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Heat-shock protein 40 is the key farnesylation target in meristem size control, abscisic acid signaling, and drought resistance

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Protein farnesylation is central to molecular cell biology. In plants, protein farnesyl transferase mutants are pleiotropic and exhibit defective meristem organization, hypersensitivity to the hormone abscisic acid, and increased drought resistance. The precise functions of protein farnesylation in plants remain incompletely understood because few relevant farnesylated targets have been identified. Here, we show that defective farnesylation of a single factor—heat-shock protein 40 (HSP40), encoded by the J2 and J3 genes—is sufficient to confer ABA hypersensitivity, drought resistance, late flowering, and enlarged meristems, indicating that altered function of chaperone client proteins underlies most farnesyl transferase mutant phenotypes. We also show that expression of an abiotic stress-related microRNA (miRNA) regulon controlled by the transcription factor SPL7 requires HSP40 farnesylation. Expression of a truncated SPL7 form mimicking its activated proteolysis fragment of the membrane-bound SPL7 precursor partially restores accumulation of SPL7-dependent miRNAs in farnesyl transferase mutants. These results implicate the pathway directing SPL7 activation from its membrane-bound precursor as an important target of farnesylated HSP40, consistent with our demonstration that HSP40 farnesylation facilitates its membrane association. The results also suggest that altered gene regulation via select miRNAs contributes to abiotic stress-related phenotypes of farnesyl transferase mutants.

[Keywords: farnesylation; heat-shock proteins; meristem; abscisic acid; drought resistance; microRNAs; Arabidopsis]

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Post-translational modification is important for the control of cellular protein activity. Lipid modifications influence the hydrophobicity of proteins and may regulate their membrane association and interactions with other proteins (Wang and Casey 2016). Prenylation, a form of lipidation in which a polyunsaturated farnesyl [C15H25] or geranylgeranyl [C20H35] group is attached to the C-terminal part of target proteins, is particularly widespread in eukaryotic organisms. Protein farnesylation and geranylgeranylation is catalyzed by deeply conserved heterodimeric enzymes that share the same α subunit but have distinct β subunits. These enzymes prenylate a C-terminal CaaX motif in target proteins (where a is an aliphatic residue, and X is any residue) by catalysis of thioether bond formation between the prenyl group and the CaaX motif cysteine (Fu and Casey 1999). In plants, the α subunit of protein prenyl transferases is encoded by the PLURIPETALA (PLP; AT3G59380) gene (Running et al. 2004). Protein farnesyl transferase contains the β subunit encoded by ENHANCED RESPONSE TO ABA1 (ERA1; AT5G40280) (Cutler et al. 1996), while the β subunit GGB (AT2G39550) confers specificity to protein geranylgeranyl transferase (Johnson et al. 2005).

The biological relevance of protein farnesylation in plants has been elucidated through the recovery of mutant alleles of era1 and plp in several forward genetic screens. Protein farnesylation is implicated in abscisic acid (ABA) signaling because era1 and plp mutants exhibit pronounced hypersensitivity to ABA (Cutler et al. 1996; Running et al. 2004). ABA is required for seed dormancy and restriction of water loss in response to drought. At the...
molecular level, ABA perception by pyrabactin resistance (PYR1)-like receptors is linked to activation of a set of protein kinases in the SNF1-related kinase 2 (SnRK2) family by inhibition of a subgroup of protein phosphatase 2C enzymes (Cutler et al. 2010). SnRK2 kinases, essential for ABA signaling [Fujii and Zhu 2009; Fujita et al. 2009], in turn phosphorylate numerous targets, including transcriptional activators of an ABA-related gene expression program and ion channels implicated in rapid closure of guard cells [Furihata et al. 2006; Geiger et al. 2009; Lee et al. 2009]. However, it is not clear at which point protein farnesylation acts in this signaling pathway, since none of its core components contains CaaX motifs at its C terminus. Despite the lack of understanding of the molecular basis of ABA hypersensitivity in farnesyl transferase mutants, drought-inducible knockdown of ERA1 has been exploited to engineer canola plants with improved performance under drought stress [Wang et al. 2005, 2009].

A recent study showed that loss of farnesylation of the cytochrome P450 CYP85A2, involved in brassinosteroid biosynthesis, leads to increased ABA sensitivity and drought resistance, but the ABA hypersensitivity of cyp85a2 mutants defective in farnesylation is substantially less severe than that of era1 mutants [Northey et al. 2016]. In addition, the CYP85A2 farnesylation site is not conserved in species that exhibit drought resistance upon ERA1 suppression [Northey et al. 2016], strongly suggesting the existence of farnesylation targets other than CYP85A2 with importance for ABA signaling and drought resistance.

In addition to ABA hypersensitivity, several developmental phenotypes have been observed in farnesyl transferase mutants. These include altered phyllotaxis and increased floral organ numbers, both of which may derive from enlarged and disorganized meristems in era1 and plp (Running et al. 1998, 2004; Yalovsky et al. 2000). Moreover, farnesyl transferase mutants are late flowering and have a rounder leaf shape. While the effect on leaf shape is probably explained by reduced brassinosteroid biosynthesis due to defective CYP85A2 farnesylation [Northey et al. 2016], the molecular basis of the other phenotypes remains unexplained. Finally, farnesyl transferase mutants show defects in heat tolerance (Wu et al. 2017), in blue light-induced stomatal opening [Jalakas et al. 2017], and in innate immune signaling via several intracellular immune receptors [Goritschin et al. 2008], but farnesylated targets responsible for these effects have also not been identified. Thus, precise molecular explanations for the many clear phenotypes of farnesyl transferase mutants are largely unknown because the relevant farnesylated proteins remain elusive.

The heat-shock protein 40 (HSP40) isoforms J2 [AT5G22060] and J3 [AT3G44110], two of >100 HSP40 proteins encoded in the Arabidopsis genome, are presumed to be farnesylated because the Atriplex nummularia J3 ortholog ANJ1 can be prenylated in vitro [Zhu et al. 1993] and because orthologs in yeast [Ydj1] and humans [DNAJA1–4] are farnesylated in vivo [Caplan et al. 1992, Kanazawa et al. 1997]. HSP40 proteins can initiate a conserved chaperone assembly line that mediates conformational changes required for the activity of many native proteins: An HSP40 dimer binds a client protein and triggers ATP hydrolysis in HSP70 to drive formation of a high-affinity HSP70–ADP–client complex [Misselwitz et al. 1998; Hernandez et al. 2002a,b]. In turn, the adaptor protein Hop mediates client transfer to HSP90 for final conformational maturation together with a host of HSP90 cochaperones [Johnson et al. 1998; Pratt et al. 2008]. Proteins with binding pockets for hydrophobic ligands constitute a well-studied class of clients of the HSP90 assembly line, exemplified by the vertebrate steroid hormone receptors [Picard 2006]. In plants, the HSP90 chaperone pathway plays a crucial role in development and immune signaling (Sangster and Queitsch 2005). Known clients include auxin and jasmonate receptors [Zhang et al. 2015; Wang et al. 2016], a class of proteins with hydrophobic binding pockets [Tan et al. 2007; Sheard et al. 2010], as well as intracellular immune receptors and effector proteins of small RNA-guided gene regulation [Hubert et al. 2003; Iki et al. 2010], two classes of multidomain proteins whose functions are associated with extensive conformational changes [Moffett et al. 2002; Elkayam et al. 2012; Schirle et al. 2014].

Similar to farnesyl transferase mutants, j3 knockout mutants exhibit late flowering [Shen et al. 2011] and were shown recently to be weakly hypersensitive to ABA [Salas-Munoz et al. 2016]. In addition, altered HSP70 and HSP90 expression confers ABA-hypersensitive inhibition of germination and compromised ABA-induced closure of guard cells [Clement et al. 2011]. We therefore hypothesized that farnesylation of J2 and J3 may be important for farnesyl transferase mutant phenotypes and show here that the lack of farnesylation specifically of J2 and J3 nearly completely recapitulates many previously described phenotypes of farnesyl transferase mutants. We also show that farnesyl transferase mutants and transgenic lines expressing farnesylation-deficient J3 fail to express an abiotic stress-related microRNA (miRNA) regulon controlled by the transcription factor SPL7. This regulon includes miR408 [AT2G47015], whose knockout is sufficient to confer drought resistance [Ma et al. 2015]. Thus, many important farnesyl transferase mutant phenotypes are due to altered function of client proteins of farnesylated HSP40 or of the HSP70–HSP90 system in general, and defective expression of an abiotic stress-related miRNA regulon is likely to contribute to the increased drought resistance observed upon loss of protein farnesylation.

Results

J2/J3 are farnesylated in vivo

J2 and J3 are 90% identical at the protein level and share identical putative farnesylation sites [CAQQ] at the C terminus [Fig. 1A]. Inspection of gene expression and coregulation data indicated that J2 and J3 are ubiquitously expressed, that they are tightly coregulated, and that J3 is the major isofrom because it is expressed to threefold to fourfold higher levels than J2 [Fig. 1B,C]. To establish
that J2/J3 are farnesylated in vivo, we first raised polyclonal J2/J3 antibodies recognizing a peptide identical in J2 and J3 (Fig. 1A). Two approaches demonstrated the specificity of these antibodies. First, transient overexpression of J3 in *Nicotiana benthamiana* produced a specific 50-kDa band that comigrated with the only protein detected in microsomal fractions of *Arabidopsis* lysates (Fig. 1D). Second, a transgenic line expressing a dexamethasone-inducible artificial miRNA targeting J3 (amiR-J3) in a j2 knockout background (*j2-2*; SALK_071563) (Supplemental Fig. S1) showed reduced intensity of the 50-kDa band specifically upon amiR-J3 induction (Fig. 1E). Thus, the 50-kDa band detected by the antibodies corresponds to J2/J3 protein.

We next used *N. benthamiana* to express His6-J3WT and His6-J3C417S with a Cys–Ser mutation in the CaaX motif, precluding prenylation (Zhang and Casey 1996). Expression was carried out in the presence of the 14C-labeled prenyl precursor mevalonate to radioactively label prenylated proteins (Fig. 2A). Autoradiograms and Western blots of Ni2+ affinity-purified fractions separated by SDS-PAGE showed clear incorporation of 14C label into His6-J3WT but not into His6-J3C417S, strongly suggesting that J3 is prenylated in vivo (Fig. 2B). Protein prenylation slightly increases protein migration rate in SDS–polyacrylamide gels (Kitten and Nigg 1991). We used this observation to further characterize J3 prenylation. A gel shift was visible between the transiently overexpressed His6-J3WT and His6-J3C417S proteins (Fig. 2B) and, more importantly, between N-terminally Flag-hemagglutinin (FHA)-tagged J3WT and J3C417S immunopurified from stable transgenic lines (Fig. 2C). These data corroborate prenylation of J3 in vivo but do not distinguish between farnesylation and geranylgeranylation. To do so, we examined gel mobility of J2/J3 in microsome samples from Col-0 wild type as well as farnesyl transferase (*era1*) and geranylgeranyl transferase (*ggb*) mutants. J2/J3 migration was slower in samples prepared from the ERA1 deletion mutant *era1-2* (Cutler et al. 1996) and from the T-DNA insertion mutant *era1-9* (SAIL_146D09) but not from ggb-1 mutants (Fig. 2D; Johnson et al. 2005). Taken together,
the gel mobility analyses and the incorporation of mevalonate-derived \(^{14}\)C label into J3\(^{WT}\), but not into J3\(^{C417S}\), establish that J3 is farnesylated in vivo.

**J3 farnesylation facilitates its membrane association in vivo**

To determine whether J3 farnesylation is relevant for its membrane association, we analyzed soluble and microsomal fractions prepared from lysates of transgenic lines expressing FHA-J3\(^{WT}\) and FHA-J3\(^{C417S}\). The ratio between J3 quantity in soluble and membrane fractions was substantially higher in the farnesylation-defective FHA-J3\(^{C417S}\) mutant than in FHA-J3\(^{WT}\) [Fig. 2E], indicating that farnesylation of J3 facilitates its membrane association.

**Farnesyl transferase interacts genetically with HSP90**

If a lack of farnesylation of J2/J3 is important for phenotypes observed in farnesyl transferase mutants, genetic interactions between chaperone and farnesyl transferase mutants may be expected. We therefore tested the genetic interaction of era1-2 with the ATPase mutant hsp90.2-3 in one of five HSP90 isoforms. This mutant displays specific defects related to immune receptor activation but does not show developmental defects [Hubert et al. 2003]. In contrast to either single mutant, era1-2/ hsp90.2-3 double mutants were completely sterile and showed defective flower and inflorescence morphology [Fig. 3A]. This striking genetic interaction is conceptually similar to synthetic-lethal interactions in yeast. Genome-wide mapping of such interactions shows that they tend to occur between ordered subsets of functionally linked genes [Costanzo et al. 2010]. Therefore, although the strong genetic interaction between era1-2 and hsp90.2-3 in and of itself does not allow unambiguous conclusions on the relation between the two genes to be drawn, it does suggest the existence of functionally important links between protein farnesylation and the HSP90 pathway. Consistent with this interpretation, we observed dramatically increased levels of J2/J3, HSP70, and HSP90 in farnesyl transferase mutants compared with wild type [Fig. 3B]. These observations reinforce the hypothesis that the molecular basis of farnesyl transferase mutant phenotypes may involve chaperone farnesylation.

**Mutants defective in J2/J3 farnesylation exhibit ABA hypersensitivity and drought resistance similar to era1**

To test the possible importance of J2/J3 farnesylation directly, we first attempted to generate double-knockout mutants in J2 and J3 and used the T-DNA insertion alleles.

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**Figure 2.** J3 is farnesylated in planta. (A) Schematic overview of transient expression of His\(_6\)-J3 in *N. benthamiana* to test Cys417-dependent incorporation of the \(^{14}\)C-mevalonate-derived label into J3 protein. (B) Ni\(^{2+}\) affinity-purified fractions of extracts from *N. benthamiana* leaves transiently expressing His\(_6\)-J3, His\(_6\)-J3\(^{C417S}\), or unfused His\(_6\) [empty vector [E.V.]]. (Top panel) Western blot analyzed with J2/J3-specific antibodies. (Bottom panel) Autoradiogram visualizing incorporated \(^{14}\)C label. (C) Flag Western blot of Flag immunoprecipitates from inflorescences of *Arabidopsis* stable transgenic lines expressing FHA-J3\(^{WT}\) or FHA-J3\(^{C417S}\). “Col-0 N.T.” refers to Flag purification from inflorescences from the nontransgenic parental line [Col-0]. (D) Western blot of microsome fractions from inflorescences probed with antibodies specific for J2/J3 and for the endoplasmic reticulum-localized small and basic intrinsic protein 2 (SIP2). Samples were separated extensively on 10% polyacrylamide gels. (E) Western blot analyses of soluble and microsome fractions from inflorescences of *Arabidopsis* stable transgenic lines expressing FHA-J3\(^{WT}\) or FHA-J3\(^{C417S}\) and from the nontransgenic wild-type control [Col-0]. Numbers below bands detected with Flag antibodies indicate intensity relative to the signal in the FHA-J3\(^{WT}\) soluble fraction lane. PEPC [phosphoenolpyruvate carboxylase] was used as a soluble protein control, and SIP2 and H\(^+/\)ATPase were used as membrane-bound protein controls. Soluble and microsome fractions were loaded on the same gel, and the separation of panels is due to removal of the lane containing the size marker during figure mounting.
and \( j_3-2 \) [SALK_141625]. The previously uncharacterized allele \( j_3-2 \) contains a T-DNA insertion close to the one in \( j_3-1 \) and showed similar loss of J3 mRNA [Supplemental Fig. S1]. No double mutants could be identified in the progeny of \( j_2-2/j_2-2; j_3-2/+ \) parents, and reciprocal crosses of \( j_2-2/j_2-2; j_3-2/+ \) to wild type showed that simultaneous transmission of \( j_2-2 \) and \( j_3-2 \) knockout alleles through the pollen occurred with dramatically reduced frequency [Supplemental Table S1]. However, it was possible to construct transgenic lines expressing either J3\textsubscript{WT} or the farnesylation-defective J3\textsubscript{C417S} in the \( j_2-2/j_3-2 \) double knockout, indicating that not all J2/J3 activities depend fully on their farnesylation. Similar lines were also constructed in a \( j_3 \) single-mutant background. We first tested the sensitivity to ABA in a germination assay. Remarkably, transgenic lines expressing J3\textsubscript{C417S} in \( j_2-2/j_3-2 \) exhibited pronounced ABA hypersensitivity, similar to era1-2 mutants (Fig. 4A). This phenotype required simultaneous loss of farnesylation of J2 and J3 because neither \( j_3-1 \) single-knockout mutants nor \( j_3-1 \) expressing J3\textsubscript{C417S} exhibited the same strong ABA hypersensitivity [Supplemental Fig. S2]. We next tested the drought resistance of mature rosettes. \( j_2-2/j_3-2 \) expressing J3\textsubscript{C417S} exhibited water retention similar to era1 mutants [Fig. 4B], significantly above the level observed in wild type. Consistent with this result, when plants were rehydrated after prolonged drought, survivors were found only among era1-2 mutants and the transgenic \( j_2-2/j_3-2 \) line expressing J3\textsubscript{C417S} [Fig. 4C]. These analyses show that lack of farnesylation of J2 and J3 is sufficient to confer ABA hypersensitivity.

**Figure 3.** Genetic evidence for functional links between ERA1 and HSP40/70/90. (A) Inflorescences of era1-2, hsp90.2-3, and era1-2/hsp90.2-3 mutants. (B) Accumulation of J2/J3, HSP70, and HSP90 protein measured by Western blotting of total protein extracts from inflorescences. (CBB) Coomassie brilliant blue staining of the membrane used for antibody incubations.

**Figure 4.** J2/J3 are key farnesylated targets for ABA signaling and drought resistance. (A, top) Germination rate measured as fraction of seeds with emerging radicals from seed coats 48 h after transfer to 21°C/light growth conditions. \( n = 100 \). (Bottom) Pictures of plates after 14 d of growth on plates with the indicated concentrations of ABA. The experiment was repeated twice with independent seed lots. Seed lots originated from parental plants that were grown side by side, and mutant and wild-type seeds were cleaned at the same time and stored together before use in the experiments. Two independent lines of J3\textsubscript{WT} and J3\textsubscript{C417S} in the \( j_2-2/j_3-2 \) background were analyzed [J3\textsubscript{WT-1}, J3\textsubscript{WT-2}, J3\textsubscript{C417S-1}, and J3\textsubscript{C417S-2}]. (B) Water retention in 5-wk-old rosettes after a 2-wk period without watering. Asterisks indicate mean values that differ significantly from those obtained in Col-0 wild type. [***] \( P < 0.001 \); [**] \( P < 0.01 \); [*] \( P < 0.05 \), two-tailed \( t \)-test, equal variance. The results obtained in era1-2, era1-9, and the two independent J3\textsubscript{C417S} lines are not significantly different. \( n = 10 \). (C) Survival of plants of the indicated genotypes 2 d after rehydration following a 12-d drought treatment. Under these conditions, Col-0 or \( j_2-2/j_3-2 \) survivors were never identified, while most, but not all, era1-2 and \( j_2-2/j_3-2 \) +J3\textsubscript{WT} plants survived. (D) Accumulation of J2/J3, HSP70, and HSP90 protein measured by Western blotting of total protein extracts from inflorescences. The same transgenic lines as those analyzed in A and C were used. (CBB) Coomassie brilliant blue staining of the membrane used for antibody incubations.
hypersensitivity and drought resistance similar to what is observed in farnesyl transferase mutants. At the molecular level, we observed strong up-regulation of HSP70 and HSP90 in j2-2/j3-2 expressing J3C417S [Fig. 4D], indicating that defective J2/J3 farnesylation is the cause of their induction in farnesyl transferase mutants. Despite the use of the endogenous J3 promoter, J3 protein was also overexpressed in these transgenic lines regardless of the presence of the Cys417Ser mutation [Fig. 4D]. Some chaperone clients are stabilized upon chaperone binding, including the TIR1 class of auxin receptors [Wang et al. 2016]. Since ABA receptors, similar to the TIR1 family, contain hydrophobic binding sites [Nishimura et al. 2009] and therefore are potential HSP40-HSP70-HSP90 clients, we tested steady-state levels of both classes of protein in farnesyl transferase mutants. While TIR1 protein indeed substantially overaccumulated in era1, plp, and j2/j3/J3C417S mutants, no clear effect on accumulation of the ABA receptor PYR1 could be detected [Supplemental Fig. S3]. We also could not detect consistent SnRK2 hyperactivation in response to ABA in era1 compared with wild type, as would be expected if ABA receptor activity were increased as a consequence of chaperone overaccumulation [Supplemental Fig. S4]. Thus, while J2/J3 are clearly key farnesylation targets, the precise point at which they act in ABA signaling remains unclear.

Farnesylation-deficient J3 mutants have enlarged meristems and developmental phenotypes similar to era1 mutants

era1 mutants exhibit developmental phenotypes, including late flowering, altered phyllotaxis, reduced fertility, and a stochastic increase in petal number [Running et al. 1998; Yalovsky et al. 2000; Ziegelhofer et al. 2000]. The underlying cause of some of these phenotypes may relate to the defective control of meristem size. Both era1 and plp exhibit enlarged meristems, suggesting that farnesylation targets somehow control the balance between cellular proliferation and differentiation in the meristem [Running et al. 1998; Yalovsky et al. 2000]. Remarkably, all of these phenotypes were apparent in j2-2/j3-2 lines expressing J3C417S, although the stochastic increase in petal number was less penetrant than in era1-2 [Fig. 5A–C; Supplemental Fig. S5]. We conclude that J2/J3 are particularly important farnesylation targets for several previously described developmental phenotypes of farnesyl transferase mutants, including defective control of meristem size.

Farnesyl transferase and farnesylation-deficient J3 mutants fail to express miRNAs controlled by SPL7

miRNAs control many aspects of plant development and stress responses, and mutants in miRNA biogenesis factors exhibit ABA hypersensitivity similar to era1 mutants [Lu and Fedoroff 2000; Hugouvieux et al. 2001; Han et al. 2004; Laubinger et al. 2008; Zhang et al. 2008]. To test whether defects in miRNA production were discernible in farnesyl transferase and j2-2/j3-2 mutants expressing J3C417S, we used small RNA sequencing [sRNA-seq] to profile small RNA populations in Col-0, era1-2, and the transgenic lines j2-2/j3-2/J3WT and j2-2/j3-2/J3C417S. The results showed that while most miRNAs accumulated normally in era1 and j2-2/j3-2/J3C417S, a small group of abiotic stress- and copper-responsive miRNAs [miR397/
SPL7SBP complemented growth defects of phenotypes and expression of SPL7-dependent miRNAs. Expression profiles of era1-2 and j2-2/j3-2/J3C417S [Fig. 6A]. Indeed, miRNA expression profiles of era1-2 and j2-2/j3-2/J3C417S were nearly identical [Fig. 6A]. Northern blots confirmed that miR398 and miR408 levels were strongly reduced in farnesyl transferase mutants, including plp and independent alleles of era1, and in farnesylation-defective J3C417S lines [Fig. 6B]. miR398 accumulation was also weakly affected in j3-2 single, but not in j2-2, mutants [Supplemental Fig. S6]. miR397/398/408/857 are all controlled by the transcription factor SPL7 [Yamasaki et al. 2009], suggesting that their transcription may be compromised upon loss of HSP40 farnesylation. Indeed, pri-miR397a, pri-miR398b/c, and pri-miR857 levels were strongly reduced in farnesyl transferase mutants and in farnesylation-defective J3C417S lines [Fig. 6C], in contrast to other pri-miRNAs that showed levels similar to wild type [Fig. 6D]. Remarkably, pri-miR398a, encoded by the only MIR398 gene whose transcription is not controlled by SPL7 [Yamasaki et al. 2009], accumulated to higher levels in farnesyl transferase mutants and in farnesylation-defective J3C417S lines than in wild type [Fig. 6E]. This observation further supports defective SPL7 function as the cause of reduced miR397–398–408–857 accumulation upon loss of J2/J3 farnesylation and may provide an explanation for residual miR398 levels detected in farnesyl transferase and farnesylation-defective J3C417S lines.

**Activation of SPL7 requires farnesylated HSP40**

SPL7 is produced as an ER membrane-bound preprotein whose activation by low copper levels involves proteolytic cleavage and translocation of the soluble product to the nucleus [Garcia-Molina et al. 2014]. We therefore asked whether the pathway directing SPL7 activation was compromised upon loss of HSP40 farnesylation—a scenario that would be consistent with our observation that HSP40 farnesylation enhances membrane association [Fig. 2E]. We first tested induction by low copper availability of a series of known direct SPL7 target genes [Yamasaki et al. 2009; Bernal et al. 2012; Garcia-Molina et al. 2014] and used an SPL7 T-DNA insertion mutant [spl7-3; SALK_125385] as a control for SPL7 dependence of induction. All of the target genes tested showed compromised, but not fully abrogated, induction in era1-2 and j2-2/j3-2/J3C417S, while SPL7 mRNA itself was normally expressed [Fig. 7A]. We next used the observation that constitutive expression of a fragment of SPL7 containing only the soluble part homologous to other transcription factors in the SPL family (SPL7SBP) is sufficient to induce SPL7 target genes in young seedlings in the presence of copper [Garcia-Molina et al. 2014]. We generated stable transgenic lines expressing YFP:SPL7SBP in wild type, era1-2, and spl7-3 genetic backgrounds and tested growth phenotypes and expression of SPL7-dependent miRNAs in pools of primary transformants. Expression of YFP: SPL7SBP complemented growth defects of spl7-3 [Fig. 7B], indicating that it retained SPL7 function. YFP: SPL7SBP was not fully functional, however, because despite expression of the YFP:SPL7SBP mRNA [Supplemental Fig. S7], only partial restoration of miR398 was observed in spl7-3 [Fig. 7C]. Importantly, expression of YFP:SPL7SBP in era1-2 fully restored the levels of mature miR398, while a more modest twofold increase was observed for miR408 [Fig. 7C]. Taken together, the defective mRNA expression of direct SPL7 targets, but not SPL7, in era1-2 and j2-2/j3-2/J3C417S and the partial restoration of miR398/miR408 expression by YFP:SPL7SBP indicate that a functional pathway for SPL7 activation requires farnesylation of HSP40.

**Discussion**

The importance of HSP40 farnesylation and its relation to other farnesyl transferase targets implicated in ABA-related phenotypes

Our study demonstrates that the requirement for farnesylation of the HSP40 chaperones J2/J3 underlies the previously described phenotypes of farnesyl transferase mutants related to meristem size, ABA responses, and drought resistance. Two obvious questions arise in the wake of this discovery: First, could other HSP40 proteins in the >100-member J domain family be implicated in explaining farnesyl transferase mutant phenotypes? Knockout of J1, a distantly related and nonfarnesylated paralog of J2/J3, does in fact lead to increased ABA hypersensitivity and drought resistance, but developmental phenotypes of J1 mutants are different from those of era1 [Park and Kim 2014]. It is possible that perturbation of several different HSP40 proteins, including J1 and J2/J3, leads to HSP70/HSP90 induction and that this is the cause of ABA hypersensitivity and drought resistance. The observation that overexpression of HSC70-1 is sufficient to cause ABA-hypersensitive seed germination [Clement et al. 2011] is consistent with this model. Second, which, if any, other farnesylated proteins play roles in farnesyl transferase mutant phenotypes? Two recent studies have yielded results on proteins whose lack of farnesylation may contribute to ABA- and drought-related phenotypes of farnesyl transferase mutants. In the first, the WD40 repeat protein ALTERED SEED GERMINATION2 [ASG2] was shown to be farnesylated, and asg2 knockout mutants exhibit ABA-hypersensitive inhibition of germination [Bassel et al. 2011; Duttleil et al. 2016]. asg2 mutants did not exhibit drought resistance at the rosette level, however, and asg2 point mutants in the farnesylation site were not examined in the seed germination assay [Duttleil et al. 2016], precluding the drawing of clear conclusions on the relevance of ASG2 farnesylation. In the second study, a screen of CaAX motif proteins regulated by ABA led to the identification of the cytochrome P450 enzyme CYP85A2, implicated in brassinosteroid biosynthesis [Northey et al. 2016]. A CYP85A2 CaAX motif mutant showed mild ABA-hypersensitive inhibition of germination and drought resistance of rosettes [Northey et al. 2016], although the ABA-hypersensitive germination was substantially less pronounced than in era1 and what we observed here for farnesylation-deficient j2-2/j3-2/J3C417S lines. One
additional observation supports the notion that J2/J3 are the main farnesylation targets to explain ABA hypersensitivity of farnesyl transferase mutants: The CYP85A2 farnesylation is not conserved in canola (Northey et al. 2016), in which ERA1 suppression leads to enhanced drought resistance (Wang et al. 2005, 2009). In contrast, the J2/J3 farnesylation site is deeply conserved in plants and indeed in eukaryotes in general.

Molecular functions of J2/J3 farnesylation

Our study shows that J3 farnesylation enhances its membrane association, consistent with a role as a membrane anchor. This may be important for some functions, including activation of SPL7 from its membrane-bound precursor. Studies on the yeast J3 ortholog Ydj1 suggest that the role of HSP40 farnesylation may not be limited to...
membrane anchoring. First, the chaperone client Ste11
fails to bind to HSP90 in the absence of Ydj1 farnesyla-
tion (Flom et al. 2008), suggesting that chaperone–client
interaction can involve farnesylation. Second, many farnesyla-
tion targets undergo cleavage of the CaaX site and
carboxymethylation to enhance membrane anchor func-
tion (Wang and Casey 2016). Ydj1 does not undergo these
additional modifications to enhance hydrophobicity
and indeed loses function upon substitution with CaaX
motifs that allow cleavage and carboxymethylation (Hil-
debrandt et al. 2016), suggesting that the role of farnesyla-
tion in the case of Ydj1 is not limited to membrane
anchoring.

A new framework for understanding the implication
of protein farnesylation in plant biology

It is a major conclusion of this study that a clear molecular
understanding of farnesyl transferase mutant phenotypes
requires identification of the relevant chaperone clients.
We show here that a functional SPL7 pathway requires
J2/J3 farnesylation, indicating that a component of this
pathway, perhaps SPL7 itself, is a chaperone client. In ad-
dition, it is likely that the previously observed require-
ment for protein farnesylation for defense activation by
R proteins (Goritschnig et al. 2008) is explained by defec-
tive HSP40/HSP70/HSP90 function because HSP70 and
HSP90 are necessary for R protein function (Hubert
et al. 2003; Takahashi et al. 2003; Noel et al. 2007). Can-
didates in ABA signaling include the ABA receptors that
may use chaperones in vivo to maintain functional hydro-
phobic binding sites for ABA. We could not find evidence
for such a model by measuring downstream SnRK2 kinase
activity as a function of ABA concentration, but note that
this approach is too crude to fully exclude the possibility
that chaperones facilitate SnRK2 activation in vivo. We
emphasize that two different effects on chaperone clients
are likely to be at play upon loss of protein farnesylation.
Some clients may lose function because they specifically
interact with farnesylated J2/J3, while others may exhibit
altered function because of induction of the HSP70–
HSP90 pathway.

Figure 7. A functional SPL7 pathway requires J2/J3 farnesylation. (A) Quantitative RT–PCR analysis of CCH (AT3G56240), FSD1
(AT4G25100), FRO5 (AT5G23990), ZIP2 (AT5G5920), YSL2 (AT5G24380), and SPL7 mRNA abundance in 16-d-old seedlings grown
on Murashige-Skoog medium either without (−Cu) or supplemented with (+Cu) 5µM CuSO4. The figure shows the results of one set of
biological samples (50 seedlings pooled per RNA preparation) with standard error between technical triplicates. (B) Four-week-old plants
grown on soil of Col-0, era1-2, spl7-3 and representative primary transformants expressing the YFP:SPL7SBP transgene. (C) RNA blot anal-
ysis of miR398 and miR408 abundance in leaves from pools of control plants and pools of five primary YFP:SPL7SBP transformants
from each line.
Implication of miRNAs in enhanced drought tolerance of farnesyl transferase mutants

The discovery that J2/J3 farnesylation is required for activation of the SPL7 pathway, including expression of miR397/miR398/miR408/miR857, provides new insights into the possible molecular basis of drought resistance of farnesyl transferase mutants. Knockout of miR408 leads to increased drought resistance in Arabidopsis [Ma et al. 2015], and miR398 and/or miR408 are repressed in response to drought in many plant species, including rice, wheat, tomatoes, peas, and cotton [Zhou et al. 2010; Kantar et al. 2011; Wang et al. 2013; Jovanovic et al. 2014; Akdogan et al. 2016; Candar-Cakir et al. 2016]. On the other hand, defective expression of these miRNAs is unlikely to cause developmental phenotypes of era1 because validated target mRNAs do not encode proteins known to control development (summarized in Arribas-Hernandez et al. 2016) and because spl7 mutants do not exhibit developmental phenotypes similar to era1 or plp (Yamasaki et al. 2009).

Materials and Methods

Plant growth conditions

Seeds were sterilized as described [Arribas-Hernandez et al. 2016] before being sown on Murashige-Skoog [MS] agar plates (4.3 g/L MS salts, 0.8% agar, 1% sucrose) or in soil containing 4% perlite and 4% vermiculite. For seedling analyses, plants were grown for 4 days at 21°C with a 16-h photoperiod (150 µmol m−2 s−1)/8-h darkness cycle. For analysis of adult leaves and inflorescences, plants were grown in growth chambers (Percival) with a 16-h photoperiod (150 µmol m−2 s−1), 21°C/16°C day-night temperatures, and 70% relative humidity. For germination tests, seeds were inoculated for 5 d at 4°C in darkness on MS plates containing 0.5, or 2.5 µM ABA. Germination frequencies of 100 seeds were counted after 48 h of incubation at 21°C with a 16-h photoperiod. For dexamethasone induction of amiR-J3, seeds were germinated on MS medium containing 10 µM dexamethasone. For tests of water retention in leaves, 3-week-old rosettes were left unwatered in growth chambers for 14 d, at which time leaf material was harvested, weighed [fresh weight], dried for 65 h at 60°C, and reweighed [dry weight]. For tests of survival upon drought, 16-d-old seedlings were left unwatered for 12 d and rehydrated, and survival was scored after an additional 2 d. For analysis of YFP:SPL7-SBP primary transformants and respective controls, the leaves of five 4-week-old plants were pooled before RNA extraction.

DNA constructs

ProJ3::3:terJ3 and ProJ3::3C417S::terJ3 constructs were made by a USER cloning strategy. PCR fragments containing 1333 base pairs [bp] of J3 [AT3G44110] promoter and 482 bp downstream from the stop codon were amplified from BAC F26G5 with the USER cloning strategy. PCR fragments containing 1333 base pairs of J3 promoter and 482 bp downstream from the stop codon were amplified from BAC F26G5 with the USER cloning strategy. The 2xFlag-2xHA cassette was amplified from an SDE3::2xFlag-2xHA construct [Garcia et al. 2012], and J3 and J3C417S fragments were amplified from the ProJ3::3::terJ3 and ProJ3::3C417S::terJ3 constructs described above. For construction of pEPAQ-J3, a J3 cDNA was amplified from oligo(dT)-primed cdNA with primers 32/33 and cloned into the AgeI–XhoI site of pEAQ (Sainsbury et al. 2009). For construction of pEPAQ-HT-J3 (His6-J3), a J3 cDNA was amplified with primers 18/19 and cloned into the AgeI–XhoI site of pEAQ-HT-J3 (His6-J3). For construction of amiR-J3, a J3 cDNA was amplified with primers 18/19, 20/21, and 23/24 and cloned into the pSMART vector by Integrated DNA Technologies. The fragment containing the amiRNA sequence was then excised and ligated into pTA7002 [Ayama and Chua 1997] using XhoI and SpeI restriction sites, was synthesized and cloned into the pSMART vector by Integrated DNA Technologies. This construct was then excised into pUTN-DEST[YPF] [Grefen et al. 2010]. Sequences of all of the oligonucleotides used for cloning are in Supplemental Table S2, and a list of transgenic lines generated in this study is in Supplemental Table S3.

Transformation of Arabidopsis

Plants were transformed by floral dipping with Agrobacterium tumefaciens strain GV3101 (Clough and Bent 1998).

Transient expression in N. benthamiana

Expression plasmids were transformed into A. tumefaciens strain GV3101. Bacterial pellets from overnight cultures were resuspended in infiltration medium [10 mM MES, 10 mM MgCl2, 100 µM acetosyringone at pH 5.6] at OD = 0.6 and infiltrated into N. benthamiana leaves with a 1-mL syringe. Leaves were harvested after 72 h of expression. For 14C-mevalonate labeling experiments, infiltrated leaves were detached with the intact petiole 48 h after infiltration and immersed in 0.5× MS medium supplemented with 20 µM lovastatin to inhibit endogenous mevalonate biosynthesis. After 3 h, the leaves were transferred to 2-mL tubes containing 500 µL [50 µCi, 60 Ci/mmol] of 14C-mevalonate and 500 µL of 0.5× MS until the entire volume had been taken up by the leaf. The leaves were then left in 0.5× MS for 12 h. Total lysates were prepared, and His6-tagged protein was purified by immobilized Ni2+ affinity chromatography.

Mutant genotyping and double-mutant construction

Uncharacterized Arabidopsis T-DNA insertion mutants [era1-9, plp-3, j2-2, and j3-2] were genotyped using primers listed in Supplemental Table S2. The deletion in era1-2 was confirmed by PCR with primers inside the ERA1 gene body and by the total absence of signal in quantitative RT–PCRs from RNA prepared from era1-2 [Supplemental Table S2]. era1-2/era1-2, hsp90.2-3/+ individuals were identified by PCR in F2 populations of era1-2 crossed to hsp90.2-3 using the absence of product with ERA1 gene body primers for era1-2 genotyping and primers 16 and 17 (Supplemental Table S2) followed by Asel digestion for hsp90.2-3 genotyping. Double-homozygous plants were identified in the F3 generation. J3/C417S double mutants expressing J3WT or J3C417S transgenes were constructed as follows: J2-2/J2-2/J3-2/J3-2+ was transformed by ProJ3::3::terJ3 and ProJ3::3C417S::terJ3 constructs. PCR screening of 200 primary transformants yielded no J2-2/J3-2 double homozygotes. T2 populations of 10 independent lines (five J2WT and five J3C417S, all with J2-2/J3-2/J3-2+ genotypes) were then rescanned by PCR. Double homozygotes were
identified in two independent lines for both J3

and J3C417S constructs at the expected frequency of one in eight, taking into account that transgenic J3 must be transmitted through the pollen. Double-homozygous plants were amplified, and individuals homozygous for J3 transgenes were identified in subsequent generations before molecular and phenotypic analyses. A CAPS marker using Pef digestion of amplified PCR products was developed to confirm the presence of J3C417S (primers 14 and 15) [Supplemental Table S2]. A list of mutants used and generated in the study is in Supplemental Table S3.

Histological analysis of meristems

Arabidopsis inflorescence meristems were isolated from the primary inflorescences of plants at the same developmental stage such that Col-0 plants were ~8 d younger than farnesyl transferase and J3 mutant lines to compensate for the later flowering of the mutants. After 2 h of fixation in Karnovsky’s fixative, material was dehydrated in a graded acetone series (30%, 50%, 70%, 90%, and 100%). Samples where then infiltrated and embedded in Spurr’s resin. Meristems were sectioned (2 µm) on a SuperNova Reichert-Jung microtome, stained with 0.05% Toluidine blue-O (pH 4.4), and visualized in bright field using a Nikon Eclipse 80i fluorescence microscope. Measurements were performed using ImageJ software.

RNA analyses

Total RNA extraction and small RNA Northern blots were performed as described [Arribas-Hernandez et al. 2016]. Sequences of oligonucleotide probes are in Supplemental Table S2. For quantitative RT–PCR, RNA was treated with DNase I (Fermentas) and converted to cDNA with Revert Aid reverse transcriptase (Fermentas) primed by oligo-(dT) according to the instructions. Quantitative PCR was performed with the SYBR Green master mix (Fermentas) on a CFX Connect real-time system (Bio-Rad). Melting curve analysis of products amplified by each primer pair showed that they amplified a single PCR product. Actin (ACT2) was used as a normalization control after verification of its robustness across the samples analyzed. The primers used are in Supplemental Table S2. Libraries for Illumina sequencing were prepared from 1 µg of total seedling RNA using the NEBNext small RNA library preparation set [Multiplex, New England Biolabs]. The quality of purified RNA and of constructed libraries was confirmed using an Agilent Bioanalyzer and sequenced on an Illumina platform [Aros].

Preparation of protein extracts and immunoblotting

Total seedling or inflorescence protein samples were prepared and analyzed by immunoblotting as described [Arribas-Hernandez et al. 2016].

Affinity purification of His6-J3 and FHA-J3

His6-J3 was purified by immobilized Ni2+-affinity chromatography using nitrilo triacetic acid (NTA)-conjugated agarose beads [Protino, Machery-Nagel] following the manufacturer’s instructions. FHA-J3 was purified by immunosaffinity chromatography using M2-conjugated agarose beads [Sigma] following the manufacturer’s instructions. For Ni2+-NTA purification, 5 g of N. benthamiana leaf tissue was ground to a fine powder and lysed in 6 mL of lysis buffer [50 mM Tris HCl at pH 7.5, 150 mM NaCl, 5 mM MgCl2, 0.1% nonidet P40, protease inhibitor cocktail Roche] and filtered through a 0.45-µm acrodisc. A bed volume of 500 µL of Ni2+-NTA agarose beads was used to bind His6-tagged proteins. Beads were collected on 15-mL disposable columns [Bio-Rad], and the flowthrough was reapplied once to the column. Columns were washed by 20 column volumes of wash buffer [20 mM Tris-HCl at pH 8, 300 mM NaCl, 20 mM imidazol, 1 mM TCEP] and eluted in 500 µL of elution buffer [20 mM Tris-HCl at pH 8, 150 mM NaCl, 300 mM imidazol, 1 mM TCEP]. A similar procedure was used for Flag immunoaffinity purification, except that 2 g of inflorescence tissue was used, and lysis, wash, and elution buffers had different compositions [lysis buffer: 50 mM Tris HCl at pH 7.5, 150 mM NaCl, 5 mM MgCl2, 0.1% nonidet P40, protease inhibitor cocktail [Roche]; wash buffer: 50 mM Tris HCl at pH 7.5, 500 mM NaCl, 5 mM MgCl2, 0.1% nonidet P40; elution buffer: 1× phosphate-buffered saline [PBS], 150 ng/µL Flag peptide]. A bed volume of 60 µL of M2-agarose beads [Sigma] per milliliter of lysate was used.

Antibodies

Rabbit antibodies against HSP40 [J2/J3] were generated by Eurogentec [Belgium] using the following peptide as an antigen: H2N-CNHPDKGDPEKFKEL-CONH2. HSP70, HSP90, SLP2, H+/ATPase, and PYR1 antibodies were purchased from Agrisera, and PEPC [phosphoenolpyruvate carboxylase] antisera was purchased from Rockland Immunochemicals. TIR1 antibodies were a kind gift from Lionel Navarro and were described in Arribas-Hernandez et al. [2016].

Microsome fractionation

Inflorescences were snap-frozen and ground to a fine powder, and 1.2 mL of microsome buffer [50 mM MOPS, 0.5 M sorbitol, 10 mM EDTA, 1% BSA, Roche protease inhibitors version 11 [one tablet/10 mL] at pH 7.6] was added to 0.2 g of ground tissue and vortexed thoroughly. Samples were spun at 8000 g for 10 min at 4°C. Supernatants were transferred to new tubes and repeatedly spun at 8000 g until no pellet was visible. Supernatants were spun at 100,000 g for 30 min at 4°C. Pellets were resuspended in wash buffer [50 mM MOPS, 0.5 M sorbitol, 10 mM EDTA, Roche protease inhibitors version 11 [one tablet/10 mL] at pH 7.6] and repelleted by centrifugation at 100,000 g for 30 min at +4°C. Pellets were resuspended in a small volume of 1× PBS buffer, and protein concentrations were measured using Bradford (Serva). Microsomes were solubilized in NuPAGE sample buffer [Invitrogen] or Laemmli sample buffer [Bio-Rad] before loading on SDS-PAGE gels.

Analysis of small RNA-seq data

Raw Illumina sequencing reads were preprocessed by trimming away the sequence of the miRNA ligation adapter [AGATCG GAAGAGCACGACGTCGAATTCC] using the program Cutadapt. Trimmed reads <21 nucleotides were discarded to be able to distinguish between mature miR398a and miR398b/c, which differ in sequence only at the 21st nucleotide. The trimmed reads were aligned against sequences of the 427 Arabidopsis thaliana miRNAs annotated in miRBase version 21 using strand-specific alignment with Bowtie 2 [Langmead et al. 2009].

Accession codes

Small RNA-seq data have been deposited at the European Nucleotide Archive under ArrayExpress accession number E-MTAB-3736.
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References


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Heat-shock protein 40 is the key farnesylation target in meristem size control, abscisic acid signaling, and drought resistance

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