZRZ expression in leaf primordia dramatically affects leaf initiation rate and plant architecture, the SAM is relatively insensitive to ZRZ activity.

Levels of CYP78A5/KLUH mRNA, encoding a putative cytochrome P450 monooxygenase and expressed in a boundary region at the periphery of the SAM (Zondlo and Irish 1999), were dramatically elevated in the amp1 mutant and its allele pt1, as well as following cytokinin treatment (Helliwell et al. 2001). However, double mutants in CYP78A5/KLUH and its close homolog CYP78A7 also resulted in reduced plastochron length (Anastasiou et al. 2007, Wang et al. 2008). We compared CYP78A5/KLUH expression in ANT> >ZRZ and wild-type plants, to test whether common targets are affected in ANT> >ZRZ and amp1/pt1. CYP78A5/KLUH1 mRNA levels were elevated in ANT> >ZRZ plants (Fig. 6). Thus, the amp1 mutation and ZRZ overexpression appear to affect partially overlapping processes.

**Discussion**

While significant progress has been made recently in elucidation of the mechanisms underlying the decision as to when, where and how frequently organs are produced from the SAM, we are far from fully understanding the complex communication network that confers both consistency and flexibility to the timing of organ formation. We show that the specific site of ectopic expression of a MATE transporter, encoded by the ZRZ gene, dramatically affects the rate of organ production, as well as internode length and apical dominance. These findings reveal that members of this MATE protein family are involved in a much broader range of biological activities than previously suspected, including developmental processes.

Our results indicate that ZRZ is involved in specifying the lateral organ initiation rate. Enhanced ZRZ expression in the
primordia results in faster organ production. In contrast, its enhanced expression in the SAM driven by the STM promoter does not affect the organ initiation rate significantly during vegetative development. These findings indicate that the specific expression domain of ZRZ affects organ initiation rate, and that leaf primordia but not the SAM are responsive to enhanced ZRZ activity.

How might a MATE transporter affect the timing of organ initiation? The timing of leaf initiation is thought to be affected by an inhibitory signal from the developing leaf (Snow and

Fig. 4 ZRZ misexpression in specific tissues leads to altered plastochron and increased numbers of axillary branches. (A and B) Sixteen-day-old seedlings. (C and D) A closer view of the rosette leaves’ axils, showing the abundance and premature growth of axillary meristems (arrow) in ANT->ZRZ plants. (E) Mature plants. (F) Siliques of wild-type (left) and ANT->ZRZ (right) plants. (G and H) Inflorescences. (I and J) Thirty-day-old plants. (K–M) Top view of the inflorescences of the indicated genotypes. In A–M, genotypes are indicated at the bottom left corner of each image. (N) ZRZ expression in 21-day-old plants, assayed by qRT–PCR. Averages (±SE, n = 3 biological repeats in ANT->ZRZ and n = 4 in Ler) of ZRZ levels, presented relative to the expression of the TUB internal control are shown for the indicated genotypes. Ler, wild type control; ANT->ZRZ, plants expressing pANT:LhG4 and OP:ZRZ; c, cotyledon; r1, r2, first and second rosette leaves, respectively. Scale bars = A–H 1 mm, I–M 2.5 mm.
Snow 1931, Miyoshi et al. 2004, Kawakatsu et al. 2006, Wang et al. 2008, Lee et al. 2009). In agreement with this, a subset of factors that have been shown to affect leaf initiation rate are expressed in developing leaves, consistent with a non-autonomous effect. Possibly, ZRZ is involved in generating a gradient of a signaling molecule, which in turn is interpreted to regulate the time of initiation. Ectopic ZRZ overexpression may disrupt the gradient, and the outcome would depend on the specific timing and location of expression. Testing of this hypothesis will require identification of the ZRZ substrates. The

Fig. 5 Quantification of the ANT>>ZRZ phenotype. (A) Number of leaves produced by wild-type (Ler) and ANT>>ZRZ plants at the indicated ages. Shown are averages ± SD (n = 10 for Ler and n = 16 for ANT>>ZRZ). (B) Number of siliques. Siliques were counted on plants that ceased producing siliques. (C) Length of the first internode. (D) Total number of elongated axillary buds. In B–D, averages ± SD are shown (n = 9 for Ler and n = 8 for ANT>>ZRZ). (E) Number of axillary buds in the axils of the cotyledons and each rosette leaf (n = 10 in Ler and n = 16 in ANT>>ZRZ).
involvement of a glutamate carboxypeptidase (Heliwell et al. 2001) and a cytochrome P450 (Miyoshi et al. 2004) in the control of the organ initiation rate supports the involvement of such a signaling molecule in the process. The up-regulation of CYP78A5/KLUH gene expression in ANT->ZRZ and amp1 genotypes, which both produce leaves faster than the wild type, is intriguing as loss of CYP78A5/KLUH function causes a similar phenotype of increased leaf initiation. This may suggest that the timing of leaf initiation responds to the relative spatial and temporal activity of CYP78A5/KLUH rather than to its absolute activity.

An alternative hypothesis might be that the activity of ZRZ is related to the cell energetic balance. The fact that ZRZ overexpression resulted in reduced labeling of mitochondria suggests an impact of ZRZ on the mitochondrion (Supplementary Fig. S2). Such an impact could result from direct activity of ZRZ in the mitochondrion or from indirect activity of ZRZ in an adjacent, biochemically related organelle of a similar size (e.g. the peroxisome). The simplest direct scenarios are: (i) ZRZ transporter activity in the mitochondrial membrane exports the MitoTracker dye molecules; or (ii) ZRZ exports other (possibly) charged molecules. In the first scenario, the low probe intensity could be explained by its reduced concentration and in the second scenario its low intensity could result from a decrease in the mitochondrial membrane potential or other essential elements, reducing the activity/vitality of the mitochondrion and thus preventing the fluorescent activity.

The ANT->ZRZ and STM->ZRZ genotypes were produced using the LhG4 transactivation system (Moore et al. 1998). This system comprises driver lines that express the synthetic transcription factor LhG4 under the control of a specific promoter (PRO:LhG4), and responder lines, in which a gene of interest is expressed under the control of several copies of the Escherichia coli operator (OP), which is recognized by LhG4 (OP:GENE). A cross between a driver line and the OP:ZRZ responder line resulted in the expression of ZRZ under the control of the specific promoter. For example, ANT->ZRZ plants express ZRZ under the control of the AINTEGUMENTA promoter, by crossing ANT:LhG4 and OP:ZRZ plants. The ANT and STM driver lines were kindly provided by Yuval Eshed (Weizmann Institute of Science, Israel).

**Materials and Methods**

**Plant material and genetics**

*Arabidopsis thaliana* plants were grown under long-day conditions (16 h light, 21°C). Arabidopsis transgenic lines were generated by the floral dip method (Clough and Bent 1998). Positive transformants were selected in soil by spraying with BASTA (0.5 mM, 2–3 times). The T-DNA insertion in zrz-1 plants (Salk 064288) was confirmed by PCR with the Zf14_sk_L primer from the ZRZ promoter and the LBa1 primer from the T-DNA. To identify a homozygote line, PCR was performed on 10 progeny. qRT–PCR was performed on mRNA from homozygous seedlings.

The ANT->ZRZ and STM->ZRZ genotypes were produced using the LhG4 transactivation system (Moore et al. 1998). This system comprises driver lines that express the synthetic transcription factor LhG4 under the control of a specific promoter (PRO:LhG4), and responder lines, in which a gene of interest is expressed under the control of several copies of the Escherichia coli operator (OP), which is recognized by LhG4 (OP:GENE). A cross between a driver line and the OP:ZRZ responder line resulted in the expression of ZRZ under the control of the specific promoter. For example, ANT->ZRZ plants express ZRZ under the control of the AINTEGUMENTA promoter, by crossing ANT:LhG4 and OP:ZRZ plants. The ANT and STM driver lines were kindly provided by Yuval Eshed (Weizmann Institute of Science, Israel).

**Plasmids, clones and RNA analysis**

The 1015 line was isolated from an enhancer trap population. The expression of ZRZ [p35S:GFP-ZRZ, the ZRZ coding sequence, kindly provided by Dr. Atsushi Kato (Kato et al. 1999)], was performed on 10 progeny. qRT–PCR was performed on mRNA from homozygous seedlings.

**Light microscopy, GUS staining and sectioning**

GUS staining was performed as described previously (Ori et al. 2000). Plants of line 1015 were stained for 2 h. Stained plants...
were photographed in 50% glycerol. For sectioning, plants were prepared and sectioned as described previously (Pekker et al. 2005) except that FAA (3.7% formaldehyde, 5% acetic acid, 50% ethanol) was used as fixative.

**Transient expression and subcellular localization of ZRZ in Arabidopsis protoplasts**

Protoplasts were isolated from Arabidopsis leaf mesophyll and the chimera GFP–ZRZ was transiently expressed using the polyethylene glycol (PEG) transformation method (Locatelli et al. 2003). Protoplasts transiently expressing cytosolic GFP were used as a control. For mitochondrial labeling, the protoplasts were incubated with 100 nM MitoTracker (MitoTracker Red CMXRos M-7512; Invitrogen) according to the manufacturer’s protocol. Imaging of GFP and MitoTracker was performed using a fully motorized epifluorescence inverted microscope (Olympus-IX8 Cell-R; Olympus) with the following features: objective lens, plan apochromat, ×60, oil immersion and a numerical aperture of 1.42. The CCD camera used was a 12-bit Orca-AG (Hamamatsu). The filter sets were GFP-3035B-000 and TXRED-4040B, with a zero pixel shift (Semrock). All images were processed using Olympus imaging software Cell-R for Windows.

**Quantification of the MitoTracker signal relative intensity in protoplasts**

The intensity levels of GFP and MitoTracker were quantified from whole protoplasts using the Olympus Cell-R 3D intensity profile; each cell was optically Z-sliced to 0.28 μm height slices. The mean signal intensity was calculated for each cell separately as the means of the slice intensities. The relative signal intensity of MitoTracker in GFP-expressing cells (ZRZ fused or free GFP) was determined as the ratio of the MitoTracker signal intensity in a GFP-expressing cell divided by the MitoTracker signal intensity in a non-expressing cell (GFP-expressing cell/ non-expressing cell). Analysis was always performed on two adjacent cells, one with high expression of GFP and a second cell with no expression.

**Supplementary data**

Supplementary data are available at PCP online.

**Funding**

This work was supported by the Israel Science Foundation [grants No. 689/05 and 60/10 to N.O., 953/07 to M.M.].

**Acknowledgements**

We wish to thank the Nottingham Arabidopsis Stock Center (NASC) for mutant seeds, A. Kato (Hokkaido University, Japan) for the ZRZ cDNA, Yuval Eshed for driver lines, and Zach Adam, Alon Samach, David Weiss and members of our laboratories for fruitful discussions and criticism.

**References**


