



## CMLs control host-plant interactions

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**In the perspective of world-wide climate changes that likely will affect the outcomes of plant interactions with pathogens, pests and symbionts, the contribution of a certain class of plant-specific calcium sensors, referred to as calmodulin-like proteins (CMLs), will play an important role in the future. Functional, genetic and biotechnological approaches in the model plant *Arabidopsis* and, subsequently as a proof of concept, in agricultural important plant species will be necessary to elucidate their function. Several members of CMLs may act as key players in both abiotic and biotic responses including plant/microbe and plant/herbivory interactions. Interestingly, using gain and loss of function strategies, these interactions can be investigated and manipulated by modulating CMLs expression in model plants. It will be important to understand how CMLs translate intracellular calcium signatures generated by various stimuli from pathogenic and beneficial microbes or pests into appropriate biological responses through the regulation of downstream components. The comprehensive analyses of CMLs will first improve our knowledge on Ca<sup>2+</sup>/CML-mediated signaling pathways but will also facilitate their application in future plant-breeding programs in order to develop highly productive crops.**

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### Introduction

Global climate changes (increased temperatures, CO<sub>2</sub> and O<sub>3</sub> levels, drought or raining periods, *etc.*) will have strong influences on plant performance and plant-host interactions, and will generate novel communities composed of new combinations of species (Lurgi *et al.* 2012). Elevated CO<sub>2</sub> levels

in the atmosphere result in higher CO<sub>2</sub> fixation rates, biomass production and carbohydrate metabolism in plants, and consequently will impact the microbial communities associated with them. The altered plant metabolism influence the biotic interactions, local species distribution (Buckley 2013, HilleRisLambers *et al.* 2013), the structure and function of roots, the diversity of rhizobial communities (Rajkumar *et al.* 2013), and the defense capacity of the plants against biotic and abiotic threats. Various models predict that long-term changes of the biological interactions of plants with their environment as well as their microbial communities have both positive and negative impacts on ecological systems, forestry and agricultural productivity. For example, a recent meta-analysis of more than 400 studies has shown that an increase in microbial and plant biomass in response to global changes should result in an alleviation of drought stress responses in both kingdoms (Kivlin *et al.* 2013). Thus, global climate warming will have unpredictable long-term consequences for crop yields, and the available studies predict predominantly negative effects what will have impacts on food productivity and security in the future. The global food production should increase by 50% to satisfy the world's population demand by 2050, but this goal appears to be difficult to achieve. Indeed, a recent review points out that crop losses due to fungal or oomycete infections, abiotic stress, injuries to plants and other pests will increase in the future due to the expected climate changes (Fisher *et al.* 2012). Selection of suitable crop plants for enhanced yield production and better tolerance to multi-stress conditions is a major task for a future world with an altered global climate (Rajkumar *et al.* 2013). These few findings highlight the importance of plant-host interactions in the context of food production and stability of ecosystems in a changing climate environment, and force scientists to (1) understand the climate change-induced effects on biological interactions at the scientific level (Weckwerth 2011), (2) decipher and enhance resistance mechanisms against pests and pathogens to preserve crop yields, and (3) to develop strategies which stabilize or even promote beneficial symbiotic plant-microbe interaction in a future world with a global climate change.

### The calcium (Ca<sup>2+</sup>) toolkit include CMLs

Among the multiple possibilities to thwart the climate change-induced effects on biological interactions and plant cell signaling, investigations of the plant «calcium (Ca<sup>2+</sup>) toolkit» are highly promising because they take part in one of the earliest events occurring in response to environmental changes, namely the free cellular Ca<sup>2+</sup> increases.

These Ca<sup>2+</sup> increases initiate appropriate signaling pathways leading to specific adaptive responses according the nature and strength of the stimulus or environmental modification perceived by the plant (Hetherington and Brownlee

2004; Hong-Bo et al. 2008; Lecourieux et al. 2006; Poovaiah and Reddy 1993; Rudd and Franklin-Tong 1999; Sanders et al. 2002; Sarwat et al. 2013). In the pathogenic interaction between *Arabidopsis thaliana* and *Pseudomonas syringae*, such Ca<sup>2+</sup> signatures are different according to whether a plant has been challenged by a virulent or avirulent strain (Grant et al. 2000). These differences in the Ca<sup>2+</sup> signatures contribute to initiate a complex signaling network leading to either susceptibility (virulent strain) or defense (avirulent strain). Indeed, if these Ca<sup>2+</sup> signatures have been proven by their spatio-temporal properties to encode a first layer of specificity (McAinsh and Pittman 2009; Ng and McAinsh 2003), they need to be decoded by downstream Ca<sup>2+</sup>-binding proteins possessing the canonical Ca<sup>2+</sup>-binding motif called EF-hand (Nakayama and Kretsinger 1994). The *Arabidopsis* genome encodes at least 250 EF-hand proteins, which is a much higher number than in mammals, arguing for higher importance of these class of proteins in plants (Day et al. 2002). These Ca<sup>2+</sup> sensors can be classified into 4 major groups, the calcineurin-B like, the calcium-dependent kinases, the calmodulin group and a closely related group, the calmodulin-like protein (CML) family, which is specific to plants and encompasses the highest number of members (50 in *Arabidopsis*; Figure 1). The sequence similarity of these Ca<sup>2+</sup> sensors with the evolutionary conserved AtCaM2 (CALMODULIN2) varies from 16% to 74.5% (McCormack et al. 2005) and the CML family members contain between one to six EF-hands. Data currently available for CML members from different plant species indicate that they participate in development but also in stress responses (cf. references in Table 1). For instance, CML9 acts as a negative regulator of drought stress showing that *cml9* mutants are able to withstand water deficit and achieve their full life cycle (Magnan et al. 2008). On the contrary, the rice CML OsMSR2 behaves as a positive regulator of drought and salinity responses (Xu et al. 2011). Overexpression of this CML in *Arabidopsis* results in enhanced tolerance to drought and salinity and increased sensitivity to exogenous ABA (Xu et al. 2011).

It has also been reported that others CMLs are responsive to different abiotic stress such as the gene CML8 which is induced by salicylic acid and NaCl (Park et al. 2010), CML24 whose transcripts level increase in response to high or low temperature (Delk et al. 2005) and CML18 reported to be involved in salt stress signaling through its interaction with the Na<sup>+</sup>/H<sup>+</sup> antiporter AtNHX1 (Yamaguchi et al. 2005).

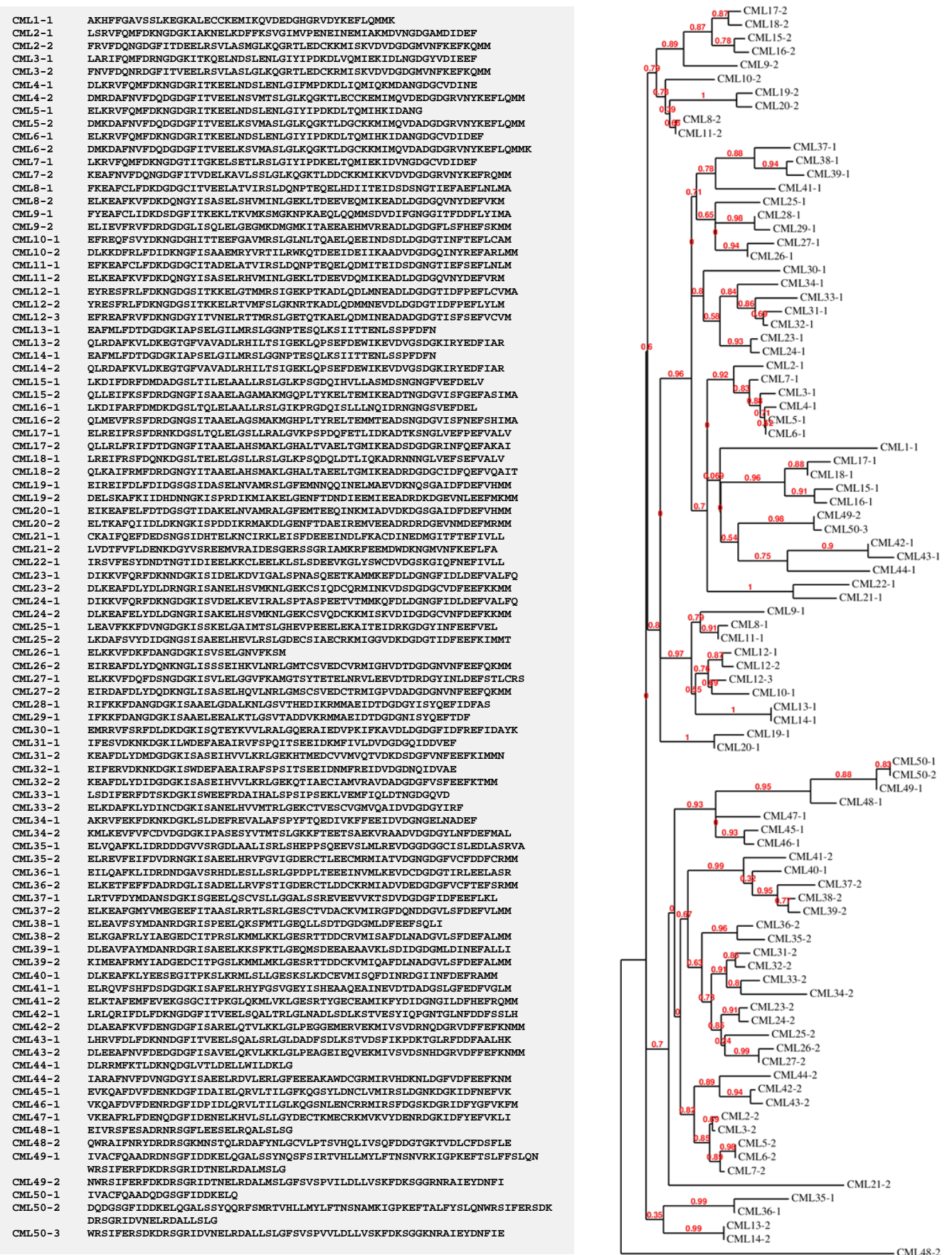
CMLs also contribute to several aspects of plant development including CML42 involved in trichome branching (Dobney et al. 2009), CML25 and -7 in root hair elongation (Lin et al. 2011; Won et al. 2009), CML39 in early seedling establishment (Bender et al. 2013), CML23 and -24 in flowering (Tsai et al. 2007), CML24 in regulating pollen tube growth by modulating the actin cytoskeleton and controlling the cytosolic Ca<sup>2+</sup> concentration (Yang et al. 2014) and CML20 regulating the cortical microtubule organization (Azimzadeh et al. 2008).

In addition, several CMLs have also been reported to participate in plant-host interactions. CML9, a stress-responsive member of the CML family in *Arabidopsis* previously shown to be a negative regulator of plant tolerance to drought (Magnan et al. 2008) is also a positive regulator of salicylic acid-dependent defense responses against the bacterial pathogen *P. syringae* pv *tomato* (Pst) DC 3000 (Leba et al. 2012). The fact that CML9 is involved both in abiotic and biotic stresses indicate that CMLs can be at the crossroads of development, biotic and abiotic stress signaling pathways

(Leba et al. 2012). Thus, as like CML9, CML24 which was already described to be involved in abiotic stress responses (Delk et al. 2005) takes also part in innate immune responses including the hypersensitive response (Ma et al. 2008), and both CML9 and CML24 play a role in the beneficial interaction of the endophyte *Piriformospora indica* with *Arabidopsis* roots in addition to two others CMLs, CML37 and CML43 (Bender and Snedden 2013; Yang et al. 2014). Expression of three of the above cited CML genes (CML42, CML37 and CML9), is also induced by a recently identified MAMP exuded by *Mortierella hyalina* which triggers cytoplasmic Ca<sup>2+</sup> elevation (R. Oelmüller, unpublished data).

Interestingly, if we consider a third type of interaction concerning pests, *Arabidopsis* defense mechanisms in response to attacks by herbivorous insects involve also Ca<sup>2+</sup> signaling. During these interactions, at least eight CML genes appeared to be up-regulated by treatments with oral secretions (OS) from the herbivorous lepidopteran insect *Spodoptera littoralis* (Scholz et al. 2014; Vadassery et al. 2012b). Among them, again CML42 and CML37 are involved but act antagonistically: while CML42 acts as a negative regulator of plant defense against herbivorous insects, CML37 acts as a positive regulator (Scholz et al. 2014; Vadassery et al. 2012a; Vadassery et al. 2012b). The latter CML is connecting Ca<sup>2+</sup> and jasmonate (JA) signaling by affecting the synthesis of jasmonic acid-isoleucine (Scholz et al. 2014). Interestingly CML37 and CML42 also act as positive and negative regulators on drought stress (Vadassery et al. 2012a; Scholz et al. 2015).

It is clear from literature that most of the above mentioned CMLs participate in growth regulation and developmental processes, in addition to their involvement in defense/adaptation processes. This pleiotropic role makes them interesting candidates to understand how calcium can control the final adaptive responses. As shown above, some of them are simultaneously involved in defense against pathogens and in beneficial interactions such as the symbiotic interaction which reinforces the plant defenses against environmental stress. Indeed, we and others have shown that symbiosis strongly promotes plant performance (which results in higher biomass and seed production), resistance to biotic and abiotic stress (Sun et al. 2014), and nutrient uptake, in particular phosphate, nitrate and sulfate (Camehl et al. 2011; Vadassery et al. 2009) by the host. The beneficial effects of *P. indica* in *Arabidopsis*, rice, barley and Chinese cabbage are strongly correlated with the promotion of lateral root development, which – in turn – increases with decrease in the phosphate concentration in the medium (Dong et al. 2013; Lee et al. 2011). In the inoculated plants, the root phenotype becomes bushier in the presence of the fungus. Genetic analyses demonstrated that benefits for the plants during the symbiosis with *P. indica* are associated with cytoplasmic Ca<sup>2+</sup> elevation which stimulates the expression of a specific subset of CML genes in *Arabidopsis* root (Vadassery and Oelmüller 2009, and unpublished data). Interestingly, in another beneficial symbiotic interaction system, the basidiomycete *M. hyalina* also promotes *Arabidopsis* performance when the fungus colonizes the roots. Strong up-regulation of the CML42, CML37 and CML9 mRNA levels both in roots and shoots suggests an important role of these proteins in the symbiotic interaction. Overall, scientific evidence makes CMLs interesting targets to develop new concepts to improve plant defense and performance which are worth to be investigated.



**Figure 1:** Phylogenetic tree built with the EF-hand motifs found in CML from Arabidopsis. (left) amino acid sequence covering EF hand pairs are shown (prediction according to PROTEIN BLAST). The first number after CML indicates the CML corresponding protein (from 1 to 50 in Arabidopsis) and the second number (1, 2 or 3) the EF-hand pair. As reported by Zhu et al. (2015), CML1 is certainly a pseudogene and cannot be considered as a CML but was conserved for the phylogenetic analysis. (right) Phylogenetic tree of the sequences.

## Ideas for future strategies

The role of CMLs in deleterious and beneficial interactions of Arabidopsis with plant pathogens, beneficial microbes and herbivores needs to be investigated in greater details. Indeed, CMLs take part in multiple interactions between Arabidopsis and bacterial [*P. syringae* (Grant et al. 2000)] and fungal pathogens such as *Alternaria brassicae* (Michal Johnson et al. 2014) and *Verticillium dahliae* (Sun et al. 2014), the herbivorous insect *S. littoralis* (Vadassery et al. 2012b) and the root-colonizing symbiotic fungi *P. indica* (Vadassery et al. 2009) and *M. hyalina* (Ludwig and Oelmüller, unpublished). The aims should be to identify (a) the CMLs involved in the decoding of the Ca<sup>2+</sup> signatures induced in the above mentioned model systems, and (b) the downstream signaling components (CMLs targets and their downstream components and effectors) required for the physiological stress adaptive responses leading to resistance (bacteria, insects) or improved symbiosis (*P. indica* and *M. hyalina*).

In each of the interactions, the participation of Ca<sup>2+</sup> variations and CMLs has already been shown. In some cases, CMLs were identified as downstream components of Ca<sup>2+</sup> variations and upstream of regulatory hormonal pathways [SA pathway in the case of *P. syringae* (Leba et al. 2012), JA pathway in the case of *S. littoralis* (Scholz et al. 2014), and putatively auxin in the case of *P. indica* (Lee et al. 2011)]. As outlined above, CMLs appear as versatile tools to integrate at the cellular levels diverse inputs (i.e. stress, pathogen attacks, growth and developmental programs). This raises the

question of their contribution in a single or several signaling pathways. Since they do not have by themselves intrinsic activities they have to play a role by modulating the activity of their downstream targets.

This finding demonstrates that CMLs play an essential and so far little recognized role in various biotic interactions of plants. Their involvement in both pathogenic and beneficial interactions may allow them to balance plant defense responses and beneficial developmental strategies. The trade-off between defense and growth is probably central and often irreversible for the plant and will have a strong influence on its performance and therefore also for agricultural yields. Investment in beneficial symbiotic interactions will stimulate growth and development, which is consistent with the observation that CMLs also participate in these regulatory processes. Therefore, the future goal should be to improve our knowledge about the role of CMLs as Ca<sup>2+</sup> sensors, considering that pathogenic and beneficial plant interactions that form our ecosystems are important determinants for the agricultural productivity and breeding strategies. The central role of CMLs for both defense and developmental strategies makes them interesting candidates in plant breeding programs. The ultimate goal is to increase resistance to pests and pathogens by down-regulating interactions with these deleterious hosts and/or by increasing plant-endosymbiotic interactions. Both reflect processes in which CMLs are involved at central signaling positions and known to be linked to each other (Sun et al. 2014).

**Table 1: Arabidopsis CML proteins and related publications for their gene expression or/and functions.**

Gene	Name	Publications
At3g59450	CML1	-
At4g12860	CML2	Wang et al. 2008; Pagnussat et al. 2005
At3g07490	CML3	Dolze et al. 2013; Chigri et al. 2012
At3g59440	CML4	Che et al. 2006; Lee et al. 2005a
At2g43290	CML5	Lee et al. 2005a; Lee et al. 2005b; Goda et al. 2004; Gepstein et al. 2003
At4g03290	CML6	Lee et al. 2005a; Bae et al. 2003; Wang et al. 2008; Becker et al. 2003; Armengaud et al. 2004
At1g05990	CML7	Lee et al. 2005a; Lin et al. 2011; Won et al. 2009
At4g14640	CML8	Hartmann et al. 2014; Park et al. 2010
At3g51920	CML9	Hartmann et al. 2014; Fischer et al. 2013; Leba et al. 2012a; Leba et al. 2012b; Vadassery et al. 2012; Magnan et al. 2008
At2g41090	CML10	Cho et al. 2015; Franco-Zorilla et al. 2005
At3g22930	CML11	Reddy et al. 2004; Oh et al. 2012
At2g41100	CML12	Antosiewicz et al. 1995; Ito et al. 1995; Rairdan et al. 2001; Wright et al. 2002; Benjamins et al. 2003; Gleeson et al. 2012; Cazzonelli et al. 2014
At1g12310	CML13	-
At1g62820	CML14	-
At1g18530	CML15	-
At3g25600	CML16	-
At1g32250	CML17	-
At3g03000	CML18	Yamaguchi et al. 2005
At4g37010	CML19	Liang et al. 2006; Azimzadeh et al. 2008
At3g50360	CML20	Liang et al. 2006; Azimzadeh et al. 2008
At4g26470	CML21	-
At3g24110	CML22	-
At1g66400	CML23	Tsai et al. 2007
At5g37770	CML24	Tsai et al. 2007; Yang et al. 2014; Ma et al., 2014; Abu-Abied et al. 2006; Delk et al. 2005
At1g24620	CML25	Wang et al. 2015; Lin et al. 2011; Won et al. 2009; Bae et al. 2003; Lee et al. 2005
At1g73630	CML26	Lee et al. 2005; Wang et al. 2008; Zhou et al. 2009
At1g18210	CML27	Lee et al. 2005; Hampton et al. 2004; Armengaud et al. 2004; Carter et al. 2004; Gepstein et al. 2003
At3g03430	CML28	Zhou et al. 2009; Wang et al. 2008; Lee et al. 2005
At5g17480	CML29	Rozwadowski et al. 1999; Zhou et al. 2009; Wang et al. 2008; Lee et al. 2005

At2g15680	CML30	Chigri et al. 2012; Lee et al. 2005
At2g36180	CML31	Lee et al. 2005
At5g17470	CML32	Lee et al. 2005
At3g03400	CML33	Wuest et al. 2010; Lee et al. 2005
At3g03410	CML34	Lee et al. 2005
At2g41410	CML35	Ascencio-Ibáñez et al. 2008; Ma et al. 2007; Wang et al. 2008; Peng et al. 2007; Lee et al. 2005
At3g10190	CML36	Le et al. 2014; Lee et al. 2005; Keates et al. 2003
At5g42380	CML37	Scholz et al. 2014; Scholz et al. 2015
At1g76650	CML38	Lee et al. 2005; Kim et al. 2008; Abercrombie et al. 2008; Marri et al. 2005
At1g76640	CML39	Bender et al. 2013; Vanderbeld et al. 2007
At3g01830	CML40	Ascencio-Ibáñez et al. 2008
At3g50770	CML41	Naydenov et al. 2015
At4g20780	CML42	Vadassery et al. 2012a; Dobney et al. 2009
At5g44460	CML43	Bender et al. 2014; Chiasson et al. 2005
At1g21550	CML44	Lee et al. 2005
At3g29000	CML45	Cho et al. 2015
At5g39670	CML46	Magnan et al. 2008
At3g47480	CML47	-
At2g27480	CML48	Abu-Abied et al. 2006
At3g10300	CML49	-
At5g04170	CML50	-

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