The co-chaperone HOP participates in TIR1 stabilisation and in auxin response in plants

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Abstract
HOP (HSP70-HSP90 organising protein) is a conserved family of co-chaperones well known in mammals for its role in the folding of signalling proteins associated with development. In plants, HOP proteins have been involved in the response to multiple stresses, but their role in plant development remains elusive. Herein, we describe that the members of the HOP family participate in different aspects of plant development as well as in the response to warm temperatures through the regulation of auxin signalling. Arabidopsis hop1 hop2 hop3 triple mutant shows different auxin-related phenotypes and a reduced auxin sensitivity. HOP interacts with TIR1 auxin coreceptor in vivo. Furthermore, TIR1 accumulation and auxin transcriptional response are reduced in the hop1 hop2 hop3 triple mutant, suggesting that HOP’s function in auxin signalling is related, at least, to TIR1 interaction and stabilisation. Interestingly, HOP proteins form part of the same complexes as SGT1b (a different HSP90 co-chaperone) and these co-chaperones synergistically cooperate in auxin signalling. This study provides relevant data about the role of HOP in auxin regulation in plants and uncovers that both co-chaperones, SGT1b and HOP, cooperate in the stabilisation of common targets involved in plant development.

KEYWORDS
Arabidopsis thaliana, auxin coreceptors, auxin signalling, HSP70-HSP90 organizing protein, HSP90 co-chaperones, SGT1b

Summary statement
This work addresses the role of HOP co-chaperones in auxin signalling through the stabilization of the auxin coreceptor TIR1, expanding the knowledge of HOP function to plant development and to auxin-dependent environmental responses.
1 | INTRODUCTION

Auxin plays an important role in many aspects of plant growth and development, including lateral and adventitious root formation, embryogenesis, maintenance of apical dominance, shoot organ and vascular development or cell elongation and division (Grey et al., 2001; Perrot-Rechenmann, 2010; Woodward & Bartel, 2005). In addition, auxin plays a critical role in plant adaptation to environmental cues, such as tropic responses and thermomorphogenesis (Quint et al., 2016; Rakusova et al., 2015). Auxin is perceived by the TRANSPORT INHIBITOR RESPONSE1/AUXIN RESPONSE F-box family proteins (TIR1/AFB), which act as auxin coreceptors. These are assembled with CULLIN/SKP1-related/RING BOX 1 to form active E3-SCF ubiquitin ligases (Salehin et al., 2015). These SCF complexes play an essential role in auxin signalling, as this hormone favours the interaction between the SCF^{TIR1/AFB} and the auxin signalling repressors Aux/IAAs, promoting Aux/IAAs degradation and, therefore, inducing the transcription of auxin-responsive genes (Del Pozo & Manzano, 2014; Leyser, 2018).

Due to their relevant role in auxin signalling, the specificity and the regulation of TIR1 and AFB auxin coreceptors have been extensively studied, although not completely understood. TIR1 and AFBs show a quite ubiquitous expression (Dharmasiri et al., 2005); nevertheless, not all these E3 ligases contribute with a similar weight to all auxin-regulated processes. Indeed, the analyses of single and higher-order mutants revealed that TIR1, AFB2 and AFB3, but not AFB1, contribute in this order of relevance to auxin signalling during root development, being TIR1 the major player in the process (Dharmasiri et al., 2005; Parry et al., 2009).

TIR1 and AFB2 are unstable proteins, whose stabilisation by the chaperone HSP90 is critical for the proper establishment of different auxin-dependent responses, including the thermomorphogenesis response. In this regard, it was demonstrated that the high accumulation of HSP90 in response to warm temperatures enhances the folding and stability of TIR1 and AFB2, promoting their accumulation and an efficient auxin signalling (Wang et al., 2016). Furthermore, pharmacological studies using HSP90 inhibitors showed that HSP90-dependent TIR1/AFB2 stabilisation is also broadly required for other auxin-regulated processes, such as gravitropism, primary root growth and lateral root formation (Wang et al., 2016). Despite the involvement of the HSP90 chaperone and of the SGT1b co-chaperone in SCF^{TIR1}-mediated auxin responses being known (Di Donato & Geisler, 2019; Grey et al., 2003; Wang et al., 2016; Watanabe et al., 2016; X. C. Zhang et al., 2015), the assumption that other HSP90 co-chaperones could also play a significant role in this process is not trivial. Indeed, it has to be taken into account that HSP90 can execute its folding activity in the absence of auxiliary proteins, suggesting that HSP90 does not always require the action of HSP90 co-chaperones. Even more, although co-chaperones are required to achieve the efficient folding and activity of specific client proteins, not all HSP90 co-chaperones participate in the folding of all HSP90 substrates; instead, the function of some co-chaperones seems to be restricted to specific subsets of client proteins. In addition, it has been described that some co-chaperones exclude the binding of other co-chaperones to the HSP70-HSP90 complex. This is the case, for example, for FKBP5, CYP40 or AHA1, whose interactions with HSP90 are inhibited in the presence of HSP70-HSP90 organising protein (HOP) (Ebang et al., 2016; Harst et al., 2005; Owens-Grillo et al., 1996), sustaining that only specific sets of co-chaperones could be part of the same HSP90 complexes. Altogether, these observations highlight co-chaperones’ specificity, making it extremely interesting, due to the relevance of auxin in plant development, the identification of the precise set of co-chaperones that modulate such important phytohormonal pathways.

HOP proteins are highly conserved HSP70-HSP90 co-chaperones, well known for participating in the folding of specific substrates of the HSP70-HSP90 cycle. HOP co-chaperones are characterised by the presence of three TPR domains that allow HOPs’ interaction with HSP70 and HSP90, interactions that were also described in plants (Chen et al., 2010; Fernandez-Bautista et al., 2017, 2018; Meena et al., 2020; Z. Zhang et al., 2003). In nonplant eukaryotes, it has been extensively demonstrated that HOP assists the folding of signalling proteins, such as transcription factors, kinases and nuclear receptors (Schoof et al., 2017); however, in plants, the role of HOP has been mainly circumvented to the response to different biotic and abiotic stresses (Toribio et al., 2020). Indeed, OsHOP was involved in the innate response to blast rice fungus through the maturation and transport of the PAMP receptor CERK1 (Chen et al., 2010). In addition, HOP was identified as a cellular determinant of Potato virus Y (PVY) symptom development in tobacco and was associated with the acquisition of basal thermotolerance in wheat (Lamm et al., 2017; Meena et al., 2020). In Arabidopsis, the three members of the HOP family: AtHOP1, AtHOP2 and AtHOP3, participate in long-term acquired thermotolerance (LAT) (Fernandez-Bautista et al., 2018), while AtHOP3 plays an essential role in plant defence against Tetranychus urticae and Botrytis cinerea, and in the alleviation of the ER stress (Muñoz et al., 2021; Fernandez-Bautista et al., 2017). Despite all these data reinforcing the role of HOP in response to stress in plants, the possible role of HOP in development remains mainly unknown.

In this paper, we show that the members of the HOP family in Arabidopsis are involved in auxin signalling. HOP binds to TIR1 and modulates TIR1 protein levels. Our data indicate that HOP and SGT1b form part of the same complexes in planta. Furthermore, the study of higher-order mutants reveals that HOPs participate along with SGT1b in auxin response. This study sheds light on HOPs’ function, expanding it to the regulation of the auxin hormonal network, and uncovers that HOPs and SGT1b participate in auxin response, most probably affecting different aspects of TIR1 folding.

2 | MATERIALS AND METHODS

2.1 | Materials, constructs and growth conditions

Arabidopsis mutants hop1-1 (GK-420A10.15), hop2-1 (GK-399G03.03), hop3-1 (Salk_00794) and edm1-1 (CS68749) were
acquired from the Arabidopsis Biological Resource Center (ABRC). The hop triple mutant hop1 hop2 hop3 (Fernandez-Bautista et al., 2018), the tir1-1 mutant (Ruegger et al., 1998), the lines pTIR1:TIR1-GUS and pAFB2:AFB2-GUS (Parry et al., 2009), pTIR1:TIR1-VENUS (Wang et al., 2016) and pDR5:GUS (Ulmasov et al., 1997) were previously described. The reporter lines pTIR1:TIR1-GUS, pAFB2:AFB2-GUS, pDR5:GUS and the mutant edm1-1 were crossed with the hop1 hop2 hop3, and the quadruple homozygous lines were used for further analyses. Except otherwise stated, seeds were surface-sterilised, stratified at 4°C for 48 h and grown at 22°C using a 16-h light photoperiod. For in vitro growth, Murashige-Skoog (MS) medium supplemented with 1% (w/v) sucrose was used in all cases.

The construct p35S:HA-HOP3 was previously described (Fernandez-Bautista et al., 2017). The different constructs were transformed into the Agrobacterium tumefaciens GV3101 strain. Bacterial cultures were used to transiently express the corresponding proteins in Nicotiana benthamiana leaves by agroinfiltration.

2.2 Thermomorphogenesis assays

Arabidopsis seedlings from the different genotypes were grown side by side in a vertical position for 4 days under short-day conditions at 20°C. After this time, the plates containing the seedlings were left at 20°C or transferred to 29°C for additional 3–8 days, as established in the figure legends. The number of analysed seedlings per condition in each experiment is described in the figure legend. When auxin treatments were combined with hypocotyl elongation assays, 4-day-old seedlings grown in short days at 20°C were transferred onto fresh MS media in the absence or presence of different concentrations of auxin and replicate plates were transferred at 29°C. Hypocotyl elongation after the transfer was measured using the image processing toolkit ImageJ (https://imagej.nih.gov/ij/index.html). Statistically significant differences were calculated using one-way analysis of variance and Newman-Keuls multiple tests.

2.3 Analyses of lateral root emergence

Arabidopsis seedlings from the different genotypes were grown side by side in a vertical position for 7 days or 10 days as established in the figure legends. At that time, pictures were taken using a Leica MZ95 stereomicroscope and the number of emerged lateral roots and main root length was measured using the image processing toolkit ImageJ. The number of analysed seedlings per condition and the statistical test used in each experiment to analyse the statistically significant differences are described in the figure legends.

2.4 Root growth inhibition assays

Arabidopsis seedlings from the different genotypes were grown in a vertical position for 4 days in long-day conditions. After this time, seedlings were transferred side by side onto fresh MS media in the absence or presence of different concentrations of auxin ranging from 0 to 1 μM IAA, 0 to 10 nM 2,4-D or 0 to 1 μM NAA. Seedlings were left to grow in such conditions for additional 3–6 days, as established in the figure legends. At that time pictures were taken and the length of roots after the transfer was measured using the image processing toolkit ImageJ. The number of analysed seedlings per condition and the statistical test used in each experiment to analyse the statistically significant differences are described in the figure legends. The percentage of growth inhibition was calculated considering that the inhibition of root growth at the highest concentration of auxin in the assay for the wild-type genotype was 100%.

2.5 Coimmunoprecipitation analyses

Coimmunoprecipitation analyses were carried out as described in (Munoz & Castellano, 2018) with minor modifications. In detail, different combinations of the indicated translational fusions of the proteins of interest with the corresponding epitope tags were transiently expressed in N. benthamiana. Around 1.2 g of the corresponding N. benthamiana leaves (harvested 3 days after the infiltration) were ground in liquid nitrogen and incubated in 4 ml/g of extraction buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5% glycerol, 0.1% (w/v) Triton X-100, 10 mM dithiotreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2% (w/v) polyvinylpolypyrrolidone (PVPP) and protease inhibitors (Sigma-Aldrich)) with end-over-end shaking for 15 min. After centrifugation at 2500 g for 20 min, the supernatants were centrifuged again at 20,000 g for 30 min. Each supernatant was collected and considered the crude extract. Its protein concentration was measured by the Bradford method (BioRad). Coimmunoprecipitation mixtures were made containing the same amounts of total protein in the same volume. Each coimmunoprecipitation mixture was incubated with constant rotation for 1.5 h with 22 μl of the corresponding anti-epitope agarose beads. Subsequently, the beads were collected by centrifugation, washed five times with 1 ml of the extraction buffer lacking polyvinylpolypyrrolidone, eluted with 120 μl of 100 mM glycine-HCl (pH 3), 150 mM NaCl and 0.1% (w/v) Triton X-100 at room temperature for 5 min with 1000 rpm shaking in a thermomixer and collected in a vial with 15 μl of 1 M Tris-HCl (pH 8). The eluted fractions and crude extracts were run in sodium dodecyl sulphate–polyacrylamide gel electrophoresis and subjected to western blot with the corresponding antibodies. For the western blots, monoclonal anti-FLAG (Sigma-Aldrich), bclonal anti-GFP (Roche), high-affinity clone 3F-10 anti-HA (hemagglutinin) (Roche) and NTHOP and HSP90 antibodies (a gift from Dr. Hans Peter Mock, IPK, Gatersleben; Fernandez-Bautista et al., 2018) were used with their respective HRP-secondary antibodies. Enhanced chemiluminescent reagent (GE HealthCare) was used to detect the proteins. Coimmunoprecipitations in Arabidopsis were carried out similarly, with slight modifications, such as an extraction buffer without PVPP, with a lower concentration of dithiothreitol (2 mM) and previous blocking of the anti-epitope beads to avoid nonspecific binding with 1 mg/ml bovine serum albumin in extraction buffer lacking PVPP for 1.5 h.
2.6 | qRT-PCR analysis

qRT-PCRs were performed as described in (Echevarria-Zomeno et al., 2015) using PP2AA3 (At1g13320) for normalisation. Each experiment was conducted using three biological replicates each including three technical replicates. Statistically significant differences were calculated using a t-test. Primer sequences are listed in Supporting Information: Table 1.

2.7 | TIR1 and AFB2 quantification assays

For each experiment, approximately 40 seedlings of the respective lines (pTIR1:TIR1-GUS or pAFB2:AFB2-GUS) in Col-0 or in the hop1 hop2 hop3 triple mutant backgrounds were grown for 7 days on MS in a vertical position. After this time, roots were collected and frozen in liquid nitrogen. β-Glucuronidase activity and total protein quantification from the root extracts were carried out as described in (Chini et al., 2018) with minor modifications.

2.8 | Analyses of auxin transcriptional response using the pDR5:GUS reporter lines

For each experiment, approximately 40 seedlings per treatment and per line from pDR5:GUS lines in Col-0 or in the hop1 hop2 hop3 triple mutant backgrounds were grown for 7 days on MS in a vertical position and then subjected to a treatment with 1 μM IAA, 1 μM 2,4-D or 0.5 μM NAA in liquid MS medium for 5 h. After this, roots were collected and frozen in liquid nitrogen. β-Glucuronidase activity and total protein quantification from the root extracts were carried out as described in the former section.

2.9 | Yeast two-hybrid analyses

Fusion proteins to the Gal4-activation domain (AD) or Gal4-binding domain (BD) were obtained in the pDEST-GBKT7 and pDEST-GADT7 vectors (Rossignol et al., 2007). Yeast transformation into AH109 and interaction analyses were carried out as described in (Castellano & Sablowski, 2008).

3 | RESULTS

3.1 | HOP family is involved in the thermomorphogenesis response

In a previous study, we demonstrated that HOP proteins are involved in LAT in plants (Fernandez-Bautista et al., 2018). Therefore, to increase our knowledge of the role of HOP in plant adaptation to heat, we focused our attention on thermomorphogenesis, a process that mediates hypocotyl and petiole elongation in response to moderate increases in ambient temperature (Quint et al., 2016). To do so, we quantified the hypocotyl elongation of seedlings from the wild-type genotype and the hop1 hop2 hop3 triple mutant after 3 days from the shift from 20°C to 29°C. As a control, hypocotyl elongation in plants maintained at control conditions was also measured. As shown in Figure 1a,b, no differences in hypocotyl length were observed between Col-0 and the hop1 hop2 hop3 triple mutant plants after the transfer from 20°C to 29°C. Seedlings were grown for 4 days under short-day conditions at 20°C. After this time, seedlings were left to grow at 20°C or transferred to 29°C for additional 3 days. Data represent mean and SE (n = 42 seedlings for each genotype and condition). Bars, 0.2 cm. (c) Quantification of hypocotyl elongation in seedlings from Col-0 and hop1 hop2 hop3 triple mutant plants transferred to 29°C in the absence or presence of different concentrations of IAA. Data represent mean and SE (n = 28 seedlings for each genotype and condition). Statistically significant differences were calculated using one-way analysis of variance and Newman-Keuls multiple tests (ns, nonsignificant; **p < 0.01; ***p < 0.001). All experiments were repeated three times obtaining similar results.

Figure 1. The hop1 hop2 hop3 triple mutant shows an altered response during thermomorphogenesis associated with possible deregulation of the auxin response. (a) Representative photographs and (b) quantification of hypocotyl elongation of seedlings from Col-0 and hop1 hop2 hop3 triple mutant plants after the transfer from 20°C to 29°C. Seedlings were grown for 4 days under short-day conditions at 20°C. After this time, seedlings were grown for 4 days at 20°C or transferred to 29°C for additional 3 days. Data represent mean and SE (n = 42 seedlings for each genotype and condition). Bars, 0.2 cm. (c) Quantification of hypocotyl elongation in seedlings from Col-0 and hop1 hop2 hop3 triple mutant plants transferred to 29°C in the absence or presence of different concentrations of IAA. Data represent mean and SE (n = 28 seedlings for each genotype and condition). Statistical significant differences were calculated using one-way analysis of variance and Newman-Keuls multiple tests (ns, nonsignificant; **p < 0.01; ***p < 0.001). All experiments were repeated three times obtaining similar results.
mutant at 20°C; however, compared to the wild type, hypocotyl elongation was significantly reduced in the hop triple mutant at 29°C. Reduction in hypocotyl elongation in the hop triple mutant was also observed at longer incubation periods at 29°C (Supporting Information: Figure S1a), suggesting that the HOP family is involved in the thermomorphogenesis response.

It has been previously shown that the members of the HOP family in Arabidopsis could display a certain degree of specialisation, with some members playing a predominant role over others in certain processes (Toribio et al., 2020). Therefore, to analyse whether this could be also the case during thermomorphogenesis, we compared hypocotyl elongation in response to moderate increases in temperature of wild type and the hop1, hop2 and hop3 single mutants. As shown in Supporting Information: Figure S1b, compared to the wild type, hypocotyl length at 29°C was significantly reduced in every single mutant. These data indicated that all members of the HOP family in Arabidopsis actively participate in the thermomorphogenesis response and, therefore, we followed our studies using the hop1 hop2 hop3 background.

3.2 | The hop1 hop2 hop3 triple mutant shows an altered thermomorphogenesis response that is partially rescued in the presence of auxin

Thermomorphogenesis is a complex process that relies on the interplay between different hormonal networks (Stavang et al., 2009). Among them, auxin has emerged as a fundamental regulator of hypocotyl elongation in response to mild heat stress (Gray et al., 1998; Wang et al., 2016). Therefore, to investigate whether the reduced hypocotyl elongation in the hop1 hop2 hop3 mutant could be associated with a defect in the auxin pathway, we carried out a similar experiment, transferring the seedlings to a medium containing 0, 0.1 or 2 µM of IAA just before the 29°C shift. As shown in Figure 2a, the analyses of root growth inhibition in 7-day-old seedlings was significantly reduced in the hop1 hop2 hop3 triple mutant compared to wild-type plants in the presence of different concentrations of indole-3-acetic acid (IAA). A similar result was also observed in response to 2,4-D (Figure 2c) and in response to 1-naphthaleneacetic acid (NAA) (Figure 2d), two auxin analogues whose uptake in the cell depends on the influx carrier or is mainly achieved by passive diffusion, respectively (Delbarre et al., 1996; Seifertova et al., 2014). Together, these data indicate that the hop1 hop2 hop3 triple mutant displays defects in auxin-associated developmental processes and shows a partial insensitivity to auxin, a phenotype that is observed regardless of the active or passive auxin intake.

3.3 | The hop1 hop2 hop3 mutant is partially insensitive to auxin

To provide additional evidence of HOPs’ involvement in auxin function, we analysed whether the hop1 hop2 hop3 mutant also displays possible defects in other auxin-dependent processes, such as lateral root development. As shown in Figure 2a, 7-day-old hop triple mutant seedlings showed a reduced number of lateral roots per primary root length and this phenotype was also maintained at later seedling stages (Supporting Information: Figure S1c), suggesting that HOP is also involved in other well-known processes regulated by auxin. Furthermore, we decided to directly analyse the auxin response of the hop1 hop2 hop3 mutant by assaying root growth auxin inhibition. As shown in Figure 2b, the percentage of root growth inhibition in 7-day-old seedlings was significantly reduced in the hop1 hop2 hop3 triple mutant compared to wild-type plants in the presence of different concentrations of naphthaleneacetic acid (NAA). A similar result was also observed in response to 2,4-D (Figure 2c) and in response to 1-naphthaleneacetic acid (NAA) (Figure 2d), two auxin analogues whose uptake in the cell depends on the influx carrier or is mainly achieved by passive diffusion, respectively (Delbarre et al., 1996; Seifertova et al., 2014). Together, these data indicate that the hop1 hop2 hop3 triple mutant displays defects in auxin-associated developmental processes and shows a partial insensitivity to auxin, a phenotype that is observed regardless of the active or passive auxin intake.

3.4 | HOP proteins interact with TIR1 and modulate TIR1 accumulation

The partial insensitivity of the hop1 hop2 hop3 mutant to IAA, 2,4-D and NAA suggests that HOP proteins could be specifically involved in auxin signalling. It has been previously reported that HSP90 modulates the stability of the auxin coreceptors (Wang et al., 2016). As Arabidopsis HOP proteins interact with HSP90 (Fernandez-Bautista et al., 2017, 2018), we wondered whether AtHOPs may assist HSP90 in promoting the folding and stability of the auxin coreceptors, modulating in such a way auxin signalling in plants.

To validate this hypothesis, we analysed whether HOP proteins interact with TIR1, the main auxin coreceptor in the root (Dharmaepiri et al., 2005; Parry et al., 2009). To do so, we transiently expressed TIR1-IgGBD-Myc along with HA-HOP3 in N. benthamiana leaves and we carried out TIR1 immunoprecipitations using immunoglobulin G (IgG) beads. As shown in Figure 3a, along with TIR1, we observed a clear band that corresponds to HOP3 in the eluate from TIR1 immunoprecipitations. This band did not appear in the eluates lacking TIR1, demonstrating that HOP3 interacts with TIR1 in vivo. This interaction was further validated by additional coimmunoprecipitation analyses in N. benthamiana using different tags and swapping the immunoprecipitated protein (in this case AtHOP3) (Supporting Information: Figure S2a) and in pTIR1:TIR1-Venus Arabidopsis plants using anti-GFP beads and anti-N. tabacum HOP antibodies previously described in (Fernandez-Bautista et al., 2018) (Supporting Information: Figure S2b). In both cases, the endogenous NbHSP90 and AtHSP90, respectively, were also detected in the immunoprecipitation eluates of HOP and TIR1, respectively.

TIR1/HOP interaction opened the possibility that HOP assists HSP90 in the maintenance of TIR1 stability. To test this hypothesis, we took advantage of the line pTIR1:TIR1-GUS in the Col-0 background. This construct was previously shown to restore wild-type auxin response in the tir1-1 mutant root (Parry et al., 2009), suggesting that it is able to recapitulate the levels and activity of the
endogenous TIR1 protein. pTIR1:TIR1-GUS was crossed with the hop1 hop2 hop3 mutant and GUS messenger RNA (mRNA) expression and GUS activity (as a measure of TIR1 protein level) were evaluated in the wild-type and hop1 hop2 hop3 mutant backgrounds. As shown in Figure 3, despite the levels of the GUS mRNA analysed by qRT-PCR being similar in Col-0 and the hop triple mutant backgrounds (Figure 3b), GUS activity (and, therefore, TIR1 protein level) was significantly reduced in the hop1 hop2 hop3 mutant (Figure 3c) compared to the wild-in planta genotype. All these data highly indicate that HOP proteins modulate TIR1 levels in planta.

As HSP90 also modulates the levels of AFB2 (Wang et al., 2016), we also carried out a similar approach to evaluate whether HOP modulates the levels of AFB2 in plants. For this, the line pAFB2:AFB2-GUS was crossed with the hop1 hop2 hop3 mutant. As shown in Supporting Information: Figure S3, in contrast to TIR1, the levels of GUS mRNA (Supporting Information: Figure S3a) and GUS activity (Supporting Information: Figure S3b), and, therefore, AFB2 quantity were similar in the hop1 hop2 hop3 mutant and the wild-type genotypes. These results suggest that HOP proteins modulate the levels of TIR1, but do not seem to significantly alter the levels of AFB2 in planta. Furthermore, this result highlights the specificity of HOP for precise sets of client proteins.

3.5 Auxin-dependent transcriptional activation of the DR5 promoter is reduced in hop1 hop2 hop3 mutant

A central step in auxin response involves the transcriptional de-repression of the auxin-responsive genes. Hence, to evaluate if the
role of HOP in TIR1 stability is relevant for the proper establishment of auxin-dependent transcriptional response, we analysed the activation of the synthetic auxin-responsive promoter pDR5 (pDR5).

With this aim, we crossed the reporter line pDR5:GUS in Col-0 background with the hop1 hop2 hop3 triple mutant, and GUS activity was analysed as a measure of pDR5 activation in response to IAA, 2,4-D or NAA. As shown in Figure 4a-c, GUS activity was significantly reduced in the presence of IAA or of different auxin analogues, which highly suggests that the transcriptional response to auxin is altered in the hop1 hop2 hop3 mutant. These data correlate with the partial insensitivity to auxin of the hop1 hop2 hop3 triple mutant.

### 3.6 HOP proteins contribute with SGT1b to TIR1 stability and auxin signalling in plants

All these results point out that HOP proteins play the main role in auxin signalling, most probably through TIR1 stabilisation, revealing, for the first time, that HOPs form part of the set of co-chaperones that regulate auxin response.

It has been previously established that the HSP90 co-chaperone SGT1b regulates TIR1 stability (Gray et al., 2003; Wang et al., 2016; X. C. Zhang et al., 2015). This, along with the provided data in this
manuscript, indicated that both co-chaperones—SGT1b and HOPs—share TIR1 as a target, giving us the unique opportunity to explore the participation of both co-chaperones in the auxin response. To start this characterisation, we decided to evaluate the specific weight of each protein within the auxin pathway. For this, we analysed in parallel the sensitivity to auxin (in terms of root growth inhibition and lateral root density) of the hop1 hop2 hop3 mutant, the sgt1b mutant (edm1-1 allele) and the tir1-1 mutant. As shown in Supporting Information: Figure S4a,b, and as expected from previous reports using different sgt1b mutants (Gray et al., 2003; Wang et al., 2016; Zhang et al., 2015), both the hop1 hop2 hop3 triple and the edm1-1 mutants show a root auxin resistance phenotype. This phenotype is milder in the case of hop1 hop2 hop3 triple mutant compared to edm1-1, which is quite close to tir1-1 (a mutant containing a single base substitution (Ruegger et al., 1998)). Remarkably, the effect on edm1-1 was fully rescued by the expression of SGT1b, which reinforces that the auxin phenotype of this mutant is due to the lack of SGT1b (Supporting Information: Figure S5). All these data suggest that, although both co-chaperones seem to participate in the auxin response, SGT1b could play a more prevalent role in this process.

As HOP and SGT1b bind to TIR1, affecting its stability, and play a nonfully redundant role in auxin response, we rationalised that they could cooperate together in auxin signalling. To test this hypothesis, we analysed whether HOP and SGT1b could be part of the same complexes, which may explain why both the sgt1b and the hop triple mutant individually show an insensitive phenotype to auxin. For this, we analysed the interaction between HOP with SGT1b by yeast two-hybrid analyses. As shown in Supporting Information: Figure S6, the three members of the AtHOP family interacted with SGT1b in this system. To verify that this interaction could also take place in vivo, we performed immunoprecipitation analyses of SGT1b in extracts from N. bethamiana leaves expressing AtSGT1b-Flag and HA-AtHOP3. As shown in Figure 5a, Hop3 is specifically coimmunoprecipitated with SGT1b, indicating that HOP and SGT1b form part of a common complex in planta.

Finally, to get a deeper insight into the possible cooperation of both co-chaperones in auxin response, we generated a quadruple mutant by crossing the hop1 hop2 hop3 triple mutant with the SGT1 edm1-1 allele and we compared the response to different concentrations of IAA of the quadruple mutant with that of the hop1 hop2 hop3 triple mutant and the edm1-1 allele. As shown in Figure 5b,c, the quadruple mutant hop1 hop2 hop3 edm1-1 displayed an enhanced insensitivity to auxin in root growth inhibition assays and a significantly reduced lateral root density than the parental lines at different concentrations of auxin, suggesting that HOPs and SGT1b cooperate together in auxin signalling. A similar phenotype was also observed at 29°C (Supporting Information: Figure S7). These results indicate that HOP and SGT1b form part of common complexes in planta and interact synergistically to modulate auxin-dependent processes in the root.

4. DISCUSSION

4.1 HOP proteins are involved in different processes controlled by the auxin pathway

Our assays indicate that HOP proteins play a role in different auxin-dependent processes associated with plant development (such as the formation of lateral roots) or associated with plant adaptation to the environment (such as the thermomorphogenesis response). In addition, our data clearly show that the lack of HOP significantly reduces TIR1 accumulation and the auxin-dependent transcriptional response. These data, along with the increased resistance to auxin of the hop1 hop2 hop3 mutant, highlight the role of this co-chaperone in the auxin pathway.

Despite the fact that HOP affects TIR1 stability, it is noteworthy that TIR1 protein levels are reduced but a certain amount of TIR1 is already found in the hop1 hop2 hop3 mutant, suggesting that in the absence of HOP part of TIR1 is still stabilised and may be functional. The fact that a portion of TIR1 seems to remain functional in the hop1 hop2 hop3 mutant is in accordance with the reduced auxin resistance of the hop1 hop2 hop3 mutant compared to tir1-1. This, along with the observation that HOPs do not seem to modulate AFB2 levels, could perfectly explain the modest but reproducible auxin-resistant phenotype compared to other auxin-related mutants. These data also suggest that HOPs contribute, not to the establishment of the auxin response per se (which is still supported in the absence of HOP), but to offer a proper response to auxin through the stabilisation of TIR1, one of the main auxin coreceptors during root development (Parry et al., 2009). This seems to be in accordance with the function of mHOP during the folding of the mGR, as in this case HOP is not strictly required for GR folding, but significantly increases the yield in the acquisition of GR’s native conformation (Morishima et al., 2000).

Remarkably, all the processes (lateral root formation, auxin-dependent inhibition of root growth and thermomorphogenesis) are also sensitive to HSP90 activity (Wang et al., 2016). Despite HSP90 being involved in the regulation of the auxin pathway and HOP being known as an HSP90 co-chaperone in plants (where the three members of the HOP family interact with HSP90 in vivo) (Fernandez-Bautista et al., 2017, 2018), no previous role of HOP was described in auxin response. In this sense, our data are the first to demonstrate that HOP proteins, like HSP90, participate in auxin signalling, interact with TIR1 and modulate TIR1 accumulation. This reinforces the hypothesis that HOP proteins assist HSP90 in the folding and stabilisation of TIR1 during auxin signalling.

4.2 HOP contributes with SGT1b to TIR1 stability and auxin signalling in plants

HOP and SGT1b were traditionally thought to be involved in specific processes (different for each co-chaperone) and to affect the folding and stability of different client proteins. Indeed, while HOP was
involved in the maturation of mammalian GR, the involvement of SGT1 in this process remains unclear (Stuttmann et al., 2008). In addition, SGT1 was associated with CBF3 kinetochore assembly in yeast and CENP-A deposition and kinetochore assembly in humans, but HOP co-chaperones have not been directly involved in these processes. In plants, HOP was involved in the maturation and transport of CERK1 (a receptor containing multiple LysM and a kinase domain), while SGT1b was found to mediate pathogen resistance by stabilising a completely different set of defence proteins, the NLR proteins, which contain nucleotide-BD and leucine-rich repeats (Austin et al., 2002; Azevedo et al., 2002; Shirasu et al., 1999; Takahashi et al., 2003). Furthermore, HOP proteins were involved in the acquisition of LAT in Arabidopsis and basal thermotolerance in wheat (Fernandez-Bautista et al., 2018; Meena et al., 2020), while SGT1b was described to have an opposite heat-associated effect (Noël et al., 2007).

In contrast to this initial assumption, our data show that HOP interacts with TIR1 and modulates its stability, supporting that HOP and SGT1b could share the same targets and participate in common processes. This observation is also reinforced by the recent description that HOP3 interacts with COI1 and contributes to Jasmonic acid-associated plant defence in Arabidopsis (as SGT1b does) (Muñoz et al., 2021). However, that latter study did not include the analysis of HOP/SGT1b interaction or the analyses of combined mutants, precluding to reach conclusions on the possible relationship between these two proteins and on the specific weight of each co-chaperone in the process under study. Our data, however, help to clarify these aspects. Indeed, our analyses highly suggest that both co-chaperones are required and contribute to efficient auxin signalling. Furthermore, we also provide genetic and biochemical data on HOP/SGT1b interaction in plants. As HOP binds and stabilises the auxin coreceptor TIR1 (as SGT1b does), the analysis

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**FIGURE 5** HOP (HSP90-HSP70 organising protein) proteins interact biochemically and genetically with SGT1b. (a) Coimmunoprecipitation analyses. Protein extracts (crude extracts) from *Nicotiana benthamiana* leaves transiently expressing, under the control of the 35S promoter, different combinations of SGT1b-Flag, HA-HOP3 and HSP90-HA (hemagglutinin) were subjected to immunoprecipitation using anti-Flag beads (IP: anti-Flag). The presence of the different proteins in the crude extracts and in the eluted fractions after SGT1b immunoprecipitation was analysed by western blot using anti-Flag, anti-HA and anti-HSP90 antibodies. This experiment was repeated three times, obtaining similar results. (b) Quantification of the percentage of root elongation inhibition in 7-day-old seedling from Col-0 and from the hop1 hop2 hop3, the edm1-1 and the quadruple hop1 hop2 hop3 edm1-1 mutant by increasing concentrations of IAA. (c) Quantification of lateral root density of the cited mutants in the above-described conditions. For (b, c) data represent the mean and SE of *n* = 3 experiments (each containing 28 seedlings for each genotype and condition). Statistically significant differences were calculated using one-way analysis of variance and Newman-Keuls multiple tests (ns, nonsignificant; *p* < 0.05; **p** < 0.01; ***p*** < 0.001).
of the mutants suggests that within the complex both co-chaperones are involved in slightly different processes in TIR1 folding and that SGT1b plays a more relevant role in this process. This may explain (1) why in the absence of HOP part of the bulk of TIR1 is still active, (2) the milder auxin-resistance phenotype of the hop1 hop2 hop3 mutants compared to tir1-1, (3) the more intense auxin-resistance phenotype of edm1-1 mutant compared to the hop triple mutant and (4) the striking and additive phenotype of the quadruple mutant. Alternatively, it could be possible that, along with TIR1, other unknown targets of SGT1b may contribute to the auxin signalling.

Despite HOP and SGT1b being part of the same complexes, whether this interaction is direct or sustained by their simultaneously binding to HSP90 is still an open question. Nevertheless, different data seem to suggest that an HOP-HSP90-SGT1b could be formed. Indeed, it is well known that HOP and SGT1b bind preferentially to the open conformation (ADP-bound form) of the HSP90, making possible these complexes. In addition, despite both proteins containing TPR motifs, HOP and SGT1 seem to bind to HSP90 using different domains. Specifically, it has been described that HOP's TPR2A mediates the interaction with the C-terminal part of HSP90, while in the case of SGT1, the interaction is held through the CS domain, which contacts with the amino-terminal part of the chaperone (Catlett & Kaplan, 2006; Noël et al., 2007). As HOP and SGT1b use different domains to interact with the HSP90 chaperone and they differ on the portion of the HSP90 they bind to, it is highly probable that both co-chaperones interact simultaneously with HSP90 at an early step in the HSP70-HSP90 cycle, forming a HOP-HSP90-SGT1b ternary complex with HSP90 acting as a bridge. In accordance with this observation, the formation of a human HOP-HSP90-SGT1b ternary complex was previously described in vitro (Catlett & Kaplan, 2006); however, the existence of these complexes in vivo or their functional relevance was not revealed. In our coimmunoprecipitation assays, we show that SGT1b interaction with HOP3 seems to increase when HSP90 is coexpressed in N. benthamiana leaves along with the co-chaperones, suggesting that HSP90 seems to favour the HOP/SGT1b association. Despite the fact that more experiments are needed to unequivocally demonstrate that HSP90 mediates the interaction between HOP and SGT1b, our data are the first that demonstrate the existence of HOP-SGT1b complex in vivo and that both co-chaperones coordinate to participate in multiple developmental and environmental responses associated to auxin pathway.

Auxin signalling is a complex process that depends on the formation of macromolecular complexes to allow Aux/IAAs degradation by the E3-SCF ubiquitin ligases SCF\textsubscript{TIR1,AFBs}. Within these latter complexes, TIR1 and AFBs are assembled with CULLIN/SKP1-related/RING BOX 1 (Salehin et al., 2015). In this sense, it is worth noting that HOP-HSP90-SGT1-SKIP1 complexes have been observed in vitro (Catlett & Kaplan, 2006), which opens the possibility that, apart from stabilising TIR1, the complex HOP-HSP90-SGT1b may also affect the auxin response through their interaction with SKP1 or by modulating TIR1 binding to the SCFs or to the AUX/IAA repressors. However, these possibilities seem improbable. Indeed, it is well known that SGT1 interacts with SKP1 through the SGT1's TPR domain (Kitagawa et al., 1999), but that this domain is not required for auxin signalling. Indeed, the expression of SGT1b constructs lacking the TPR domain are able to complement the auxin resistance phenotypes of sgt1b-1 mutant (Azevedo...
et al., 2006), suggesting that the TPR and, thus, the SGT1 interaction with SKP1 may not be relevant for SGT1b function in auxin signalling. In addition, it has been reported that the SGT1b allele eta3, which also displays an auxin resistance phenotype and lower levels of TIR1, does not show alterations in TIR1 interaction with CUL1, with ASK1 or with AXR3 (Gray et al., 2003). These data seem to favour that SGT1b modulates the stability of TIR1 over the hypothesis that SGT1b-mediated TIR1 folding could be strictly required for the binding of TIR1 to other components of the SCF\(^{TIR1}\) complexes or with the AUX/IAA proteins. Nevertheless, future experiments are required to fully discard these hypotheses.

Compiling our results, the information about HOP in other eukaryotes and the role of SGT1b in plants, it is possible to speculate that HOP, consistently with its function in other eukaryotes, may facilitate TIR1 loading to HSP90 (Figure 6). Interaction with HSP90 may expose the substrate to a large surface scattered with hydrophobic and charged amino acids, increasing the hydrophobicity and promoting its progression in the folding with the help of SGT1b, which may impinge an additional conformational change to favour TIR1 stability. In this scenario, HOP, as described in mammals, would increase the acquisition of TIR1 active conformation, while SGT1b may participate in a more relevant aspect of the folding and functional activity of the SCF-TIR1 complex.

Due to the relevance of the auxin pathway in plant development and stress response, these data highly contribute to understanding auxin signalling regulation in plants. Furthermore, our data exceeds the importance that this finding has in the plant field as it also opens the possibility that HOP and SGT1 cooperate in the folding of other signalling proteins in other eukaryotes.

AUTHOR CONTRIBUTIONS
Alfonso Muñoz designed and performed the communoprecipitation experiments, carried out the different crosses of the reporter lines with the hop mutants and performed GUS activity. Silvina Mangano carried out the phenotypical assays and performed the crosses with SGT1b mutants. René Toribio carried out expression analyses and prepared the figures. Lourdes Fernández-Calvino carried out the initial thermomorphogenesis assays. Juan C. del Pozo contributed to the design of experiments and in the writing/revision of the article. M. Mar Castellano planned the research, participated in the design, supervised the experiments and wrote the article with the contribution of all the authors.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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HOPS ARE INVOLVED IN AUXIN SIGNALLING

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