TIR1-like auxin-receptors are involved in the regulation of plum fruit development

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Abstract

Ethylene has long been considered the key regulator of ripening in climacteric fruit. Recent evidence showed that auxin also plays an important role during fruit ripening, but the nature of the interaction between the two hormones has remained unclear. To understand the differences in ethylene- and auxin-related behaviours that might reveal how the two hormones interact, we compared two plum (Prunus salicina L.) cultivars with widely varying fruit development and ripening ontogeny. The early-ripening cultivar, Early Golden (EG), exhibited high endogenous auxin levels and auxin hypersensitivity during fruit development, while the late-ripening cultivar, V98041 (V9), displayed reduced auxin content and sensitivity. We show that exogenous auxin is capable of dramatically accelerating fruit development and ripening in plum, indicating that this hormone is actively involved in the ripening process. Further, we demonstrate that the variations in auxin sensitivity between plum cultivars could be partially due to PslAFB5, which encodes a TIR1-like auxin receptor. Two different PslAFB5 alleles were identified, one (Pslafb5) inactive due to substitution of the conserved F-box amino acid residue Pro61 to Ser. The early-ripening cultivar, EG, exhibited homozygous endogenous auxin levels and auxin hypersensitivity during fruit development, while the late-ripening cultivar, V98041 (V9), displayed reduced auxin content and sensitivity. We show that exogenous auxin is capable of dramatically accelerating fruit development and ripening in plum, indicating that this hormone is actively involved in the ripening process. Further, we demonstrate that the variations in auxin sensitivity between plum cultivars could be partially due to PslAFB5, which encodes a TIR1-like auxin receptor. Two different PslAFB5 alleles were identified, one (Pslafb5) inactive due to substitution of the conserved F-box amino acid residue Pro61 to Ser. The early-ripening cultivar, EG, exhibited homozygous genotype. Our results highlight the impact of auxin in stimulating fruit development, especially the ripening process and the potential for differential auxin sensitivity to alter important fruit developmental processes.

Key words: Auxin-receptors, plum fruit development, Prunus salicina, subcellular localization, protein–protein interaction, auxin/ethylene crosstalk.

Introduction

Fruit and seeds are essential for human nutrition. Post-fertilization, fruits develop rapidly through a coordinated programme of molecular, biochemical and structural changes that optimize the potential for the development and dissemination of the seeds within. The fundamental importance of these processes has prompted considerable research into how they are governed. Research into fruit developmental processes has been greatly aided by analysing the outcome of both naturally occurring and induced genetic diversity (Kelly and Bradford, 1986; Lau et al., 2008). One outcome of this research has been the identification of phytohormones as master regulators of the many processes involved (McAtee et al., 2013). Ethylene, auxin, GA, ABA, and cytokinin have all been shown to play important integrative roles (Crane, 1969; Srivastava and...
Given its almost ubiquitous importance, it was not unexpected that auxin would play a prominent role in reproductive growth (Friml, 2003). Collectively, data from auxin application, quantification, and molecular genetic studies have shown that auxin is integral to most reproductive processes (Sedgley and Griffin, 1989; Sundberg and Østergaard, 2009). For example, auxin is required for floral meristem formation and acts with homeotic genes in determining floral organogenesis (Coen, 1992). Auxin is critical for the cell divisions that occur in response to fertilization (fruit-set), to the subsequent expansion and development of these cells (Mapelli et al., 1978), and in regulating the onset and coordination of ripening processes (Gillaspy et al., 1993; Trainotti et al., 2007; El-Sharkawy et al., 2010). Recent studies have shown that exogenous auxin accelerates fruit development and ethylene production, acting at least partially by triggering the expression of several genes, such as ACS and ERF, encoding ethylene-synthesis and -response components (Trainotti et al., 2007; El-Sharkawy et al., 2008, 2009).

Auxin responses are regulated at multiple levels, but they basically fall into two categories: responses that affect the hormone content and responses that regulate the response capacity (Lau et al., 2008). Previous studies in tomato and Arabidopsis have identified auxin-signalling components, such as a member of the Aux/IAA family (IAA9) and two members of the Auxin Response Factor protein family, ARF7 and ARF8, that are clearly important for early fruit development (Wang et al., 2005; Goetz et al., 2006, 2007; de Jong et al., 2009b). Downregulation of these transcriptional regulators results in pleiotropic phenotypes, including fertilization-independent fruit-set.

The discovery that certain F-box proteins are auxin-receptors considerably improved the understanding of how auxin mediates cellular responses (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). Basically, Aux/IAAs and ARF-transcriptional regulators interact in homo- and heterodimers, forming complexes that repress auxin-dependent changes in gene expression and therefore auxin action. Auxin binding to an F-box receptor promotes the binding of Aux/IAAs to the SCF$^{TIR1/AFB}$ complex, leading to the ubiquitin-dependent proteolysis of the Aux/IAA (Dharmasiri and Estelle, 2004; Leiser, 2006). Loss of Aux/IAA repressors allows ARF-mediated auxin-responsive changes in gene transcription. Although this archetypal response mechanism is widespread, genetic studies have demonstrated alternative mechanisms. For example, TIR1- and AFB2-type auxin-receptors have been shown to be positive regulators of the auxin-response; however, loss-of-function studies revealed that members of the distinct AFB4 group act as negative regulators (Dharmasiri et al., 2005b; Parry et al., 2009; Greenham et al., 2011). Given that auxin is a key regulator of early fruit development, it might be expected that TIR1-like receptors also regulate the process (Ren et al., 2011).

In this study, we evaluated the role of auxin in flower and fruit development of two plum cultivars that vary in their flowering and ripening dates. We showed that the diversity in fruit development behaviours and auxin-responsive-ness between the early-ripening/auxin-hypersensitive EG cultivar, and the late-ripening/reduced auxin-sensitivity V9 cultivar, could be partially due to a mutation occurred within the essential F-box domain of the auxin-receptor PslAFB5. This mutation resulted in a substitution in the highly conserved residue Pro$^{61}$ to Ser. Using yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) strategies we provided evidence that the allele with Pro$^{61}$ (PslAFB5) encodes a functional auxin-receptor and the allele with the substitution Ser$^{61}$ (Pslafb5) is inactive. Interestingly, genetic analysis of the two plum cultivars revealed that the early cultivar, EG, exhibited homozygosity for the inactive allele gPslafb5; however, the late cultivar, V9, displayed a gPslAFB5/afb5 heterozygous genotype. The data presented here suggest that the auxin-hypersensitivity in the EG cultivar is at least partially due to the loss of PslAFB5 activity. Collectively, our results highlight the importance of auxin in coordinating the progression of fruit development, particularly in terms of the developmental phase transition that leads to the onset of ripening.

**Materials and methods**

**Plant materials and post-harvest treatments**

Reproductive structures from flower buds to ripening fruit were collected from two Japanese plum (Prunus salicina L.) cultivars, Early Golden (EG) and V98041 (V9). These were frozen in liquid-N$_2$ and stored at -80°C until analysis. These two varieties were chosen according to the diversity of their responses to auxin application.

Fruit were picked at stages S1 (first exponential growth phase), 16–34 days after bloom (DAB); S2 (pit hardening), 36–54 and 32–50 days after bloom; S3 (second exponential growth phase), 57–77 and 74–108 DAB for EG and V9, respectively. Fruits from S4 (climacteric ripening) were collected from EG between 78 and 83 DAB, and from V9 between 110 and 128 DAB. To evaluate the effects of exogenous auxin in flower and fruit development, EG and V9 trees were sprayed during the reproductive cycle (flower bud and immature expanded fruit, stage 3) with two different concentrations of the synthetic auxin naphthalene acetic acid (NAA) (10 and 100 μM). Mature fruits of EG (76 DAB) and V9 (108 DAB) were harvested, surface sterilized, and subjected to various treatments, including: propylene (1000 μl l$^{-1}$); the ethylene-inhibitor 1-MCP (1 μl l$^{-1}$); NAA (10 and 100 μM) for EG and V9, respectively); and the auxin transport-inhibitor TIBA (10 μM). Non-treated fruit were used as controls. See supplementary material for other details concerning hormone quantification, PslTIR1/AFBs sequence isolation and analysis, nuclear acid extraction, qPCR assays, and subcellular localization.

**Generation of truncated and mutated versions of PslTIR1-like proteins**

Fragments containing N-terminal (∆N) and C-terminal (∆C) deletions of PslTIR1, PslAFB2, and PslAFB5 were obtained by PCR using gene-specific primer pairs (primers 11–22, Supplementary Table S1 available at JXB online). Internal deletions of the F-box domain (AF) from each sequence were created using two different strategies depending on the variation in N-terminal length (see supplementary material for further details). Mutants of PslTIR1 (Pro$_{1}$ to Ser) and PslAFB5 (Pro$_{61}$ to Ser) were created using the QuikChange site-directed mutagenesis kit (Strategene, San Diego, CA, USA).

**Y2H assays**

Yeast two-hybrid (Y2H) assays were performed using the Matchmaker Gold Yeast two-hybrid System (Clontech, Palo Alto, CA, USA). Full-length and modified PslTIR1, PslAFB2,
and PslAFB5 ORFs were cloned into the BamHI-SalI site of the pGBK77 bait vector (GAL4 binding-domain; DBD). Tomato and Arabidopsis cDNAs were fused into either the NdeI-BamHI site (AtIAA7) or the BglII-BamHI site (ASK1, SIIA3, and SIIA49) of the pGADT7 prey vector (GAL4 activation-domain; AD). Bait and prey vectors (100 ng) were then introduced into Y2HGold and Y187 yeast strains, respectively, using Yeastmaker yeast transformation system 2. Interactions between the proteins were assayed by the mating method, according to the manufacturer’s instructions, in 96-well plates containing medium with or without 100 µM IAA. All assays were repeated at least three independent times.

BiFC assays

Full-length and mutated PstTIR1 and PstAFB5 ORFs were fused into the Sall-I BamHI site of the PSAT1-N vector, containing N-terminal YFP (NY). ASK1 and AtIAA7 sequences were cloned into the BglII-BamHI and XhoI-BamHI sites, respectively, of the PSAT1-C vector containing C-terminal YFP (CY). The different combinations of constructs encoding NY and CY at similar concentrations were mixed and then co-transfected into protoplasts obtained from suspension-cultured tobacco BY-2 cells in the presence of 100 µM IAA, as previously described (El-Sharkawy et al., 2014). Empty BiFC vectors (NY/CY), (NY:gene /CY) and (NY/CY:gene) were used as negative controls. All assays were repeated at least three times and visualized using confocal microscopy.

Results

Auxin enhances fruit development

To assess the role of auxin in fruit development, a range of plum genotypes that vary in their flowering and ripening dates were selected and treated with NAA (Supplementary Table S2 available at JXB online). Two cultivars, early-ripening, EG, and late-ripening, V9, were selected for further analysis based on the diversity of their responses to the auxin treatment. Application of auxin to floral buds considerably enhanced flower development in both cultivars as determined by flower sizes at different developmental stages (Supplementary Table S3 available at JXB online). In EG, the maximum response to auxin in terms of flowering occurred at an NAA concentration of 10 µM. However, higher concentrations of NAA diminished responses in EG flowering (Fig. 1A, B; Supplementary Table S3 available at JXB online). In contrast, a flowering response to auxin application occurred only at a concentration of 100 µM in V9. In both cultivars, auxin treatment accelerated the fruit-set by 5 ± 1 days compared to the untreated controls. Moreover, treatment of mature fruits from both cultivars with auxin resulted in significant increases in fruit size (~28%) and weight (~35%) over the untreated controls (Fig. 1C, D, E), indicating the potential for auxin to affect fruit development.

Ripening is the final stage of fruit development. Many of the processes integral to the ripening of plums and other climacteric fruit are known to be triggered by ethylene (Lelièvre et al., 1997). To examine the contribution of auxin to the coordination of fruit development, the onset of ethylene production, and ripening, we monitored the ethylene production and endogenous IAA levels during EG and V9 fruit development. In stone fruit, including plum, the fruit growth followed a typical double-sigmoidal curve (Fig. 2A, B).
in which four developmental stages (S1–S4) can clearly be recognized (El-Sharkawy et al., 2007). V9 exhibited significant delays in fruit developmental progression compared to EG, due to a delay in flowering (16 ± 2 days later than in EG) and extended S3 duration (14 ± 2 days longer than in EG), resulting in prolonged fruiting period (Supplementary Table S2 available at JXB online). Ethylene was undetectable in immature fruit, but both cultivars produced significant amounts of ethylene during ripening (Fig. 2C, D). EG fruit, in particular exhibited a sharp increase in ethylene production during ripening. In contrast, the onset of ripening was delayed in V9 (22 ± 2 days later than in EG) with lower ethylene levels, resulting in slower ripening process (13 ± 2 days longer than in EG).

The IAA accumulation patterns were similar in the two cultivars during the early stages of fruit development (Fig. 2E, F). Endogenous IAA levels were low at anthesis, but rose rapidly in flowers post-fertilization. IAA levels gradually declined thereafter in both cultivars to low or undetectable levels by S2. A clear divergence in IAA accumulation profiles between the two cultivars occurred subsequent to this minimum. In EG, the concentration of endogenous IAA increased rapidly as the fruit matured, reaching maximal levels during climacteric ripening. In stark contrast, the levels of IAA increased only slightly in V9, and remained low through to the end of the ripening phase.

Auxin application accelerated the onset of ripening; thus, to ascertain the relationship between the auxin treatment and ethylene production and ripening, ethylene was assessed during the ripening of EG and V9 fruits pre-exposed to different treatments designed to alter auxin- or ethylene-responses. EG and V9 fruit displayed a climacteric peak of ethylene production at ~5 days and ~13 days after the onset of ethylene production, respectively. Ethylene emission at the peak of the climacteric was 48.2 ± 4.2 and 5.7 ± 0.9 nl g⁻¹ h⁻¹ for EG and V9, respectively. Propylene-treatment accelerated ripening and increased ethylene production in both cultivars. Maximal levels of ethylene were observed at ~2 days (EG) and ~5 days (V9) post-treatment with a correspondingly increased ethylene production of 80.5 ± 5.1 and 58.6 ± 3.2 nl g⁻¹ h⁻¹ for EG and V9, respectively (Supplementary Figure S1A, B available at JXB online). As expected, all fruit treated
with the ethylene response inhibitor, 1-MCP, were unable to ripen autonomously and their ethylene production remained low (Supplementary Figure S1C, D available at JXB online).

Auxin dramatically accelerated the ripening of both plum cultivars, but in a slightly different manner for each one. Ethylene production in the auxin-treated EG fruit peaked at a high level (69.5 ± 5.5 nl g⁻¹ h⁻¹) ~3 days after treatment. Relative to NAA-treated EG fruit, a lower climacteric ethylene peak of 30.6 ± 2.2 nl g⁻¹ h⁻¹ was observed in NAA-treated V9 fruit and this peak occurred much later, ~15 days after the treatment (Supplementary Figure S1E, F available at JXB online). Treatment with the auxin transport inhibitor TIBA had almost no effect on ethylene production in EG, but had more pronounced effects in V9 (Supplementary Figure S1G, H available at JXB online), as determined by the time necessary to have the climacteric peak after the treatment and the levels of ethylene at the peak.

Identification and characterization of PslTIR1/AFB genes

To investigate the molecular basis of auxin action in fruit development, genes encoding three novel proteins closely related to the F-box TIR1-like gene family of auxin-receptors were isolated from plum. The isolated sequences were designated as PslTIR1, PslAFB2, and PslAFB5 to comply with the names retained for the homologues from other plant species. The relationships between the predicted plum and Arabidopsis amino acid sequences (Dharmasiri et al., 2005b; Walsh et al., 2006), as indicated by percentage similarity over the whole sequence, are presented in Supplementary Table S4 available at JXB online. Despite strong sequence diversity among the different gene members, there were also highly homologous sequences putatively coding for closely related proteins. Strong sequence similarity between specific plum and Arabidopsis sequences (80–89%) indicates that these sequences are likely to be orthologous. The derived PslTIR1/AFB sequences comprise three typical domains that are associated with the F-box subunit protein of SCF (E3) ubiquitin–ligase (Supplementary Figure S2 available at JXB online). The deduced amino acid sequences of PslTIR1 and PslAFBs start by an N-terminal domain with unknown function that exhibits high sequence divergence in length [3–58 amino acids (aa)] and homology (12–66% similarity), followed by the characteristic F-box domain (42–48 aa), which shows higher sequence similarity, ranging from 65–81%. Finally, the C-terminal domain, which is composed of several LRRs, occupies the major part of the predicted protein (521–535 aa; 64–90% similarity). The number of LRRs varied among the different sequences [predicted to be 5- (PslAFB5), 6- (PslTIR1), and 7-LRRs (PslAFB2)]. See supplementary material for gene structure and organization.

PslTIR1/AFBs are components of an SCF complex that interacts with Aux/IAAs

F-box subunits are anchored into SCF complexes via binding of the F-box domain to Skp1 (Zheng et al., 2002). If PslTIR1/AFB proteins are part of a functional SCF complex, they should interact with one or more Skp1 homologues (Gray et al., 1999). We tested for the capacity of PslTIR1/AFBs to interact with the Arabidopsis Skp1-related protein (ASK1) using Y2H. Consistent with the Arabidopsis TIR1-like proteins, all PslTIR1/AFBs interacted strongly with ASK1 in a manner independent of auxin action (Fig. 3). We next sought to determine if Aux/IAA proteins are potential substrates of the SCF complex ubiquitin–ligase by assessing interactions between PslTIR1/AFBs and several Aux/IAAs using the Y2H system in the presence or absence of IAA (Fig. 3). The well-characterized Aux/IAA proteins chosen for this study were the Arabidopsis IAA7 (AtIAA7), and the tomato IAA3 and IAA9 (SIIA3 and SIIA9), which represent three different sub-clades of Aux/IAAs and play distinct roles in mediating auxin responses in planta (Nagpal et al., 2000; Wang et al., 2005; Chaabouni et al., 2009). The analysis revealed a clear difference in the binding specificity and auxin-dependence of the PslTIR1/AFB proteins for the various Aux/IAAs. The binding results confirmed the auxin-induced assembly of stable IAA::PslTIR1/AFBs::Aux/IAAs co-receptors in yeast.

Characterization of PslTIR1/AFBs domains

All TIR1/AFB gene family members are structurally conserved across land plant lineages. Their predicted proteins comprise three typical domains—N-terminal, F-box, and C-terminal—that are commonly associated with the F-box subunit protein of SCF (E3) ubiquitin–ligase (Supplementary Figure S2 available at JXB online). To explore the structural contribution of each domain in mediating auxin signalling, we performed a series of experiments, including subcellular localization and Y2H assays of full-length ORFs and ORFs containing independent deletions of the three PslTIR1, PslAFB2, and PslAFB5 domains. The full-length proteins are referred to
as wild-type (WT). Proteins lacking the N-terminal, F-box, or C-terminal domains are referred to as ∆N, ∆F, or ∆C, respectively. Analysis of TIR1/AFB sequences from different plant species revealed the absence of any feasible targeting sequence that can signify the localization of such protein in the plant cell. Fluorescence microscopy revealed, however, that as expected the full-length PslTIR1/AFB::GFP fusions were localized exclusively in the nucleus (Fig. 4A). Treatment of cells with IAA before transfection of the GFP fusion construct did not alter the protein localization (data not shown). Deletion of the N-terminal (∆N) domain did not visually affect nuclear localization. By contrast, removal of the C-terminal domain (∆C) noticeably altered the protein localization. Without the C-terminal domain, the GFP signal was uniformly spread throughout the cytoplasm and nucleus. An even more dramatic disruption of protein localization occurred when the F-box domain was eliminated (∆F). Small amounts of the protein localized in the nucleus or cytoplasm; however, most of the GFP signal was observed on the outer surface of the nuclear envelope. To further assess the contribution of each domain in forming SCF-like complexes and activating auxin signalling, the different ORFs were challenged to interact with ASK1 and AtIAA7 in the presence of auxin using a Y2H approach (Fig. 4B). Compared with full-length ORFs, the lack of an N-terminal domain did not noticeably affect the interaction of ∆N with ASK1. Interestingly, although PslTIR1ΔN and PslAFB2ΔN were able to assemble a co-receptor complex with AtIAA7, the interaction activity of PslAFB5ΔN was visibly reduced. The ∆C-derivative remained able to interact with ASK1, albeit at a significantly lower efficiency. In contrast, lack of a C-terminal domain resulted in a complete failure to interact with AtIAA7. Deletion of the F-box domain completely abolished the ability of the protein to interact with ASK1 and AtIAA7. Therefore, although the F-box domain appears to be the key factor in successful Skp1-like protein complex formation, the loss of the C-terminal domain is similarly critical for proper function.

**PslTIR1/AFB expression during fruit development**

To investigate the contribution of the plum auxin receptors in fruit development, the accumulation profiles of the three genes were determined at different developmental stages in EG and V9 fruit. During the immature stages (flowering to S3), both cultivars exhibited similar transcript accumulation profiles for all three genes, with slightly, but consistently higher levels, in EG compared with V9 (data not shown).

All transcripts were initially present at low levels in flower buds, but accumulated during flower development, peaking soon after fertilization at ~7 DAB (Fig. 5A). The levels detected at this stage were the highest detected over the experiment, with the transcript levels for all three genes declining to very low levels by the S2 stage (~52 DAB). PslTIR1 and PslAFB2 transcript levels, however, increased markedly during S3, with the progression to maturity. By contrast, those of PslAFB5 were barely detectable during this phase.

Transcript levels for the three genes were closely assessed during the S4 stage, where most ripening-related metabolic changes take place. Clear differences in transcript accumulation profiles were observed during this stage (Fig. 5B). In EG, PslTIR1 and PslAFB2 levels were low before the onset

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**Fig. 4.** Functional characterization of PslTIR1, PslAFB2, and PslAFB5 domains. Constructs containing full-length ORFs are referred to as WT. Truncated derivatives, including independent deletion of the N-terminal, F-box, and C-terminal domains, are referred to as ∆N, ∆F, and ∆C, respectively. (A) Subcellular localization of WT and truncated derivatives fused to the GFP tag. All constructs were transiently transformed for the assay into *Nicotiana tabacum* protoplasts. NLS-mCherry was included in each transfection to indicate the location of the nucleus. GFP fluorescence is shown as green; the merged image is a digital merge of bright field and fluorescent images to illustrate the protein compartments. All experiments were repeated a minimum of three independent times. Bars, 10 μm. (B) Y2H assays were performed using previously indicated derivatives with ASK1 and AtIAA7 in the presence of IAA. Other details as described in Fig. 3.
of the climacteric phase (~78 DAB), but steadily increased with the progression in ripening. In contrast, PslTIR1 and PslAFB2 transcript levels during ripening of V9 (~110–128 DAB) remained constitutively low throughout the process. PslAFB5 transcripts were undetectable during ripening in both cultivars.

Identification of PslTIR1/AFB alleles among plum cultivars

In an attempt to further unravel the effects of auxin during fruit development and links between auxin receptors and the variation in auxin sensitivity among cultivars, the full-length genomic sequences of the three genes were isolated and sequenced from EG and V9. All three gPslTIR1/AFB genes in both cultivars have open reading frames comprising three exons and two introns (Supplementary Figure S4 available at JXB online) with the exon/intron boundaries occurring in the same positions in both cultivars (Supplementary Figure S2 available at JXB online). The two cultivars were homozygous for gPslTIR1 and gPslAFB2; however, two different alleles were identified for gPslAFB5, with the alterations in nucleotide composition leading to four predicted changes in amino acid residues (Supplementary Figure S5 available at JXB online). One alteration in particular occurred within the F-box domain, causing a predicted substitution in the highly conserved residue Pro61 (Pro to Ser). Pro61 has been shown to be essential for TIR1-like function (Gray et al., 1999; Kepinski and Leyser, 2005). Given the nature of the substitution, we predicted that the allele form with Pro61 (designated gPslAFB5) would be functional and the allele with the substitution Ser61 (designated gPslafb5) would be inactive, or at best differentially functional. Strikingly, the auxin-hypersensitive cultivar, EG, was homozygous for gPslafb5; and the reduced auxin-sensitivity cultivar, V9, had the gPslAFB5/afb5 heterozygous genotype (Supplementary Figure S6 available at JXB online).

To confirm the distribution of gPslAFB5 alleles among cultivars, we performed a cleaved-amplified polymorphic sequence (CAPS) analysis (Fig. 6). Sequence analysis revealed that the exon-1 fragment of PslAFB5 (632 bp) is interrupted by a unique AccIII-restriction site that divides it into two smaller fragments (121 and 511 bp). Due to a nucleotide substitution, the AccIII-site is not present in the gPslafb5 allele. Direct digestion of the amplified gPslAFB5 exon-1 from the two cultivars confirmed that gPslAFB5 was represented by a single allele (gPslafb5) in EG and by two different alleles in V9.

The Pslafb5 protein is an inactive auxin-receptor

To determine whether the observed Pro61-to-Ser mutation in Pslafb5 influences SCF assembly and/or subsequent co-receptor formation with Aux/IAAs, the corresponding proline residues in PslTIR1 (Pro9) and PslAFB5 (Pro61) were mutated into serine. The consequences of these mutations were evaluated by assessing the interaction capacity of the full-length WT ORFs (as a positive control) and ORFs
carrying mutations, using both Y2H and BiFC approaches (Fig. 7). Relative to WT ORFs, all mutated proteins were unable to interact with ASK1 or AtIAA7 in yeast or tobacco protoplasts, providing strong evidence that the Pslafb5 allele encodes an inactive TIR1-like protein.

**Discussion**

Fruit growth is a complex process where cell division, expansion and differentiation are coordinated by a diverse range of hormones. Auxin is a regulator of patterning and tissue specification in many processes in plants, including the development of reproductive and fruiting tissues (Sundberg and Østergaard, 2009). In flowers, auxin specifies the site of floral primordium initiation and regulates floral organogenesis and patterning (Reinhardt et al., 2000; Cheng and Zhao, 2007). Post-fertilization, an interaction between auxin and gibberellin triggers fruit-set, the term given to the onset of rapid cell division necessary for early embryo development and fruiting structure enlargement (de Jong et al., 2009a; El-Sharkawy et al., 2014). In both berry- and stone-fruit, auxin has also been shown to be important during the immature fruit development and ripening processes (Miller et al., 1987; Agustí et al., 1999; Jones et al., 2002; El-Sharkawy et al., 2010, 2012; Seymour et al., 2013). Our results show that the IAA content in plums is high during flowering and in most phases of fruit development. The major exception is the S2 phase of fruit growth where the fruit appears to fall into a quiescent phase. Whereas auxin was once thought to be exclusively involved in the early stages of fruit development (S1 and S3), it is now understood also to be important during the S4 phase, where autocatalytic ethylene production is critical for the suite of changes that comprise the ripening process (Miller et al., 1987; Trainotti et al., 2007). We have previously shown the effect of auxin in enhancing ethylene production and ripening in Shiro, a late plum cultivar (El-Sharkawy et al., 2008, 2009, 2010, 2012). This finding led us to hypothesize that the differences in ripening-associated ethylene production observed in different plum cultivars are at least partially due to variations in the auxin-content and/or -responsiveness in fruiting tissues. The early ripening cultivar, EG, exhibited a sharp increase in auxin levels during fruit ripening and a strong auxin-sensitivity. Treatment with auxin elicited an enhanced ethylene climacteric comparable to that observed in fruit treated with propylene, a known ethylene inducer (McMurchie et al., 1972). Together, the data indicate that auxin plays a key role in progressing fruit development towards the transition phase that leads to the initiation of autocatalytic ethylene production. It also indicates that auxin regulates ethylene production and the progression of ripening. A capacity to advance and enhance ripening in EG suggests that the relative lack of auxin and/or reduced auxin-sensitivity in V9 is, at least partially, responsible for the suppressed ethylene production and prolonged ripening in this cultivar.

To investigate the molecular basis of auxin action in fruit development, three auxin-receptors, belonging to the F-box TIR1-like gene family, were isolated and characterized. TIR1-like proteins are components of an SCF–ubiquitin ligase complex that targets the Aux/IAA auxin-repressors for ubiquitination and subsequent degradation (Gray et al., 2001; Dharmasiri et al., 2003). Consistent with published data on TIR1-like proteins, we demonstrated that the isolated PsTIR1/AFBs were able to interact with the receptor complex component, ASK1 and that they were differentially responsive to various Aux/IAA proteins. Interestingly, the absence of auxin did not prevent the
assembly of PslTIR1/PslAFB2::AtIAA7; however, the tomato Aux/IAA (SIIAA3) interacts poorly with PslAFB5, even in the presence of auxin. Auxin orchestrates a broad range of processes and many levels of regulation are known to be involved in determining responses (Ljung, 2013), including biosynthesis/metabolism, transport, and response. Our data and those of others (Parry et al., 2009; Calderón Villalobos et al., 2012) show that another level of regulation exists at the level of receptor::auxin::Aux/IAA-binding capacity.

Assessing the role of different PslTIR1/AFBs domains in protein function confirmed the essential contribution of the F-box and C-terminal domains in ensuring appropriate receptor activity. Loss of the N-terminal domain in PslTIR1 and PslAFB2 had little effect on ligand–receptor complex formation and function; however, the long N-terminal region of PslAFB5 was required for full protein function. Similarly, other studies have also shown the importance of sequences outside the F-box for Skp1 association (Li and Johnston, 1997; Gray et al., 1999).

An analysis of transcripts for three PslTIR1/AFB mRNAs during EG and V9 fruit development did not correlate with the observed cultivar differences in terms of auxin-sensitivity and fruit development. Sequence data indicated, however, that while only one type of allele was present for two of the receptor genes, gPslTIR1 and gPslAFB2, two different allele types were identified for gPslAFB5. The auxin-hypersensitive cultivar, EG, is homozygous for the gPslafb5 allele, whereas V9 is gPslAFB5/gafb5 heterozygous. In the gPslafb5 allele, a serine residue is substituted for the highly conserved Pro residue located within the F-box domain. A targeted mutation in the corresponding proline (Pro10 to Ala) of the Arabidopsis TIR1 abolished auxin responses by inhibiting its capacity to interact with Skp1-like and Aux/IAA proteins (Gray et al., 1999; Kepinski and Leyser, 2005). This suggests that the Pslafb5 allele encodes an inactive auxin-receptor. In Arabidopsis, the loss of the closest homologues of PslAFB5, AFB4, and/or AFB5 resulted in a range of growth defects consistent with auxin hypersensitivity, suggesting that this class of auxin receptors act as negative regulators of auxin responses (Greenham et al., 2011). EG is therefore associated with the homozygous presence of the inactive Pslafb5 allele, and conversely, V9 is associated with the presence of a functional PslAFB5 allele. Further, we were able to show that a substitution of a highly conserved F-box proline residue (PslTIR1–Pro10 and PslAFB5–Pro10) into serine, simulating the naturally occurring sequence in the Pslafb5 allele, completely abolished auxin responses by inhibiting interactions with ASK1 and AtIAA7. This provides strong evidence that this proline residue is essential for protein function and therefore that the Pslafb5 allele in the EG cultivar is non-functional.

In the auxin-hypersensitive Arabidopsis afb4-2 mutant, IAA levels are not significantly altered compared to the wild type (Greenham et al., 2011). Intriguingly, EG also exhibited auxin hypersensitivity. Taken together, the results suggest that the active PslAFB5 is involved in the auxin-signalling network that negatively regulates fruit development processes, particularly from flowering until the end of the S1 stage. Thus, the loss of PslAFB5 function may increase auxin responses and consequently accelerate early fruit development processes in EG. Auxin can also be a part of the mechanisms that control the capacity of the fruit to ripen. However, the absence of PslAFB5 transcripts during late fruit growth and ripening phases (S3 and S4) in both plum cultivars suggested that the auxin content character should be more important than the auxin hypersensitivity feature for determining the auxin-dependence of fruit development and ripening. Auxin acts upstream of ethylene to control the initiation of autocatalytic ethylene production and the transition of a fruit from the end of growth into the onset of ripening. So the high auxin levels in EG should accelerate fruit growth and force the fruit to early transit into ripening; however, the lack of sufficient auxin in V9 delays both processes. Once autocatalytic ethylene production is initiated during ripening, the process will progress in both auxin- and ethylene-dependent manners (El-Sharkawy et al., 2008, 2009; Ziliotto et al., 2008).

The data presented here provide further evidence of the integral role played by auxin in fruit development, and provide a basis for continuing research to determine the precise nature of the many complex regulatory processes involved.

**Supplementary material**

Supplementary data can be found at JXB online.

**Supplementary Table S1.** Oligonucleotide primers used in this study.

**Supplementary Table S2.** Flowering, maturation dates and fruiting duration in different plum genotypes.

**Supplementary Table S3.** Changes in plum flower developmental characteristics due to auxin treatment.

**Supplementary Table S4.** Amino acid sequence comparison between the predicted full-length plum and Arabidopsis auxin receptors.

**Supplementary Figure S1.** Alteration in ethylene production during the ripening of fruits from early and late cultivars pre-exposed to different treatments designed to alter auxin- or ethylene-responses.

**Supplementary Figure S2.** Amino acid sequence alignment of plum and Arabidopsis TIR1/AFB proteins.

**Supplementary Figure S3.** Phylogenetic relationships between TIR1/AFB genes from plum and other plant species.

**Supplementary Figure S4.** Genomic structure of PslTIR1/AFB genes.

**Supplementary Figure S5.** Amino acid sequence comparison of the two plum AFB5 alleles (PslAFB5 and Pslafb5).

**Supplementary Figure S6.** Sequencing traces of PslAFB5 allelic genotypes in EG and V9 plum cultivars.

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