BYPASS1: synthesis of the mobile root-derived signal requires active root growth and arrests early leaf development

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Abstract

Background: The Arabidopsis bypass1 (bps1) mutant root produces a biologically active mobile compound that induces shoot growth arrest. However it is unknown whether the root retains the capacity to synthesize the mobile compound, or if only shoots of young seedlings are sensitive. It is also unknown how this compound induces arrest of shoot growth. This study investigated both of these questions using genetic, inhibitor, reporter gene, and morphological approaches.

Results: Production of the bps1 root-synthesized mobile compound was found to require active root growth. Inhibition of postembryonic root growth, by depleting glutathione either genetically or chemically, allowed seedlings to escape shoot arrest. However, the treatments were not completely effective, as the first leaf pair remained radialized, but elongated. This result indicated that the embryonic root transiently synthesized a small amount of the mobile substance. In addition, providing glutathione later in vegetative development caused shoot growth arrest to be reinstated, revealing that these late-arising roots were still capable of producing the mobile substance, and that the older vegetative leaves were still responsive.

To gain insight into how leaf development responds to the mobile signal, leaf development was followed morphologically and using the CYCB1,1::GUS marker for G2/M phase cells. We found that arrest of leaf growth is a fully penetrant phenotype, and a dramatic decrease in G2/M phase cells was coincident with arrest. Analyses of stress phenotypes found that late in development, bps1 cotyledons produced necrotic lesions, however neither hydrogen peroxide nor superoxide were abundant as leaves underwent arrest.

Conclusions: bps1 roots appear to require active growth in order to produce the mobile bps1 signal, but the potential for this compound’s synthesis is present both early and late during vegetative development. This prolonged capacity to synthesize and respond to the mobile compound is consistent with a possible role for the mobile compound in linking shoot growth to subterranean conditions. The specific growth-related responses in the shoot indicated that the mobile substance prevents full activation of cell division in leaves, although whether cell division is a direct response remains to be determined.

Background

Plants synthesize a wide array of metabolites, and a major goal of metabolomics is to identify natural plant metabolites and their associated functions (reviewed in [1-3]). Recent advances facilitating identification of metabolites [4,5] have led to identification of groups of metabolites that correlate with important plant traits, such as growth rate and biomass [6,7], and identified metabolic regulators such as leucine [8]. However, how specific metabolites other than characterized hormones function in signaling and development is largely unknown. One approach to learning about alternate signaling molecules is to study mutants with signaling-related defects.

The Arabidopsis bypass1 (bps1) mutant might be an important tool for identifying a metabolite functioning as a long-distance signal. The bps1 mutant produces small abnormal roots and shoot development arrests
soon after germination. This phenotype is linked to a mobile substance as the bps1 mutant root is necessary to induce arrest of bps1 shoots, and in graft chimeras, the bps1 root is sufficient to induce arrest of the wild-type shoot [9]. These observations led to a model featuring BPS1 as a negative regulator that was required to prevent the excess production of a mobile substance. The mobile compound appears to be novel, and its synthesis requires carotenoid biosynthesis [10]. The pathway producing the bps1 mobile compound appears to be conserved in plant lineages, as knock-downs of conserved BPS-like genes in tobacco produced similar phenotypes [11]. Critical questions include whether this mobile compound is an endogenous developmental regulator, and how it modifies shoot growth.

Control over shoot branching by a root-derived signal has been elegantly analyzed in pea, rice, and Arabidopsis [12-15]. In these systems, mutations disrupting biosynthetic enzymes lead to reduced production of a mobile compound that controls auxin transport in the shoot [16,17]. Recently, this substance was identified as strigolactone [18,19]. Additional unknown root-to-shoot signals have been implicated by studies of drought (reviewed in [20]), soil compaction [21], nutrient depletion [22-24] and low-fluence UV-B light [25]. The identities of the mobile compounds elicited by these treatments are unknown; it is also unknown whether the bps1 mobile substance is related to any of these pathways, but its root-to-shoot mobility make it an attractive candidate.

It is also possible that the bps1 mobile compound could instead be an intermediate molecule that normally doesn’t accumulate. For example, a biosynthetic pathway might be blocked in bps1 mutants, resulting in build-up of a precursor that happens to be mobile, and happens to have biological activity. For example, in superroot1 mutants, a defect in glucosinolate biosynthesis causes a build-up of precursors that spills over into auxin biosynthesis, resulting in a high-auxin phenotype [26].

Here, we evaluate the conditions under which bps1 roots produce the mobile compound, and the characteristics of shoots undergoing arrest from this substance. We find that bps1 roots produce and transport the mobile substance in actively growing roots, but that arrest of cell division leads to cessation of signaling to the shoot. Shoot responses include growth cessation, and in particular, arrest of cell division.

Results

The bps1 root: shoot growth inhibition requires root growth

The central feature of bps1 mutants is that a growth-arresting mobile compound arises in the root [9]. However, the experimental basis for this assignment required wounding, and it was only tested in very young seedlings. To expand our understanding of the root’s role in producing the bps1 signal, we examined how leaf development responded when bps1 root growth and development was blocked after embryogenesis. Post-embryonic root growth and development requires glutathione (GSH, [27]). Root development can therefore be blocked by either supplying germinating seeds with L-buthionine sulfoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase, or by generating double mutants between bps1 and root meristemless-1 (rml1-1), which has a defect in the gene encoding γ-glutamylcysteine synthetase and lacks post-embryonic root development [27,28].

In agreement with previous publications, the roots of BSO-treated wild type appeared to arrest development at germination. In addition, these plants produced small, but flattened, leaves with distinct blade and petiole, a modest reduction in leaf vein pattern, and bleached cotyledons (Figure 1). By contrast, bps1 mutants grown
on BSO-supplemented medium produced leaves with two distinct shapes (Figure 1). The first leaf pair was small, radially symmetric, and contained only a single unbranched vein, while subsequently produced leaves were broad, flat, showed distinct blade and petiole, and contained both primary and secondary veins. This partial rescue of leaf development in BSO-treated bps1 mutants suggested that post-germination arrest of the bps1 root led to reduced synthesis of the root mobile signal.

Similarly, the roots of rml1-1 mutants appeared to arrest development at germination, and they produced small, flattened leaves with distinct blade and petiole (Figure 1). Growth of rml1-1 mutants on GSH-supplemented medium restored post-embryonic root growth, as reported previously [27] (Table 1). The bps1 single mutants and F2 seedlings derived from rml1-1/+ bps1-2/+ parents, grown on GSH-supplemented media, were indistinguishable from bps1 controls (Table 1). By contrast, F2 seedlings derived from rml1-1/+ bps1-2/+ parents grown on standard growth medium (lacking GSH), segregated for four different phenotypes: wild type; rml1-1; bps1-2; and a phenotype similar to BSO-grown bps1 mutants (Figure 1). This last phenotype appeared at numbers consistent with it being the rml1-1 bps1-2 double mutant (Table 1). As with rml1-1, the bps1 rml1-1 double mutants produced roots that showed no sign of post-embryonic cell divisions. However, their first pair of leaves were radial and contained a single unbranched vein; these were similar to the bps1 single mutant, but much longer (Figure 1). Strikingly, leaf 3 and subsequently produced leaves were broad and flattened, with distinct petiole and blade, and contained both primary and secondary veins, much like the leaves of rml1-1 single mutants and BSO-treated bps1 (Figure 1). Thus, these data indicate that post-embryonic root growth and development is required for continuous production and delivery of the leaf-arresting substance and further support the root as the source of this molecule [9].

Because imposing a GSH deficit (using either BSO or rml1-1) led to partial rescue of the bps1 shoot phenotype, it was a formal possibility that the bps1 root-derived compound could be GSH itself. To test this, we supplied GSH to excised bps1 shoots, and monitored subsequent leaf development. Typically, root excision leads to partially rescued shoot development in approximately 75% of bps1 single mutants [9]. We reasoned that if the mobile signal was GSH, supplying it to bps1 mutants following root excision would reduce the number of bps1 mutants that were rescued by root excision. However, supplying GSH did not diminish shoot developmental rescue, and no regimen of GSH provision (prior to cut, after cut, or both prior and after cut) yielded a statistically significant reduction of developmental rescue. Moreover, shoot rescue resulted in leaves reaching similar sizes, whether or not excised shoots were supplemented with GSH (Table 2). These data indicated that the mobile compound was not GSH.

Arrested roots provide a transient source of the bps1 signal

Arrest of post-embryonic root growth in bps1 caused strikingly different responses in the first leaf pair as compared to leaf three. Both BSO-grown bps1 mutants and rml1 bps1 double mutants initially produced a pair of radialized leaves, yet rescued development was observed in subsequently produced leaves (Figure 1). In addition, the rml1-1 bps1-2 first leaf pair was consistently larger than that of BSO-grown bps1-2 mutants. These results contrast to root excision (carried out at day 4), where the strongest rescue was observed in the first leaf pair [9], and suggest that the arrested bps1 root might be a transient source of the bps1 mobile compound.

We tested this possibility using the bps1 temperature dependent phenotype [9]. We compared the first leaf pair of bps1 rml1-1 double mutants grown at 16, 22, and 29°C. For bps1 single mutants, leaf development is temperature dependent: severe arrest occurs at low temperatures, and small but flattened leaves are produced at high temperatures. Thus, if the radialized first leaf pair of bps1 rml1-1 double mutants was due to the bps1 mobile signal, then we expected its development to be similarly dependent on growth temperature.

Growth of bps1 rml1 double mutants at 16°C led to production of a narrow radially-shaped first leaf pair that was much longer than the bps1-2 control (Figure 2). In bps1 rml1 double mutants grown at 22°C, the first leaf pair was long, but very narrow. Its narrowness was

Table 1 The bps1 shoot phenotype requires post-embryonic root development

| Plants analyzed | GSH | Total shoot (n) | Shoot Phenotypes Observed | $\chi^2$ |
|----------------|-----|----------------|---------------------------|--------|
|                |     | wild type (n)  | rml1 (n)  | bps1 (n)  | bps1 rml1 (n)  |              |
| rml1-1         | -   | 240            | 189        | 51        | 0            | 0            | 1.8a        |
| rml1-1         | +   | 70             | 70         | 0         | 0            | 0            | n/a         |
| bps1-2         | -   | 224            | 164        | 0         | 0            | 0            | 0.381a      |
| bps1-2         | +   | 111            | 85         | 0         | 26           | 0            | 0.147a      |
| rml1-1 bps1-2  | -   | 1258           | 728        | 236       | 210          | 84           | 3.793b      |
| F2            |     |    |              |            |     |              |             |
| rml1-1 bps1-2  | +   | 590            | 437        | 0         | 153          | 0            | 0.2734c      |
| F2            |     |    |              |            |     |              |             |

n/a, not applicable.

a Critical $\chi^2$ values at 95% confidence is 3.841 when df = 1.

b Critical $\chi^2$ values at 95% confidence is 7.815 when df = 3.
similar to the age-matched \textit{bps1} single mutant, but it was much longer. Finally, in \textit{bps1 \text{rml1}} double mutants grown at 29°C, the first leaf pair was flattened and had a distinct blade, very similar to the \textit{bps1} control. The similar effects of growth temperature on the first leaf pair of \textit{bps1} single and \textit{bps1 \text{rml1}} double mutants indicates that the shape of the first leaf pair is due to the \textit{bps1} mobile root-derived compound. This suggests that the first leaf pair of \textit{bps1 \text{rml1}} double mutants was exposed to the root-derived compound, while the later-arising rescued leaves were not.

**Competence to synthesize and respond to the \textit{bps1} signal is retained in older seedlings**

Although the molecular target of the \textit{bps1} root-derived mobile compound is unknown, root-dependent arrest of early shoot development in \textit{bps1} seedlings indicates that the target is present at this early stage. However, we do not know if the molecular target is present later in development, nor do we know whether an older root retains the capacity to synthesize the mobile compound.

To test this, we followed up on the observation that \textit{rml1} root development is rescued by supplying glutathione (GSH) [27]. We reasoned that supplying GSH to an older \textit{bps1 \text{rml1}} double mutant might restore growth of a \textit{bps1}-like root. If this root retained the ability to synthesize and deliver the mobile compound, and its molecular target was present in older shoots, then we would expect to observe arrested leaf growth.

Seeds segregating for both \textit{bps1} and \textit{rml1-1} were plated on standard growth media, and at 10 days, 90 seedlings with \textit{rml1} root phenotype were transferred to GSH-supplemented medium (approximately 22-23 were expected to be \textit{bps1 \text{rml1}} double mutants). At 18 days after transfer (28 total days), we analyzed their phenotypes. Most of the plants looked the same as \textit{rml1-1} controls; they produced normal-appearing roots and large flat leaves (Figure 3). However, 18 of the seedlings

| Growth Medium | Total \textit{bps1} with excised root (n) | Percent producing broad leaves with distinct blade and petiole (n) |
|---------------|---------------------------------------|---------------------------------------------------------------|
| Pre-excision  | Post-excision                         |                                                               |
| GSH-          | GSH-                                  | 31                                                            | 77% (24)                                      |
| GSH-          | GSH+                                  | 40                                                            | 83% (33)                                      |
| GSH+          | GSH-                                  | 27                                                            | 70% (19)                                      |
| GSH+          | GSH+                                  | 34                                                            | 94% (32) **                                   |

**The number of plants that produce leaves under GSH+/GSH+ conditions is statistically greater than the number that produces leaves under GSH-/GSH- conditions (p value = 0.009).**
produced roots that were short, blunt, and very swollen, and had produced lateral roots somewhat similar to bps1. These plants also produced shoots with variable leaf shapes. Their first leaf pair was long and radial, the subsequently produced 5-9 leaves appeared flat (partially rescued), and with distinct petiole and blades. Finally, the newest arising leaves were short and radially shaped (bps1-like). This range of leaf phenotypes is consistent with restored synthesis and delivery of the bps1 mobile compound upon induction of root growth, and response in these later-arising vegetative leaves. These results indicate that roots retain the capacity to synthesize and deliver the bps1 mobile substance to the shoot, and that the shoots of older seedlings retain the ability to respond.

The bps1 mobile compound: synthesis and delivery require neither the phloem nor endodermis

We next developed double mutants that combined bps1 with altered phloem development (apl), shortroot (shr), and scarecrow (scr) mutants. The apl mutant lacks phloem, shr lacks endodermis, and scr replaces endodermal and cortical cell layers with a single layer of mixed identity [29-33]. We predicted that if the phloem or endodermis were the sole site of synthesis of the bps1 mobile compound, or required for its transmission, then leaf development would be at least partially restored in the double mutants.

The bps1 apl double mutants showed an arrested shoot phenotype that was indistinguishable from bps1 (Figure 4), indicating that phloem was dispensable for synthesis and delivery of the bps1 mobile compound, at least in these very small mutants. Similarly, both shr bps1 and scr bps1 double mutants resembled the bps1 single mutant (Figure 4, Table 3). Taken together, these data indicate that normal root development, including formation of the phloem and the endodermis, is not required for production and delivery of the bps1 signal.

Shoot responses to the bps1 mobile root-derived compound

The reversible arrest of shoot development in bps1 mutants correlates with a loss of auxin responses [9], but the underlying mechanism of arrest is unknown. To broaden our understanding of shoot responses in bps1, we carried out a series of time-course analyses where we analyzed leaf size, shape, the distribution of dividing cells, and stress responses (necrotic lesion formation and appearance of ROS).

Dividing cells were identified using the CYCB1;1::GUS reporter, a cell cycle reporter expressed in cells at the G2/M phase [34]. Patterns of CYCB1;1::GUS expression in Arabidopsis are well characterized; early leaf development shows a nearly uniform distribution of GUS-staining (i.e. dividing) cells, while later in development cell divisions become restricted to the leaf base and provascular tissue [35] (Figure 5). In bps1 mutants, the three-day leaf primordia largely matched wild type in terms of size, shape and CYCB1;1::GUS expression patterns, however there were pronounced differences by day four. The four-day wild-type leaf was much larger than that of bps1 and CYCB1;1::GUS-staining cells were distributed throughout, while the small four-day bps1-2 leaf had only a few CYCB1;1::GUS-staining cells. At day five, the wild-type leaf started to show distinct lamina expansion, and a slight tendency for there to be more GUS-staining cells toward its proximal end. By contrast, the five-day bps1 leaf showed no sign of lamina expansion, and few CYCB1;1::GUS-staining cells. The six-day wild-type leaf showed a strong reduction of CYCB1;1::GUS-staining cells at the distal end, and was much larger than the five-day wild-type leaf, while the corresponding bps1

![Figure 4](http://www.biomedcentral.com/1471-2229/11/28)

**Figure 4** Neither phloem nor endodermal cell types are required for production or transmission of the bps1 mobile compound. Top row: shoot and root phenotypes of 15-day Col-0 (left), the phloem-deficient apl (middle), and scarecrow (scr, right). Bottom row: shoot and root phenotypes of bps1 (left), the bps1 apl double mutant (center), and bps1 scr double mutant (right). The double mutants show a leaf arrest phenotype that is similar to that of bps1, indicating that the root-derived mobile compound is still synthesized and transmitted to the shoot. Seedlings shown here were grown at 22°C and photographed at 15 days. Size bars: 1 mm.

### Table 3 shr and scr radial patterning defects do not suppress the bps1 shoot phenotype

| Plants analyzed | Total (n) | wild type (n) | shr (n) | bps1 (n) | Double mutant (n) | \( \chi^2 \) |
|----------------|----------|---------------|--------|---------|------------------|---------|
| sc3-9 bps1-2 F3 | 721 | n/a | 545 | n/a | 176 | 0.134* |
| shr bps1-2 F2 | 1040 | 576 | n/a | 194 | 270 | n/a | 0.528b |
| shr | 167 | 129 | n/a | 38 | n/a | n/a | 0.449a |
| bps1-2 | 191 | 146 | n/a | 45 | n/a | n/a | 0.211c |

n/a, not applicable

* Critical \( \chi^2 \) values at 95% confidence is 3.841 when \( df = 1 \).

** Critical \( \chi^2 \) values at 95% confidence is 5.991 when \( df = 2 \).
leaf was largely unchanged. By day seven, the wild-type leaf was even larger and the few CYCB1;1::GUS expressing cells were at the leaf base. Similarly, the bps1 seven-day leaf had only a few CYCB1;1::GUS-staining cells, and most were restricted to the leaf base. This analysis revealed a fully penetrant leaf arrest phenotype. In addition, despite the striking reduction in numbers of dividing cells, the apical/basal spatial control of cell divisions appeared to be intact.

While carrying out this analysis of leaf development, we also compared patterns of CYCB1;1::GUS-staining in roots (Figure 5). In the wild type, CYCB1;1::GUS-staining patterns were restricted to the root meristem, as has been described previously [36], and a similar pattern was observed between days three and seven. By contrast, at all time points, the bps1 root had fewer CYCB1;1::GUS-staining cells.

The bps1 mutant analysis revealed occasional necrotic lesions on bps1 cotyledons (Figure 6A). To assess a possible relationship between these lesions and leaf arrest, we carried out another time-course analysis, this time examining wild type and bps1 mutants for necrotic lesion formation. In bps1 mutants, necrotic lesions began to appear between 8 and 10 days. They were restricted to cotyledons, and never observed on leaves, hypocotyls, or roots, and necrotic lesions were never observed on the wild type (Col-0 or L. er) (Figure 6B). More bps1-1 seedlings formed necrotic lesions than bps1-2, and by day 18, 92% of the bps1-1 plants had at least one necrotic lesion. The average lesion number per plant was highly variable, and increased over time (Figure 6C); by day 18 the bps1-2 mutants had between zero and seven necrotic lesions. Both bps1-1 and bps1-2 are null alleles [1], and so we attribute the difference in lesion formation to their genetic backgrounds (Col-0 for bps1-2 and L. er for bps1-1).

Because lesion formation is typically preceded by reactive oxygen species (ROS) [37-40], we compared ROS in bps1 and wild type shoots using diaminobenzidine (DAB) to assay for hydrogen peroxide (H₂O₂) and nitroblue tetrazolium (NBT) to assay for superoxide. Because the bps1-1 allele showed a more robust necrotic lesion
phenotype, these analyses used bps1-1 and L. er. Both staining procedures produced a strong reaction in the vascular tissue, consistent with a role for ROS in lignification [41,42]. We found H$_2$O$_2$ in the 14-day bps1 cotyledons, typically in positions surrounding the developing necrotic lesions (Figure 7A), but did not observe any nonvascular staining in the wild type cotyledon (data not shown). Additionally, we did not detect H$_2$O$_2$ in bps1 leaves. Similarly, superoxide was primarily associated with vascular tissue in wild type leaves, and it was nearly absent from the leaves of bps1 mutants (Figure 7B). Because accumulation patterns of these two ROS were similar for the wild type and bps1 mutants, severe oxidative stress does not appear to cause the bps1 leaf developmental arrest.

**Discussion**

Physiological studies have implicated long distance signaling as a link between the development and physiology of roots and shoots [20]. However, only a small number of long-distance signaling pathways have been verified molecularly. In Arabidopsis bps1 mutants, the non-cell-autonomous activity of mutant roots suggests that BPS1 might function to limit the synthesis of a root-derived mobile signaling molecule [9].

**Capacity to Synthesize the BPS1 Mobile Compound**

A central feature of bps1 mutants is that the root is the source of a biologically active mobile compound, which we refer to as the bps1 signal. Here, we extended our understanding of the conditions under which the mutant root produces this compound. Previously we showed that cutting off the root led to rescue of the first leaf pair [9]. Indeed, we have now found that arresting post-embryonic bps1 root growth also resulted in rescue of leaf development. However, in contrast to root excision, the first leaf pair was only mildly rescued, and strong rescue was delayed until leaf three. These observations indicate that the bps1 root, despite post-embryonic arrest, retained a transient ability to supply the bps1 signal to the shoot.

We used two related approaches to arrest post-embryonic root growth: we caused arrest through the depletion of GSH either genetically (using the rml1-1 mutant) or chemically (using BSO). In both cases, the first leaf pair in GSH-depleted bps1 was larger than that of untreated bps1 mutants, and the first leaf pair of bps1 rml1-1 double mutants were consistently larger than that of BSO-grown bps1. Here, a larger leaf size probably reflects an earlier block to GSH synthesis in the mutant, and therefore an earlier reduction in bps1 signal synthesis.

Similarly, we found that restoring development of bps1 roots (by GSH provision to bps1 rml1-1 seedlings) reinstated arrest of leaf development. The extended capacity to produce and respond to the mobile compound is in line with physiological studies of drought-evoked long distance signaling, which has been documented in diverse plants, and at varying developmental stages [4].

A possibly less obvious question is why growth-arrested roots (i.e. bps1 rml1-1 double mutants and BSO-grown seedlings) show a decreased ability to arrest shoot growth. One possibility is that bps1 signal synthesis has a direct requirement for GSH. Alternatively, either synthesis or transmission to the shoot requires active root growth and cell division.

The maintenance of shoot arrest in apl bps1 double mutants is consistent with a link to root growth. Although apl mutants have determinate roots [29], growth ceases later than for rml1-1 or BSO-treated

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**Figure 7** ROS is not increased in arrested bps1 leaves

(A). Hydrogen peroxide, visualized using DAB, was found in necrotic lesions and associated with vascular tissue, but it was not elevated in the bps1 leaf. (B) Superoxide, visualized using NBT staining, was found associated with vascular tissue, but it was not elevated in the bps1 leaf. Bars = 200 μm.
plants, and the *apl bps1* analysis was carried out prior to evidence of root cell division arrest. However, if root growth is a requirement for *bps1* signal synthesis, then we would need to be able to explain constitutive synthesis of *bps1* signal in *bps1* mutants, which show primary root arrest soon after germination. One possibility is that synthesis is sustained by lateral roots, which initiate repeatedly. Alternatively, *bps1* roots (including the primary) expand radially, and this radial growth might also sustain synthesis of the *bps1* signal.

**bps1** signal transmission

Movement of the *bps1* signal from the root to the shoot is likely to use the plant’s vascular system. Two vascular tissues are specialized for long-distance movement: the phloem, which transports photosynthetic products; and the xylem, which primarily transports water and dissolved nutrients. Here, we found that the shoot undergoes arrest in *bps1 apl* double mutants, which lack phloem [29]. The simplest conclusion is that the *bps1* signal moves in the xylem. However, this conclusion is not definitive, because the very small size of *bps1 apl* double mutants doesn’t preclude movement by diffusion.

**Shoot responses to the mobile bps1 signal**

The small leaf size and reduced number of CYCB1;1::GUS expressing cells are a fully penetrant *bps1* phenotype. Strikingly, although reduced in number, the pattern of CYCB1;1::GUS-expressing cells mimicked the wild type pattern: leaf primordia showed an even distribution of dividing cells, but as the mutant leaves matured, dividing cells were restricted to the base of the leaf. The retention of a normal pattern of dividing cells shows that some aspects of leaf developmental programming persist in *bps1* mutants. This result hints that instead of altering development, the *bps1* signal might instead disrupt the link between development and cell cycle control.

Another phenotype in *bps1* mutants is the formation of necrotic lesions. These were late-appearing and not fully penetrant. Necrotic lesions have been observed in a wide range of Arabidopsis mutants. These include plants with defects in syntaxin genes [43], and mutants with defects in the cytochrome P450 gene *CYP83B1*, which results in excess auxin synthesis [44]. Necrosis is typically associated with plant defense responses, and can be a secondary consequence of elevated expression of defense genes, such as observed in the developmental mutant *asymmetric leaf 1* [45] and in response to phosphate deficiency [46,47].

**Conclusions**

The results presented here support the phenomenon of shoot arrest by a root-derived molecule in *bps1* mutants. A key question raised by discovery and characterization of this mutant is whether the *bps1* mutation exposes a novel root-to-shoot signaling molecule or a metabolic intermediate with toxic effects on shoot development. The crucial difference between these two concepts is that a novel root-to-shoot signaling molecule would be present in the wild type, while a metabolic intermediate would only accumulate in *bps1* mutants. Because the synthesis of the root-derived molecule requires post-embryonic root development and aerial organs appear to arrest growth prior to showing any signs of toxicity (necrosis), we tend to favor the hypothesis that *bps1* reveals a novel root-to-shoot signaling pathway. A full resolution of this issue awaits biochemical identification of this mobile molecule. Regardless of the nature of the root-derived compound, it should be pointed out that under either scenario the *bps1* mutation has unveiled a molecule with potent biological activity. Despite the impact of root-to-shoot communication on plant productivity, the molecular mechanisms involved are poorly understood. The *bps1* mutation could be utilized as a tool to begin to dig into the pathways that both synthesize and respond to root-derived growth modulators.

**Methods**

**Plant Growth**

All seeds were cold-shocked for 2-4 days in darkness at 4°C, and most grown in 24 hour light at the 22°C, unless noted otherwise. Growth media composition is 0.5X MS salts (Caisson labs), 1% sucrose, 0.5g/l MES, pH 5.8, 0.8% phytoblend agar (Caisson Laboratories). Seedlings were grown in Conviron TC30 growth chambers under light and temperature regimes as described.

**Plant Materials**

Mutant alleles used: *bps1-2* (Col), *bps1-1* (*Ler*), *rml1-1* (Col, received from Z.R. Sung), *scr-3* (Col, CS3997), *shr* (Col, SALK_002744), *apl* (Col, received from M. Bonke), and CYCB1;1::GUS seeds were received from J.L. Celenza.

**GUS Staining**

The CYCB1;1::GUS transgene was crossed into both *bps1-1* and *bps1-2*, and F3 lines homozygous for the transgene and segregating for *bps1* were identified. We plated these lines (and control wild-type transgenic) on normal growth media, and subjected them to a 2-7 day cold shock (4°C). Each day, plates were transferred to a 22°C growth chamber. GUS staining followed previously published protocols [48].

**Conditional Root Arrest**

Arrest of roots using BSO was carried out by making our standard GM (above), and supplementing it to 2.5 mM BSO (DL-Buthionine-[S,R]-sulfoximine, Sigma),
and bps1-2 rml1-1 double mutants were generated by standard methods. For both BSO and rml1 experiments, plants were grown in short day (8 hours light/16 hours dark) at 22°C. To reinstate root growth of rml1-1, we supplemented the media to 750 μM glutathione (Acros Organics) [27]. To test whether the number of plants producing broad leaves upon root excision in the presence of GSH was statistically different from that observed in the absence of GSH (Table 2), we performed hypothesis testing for proportions using the Z-score method. If GSH were the root-shoot signal, we would predict that the number of plants forming broad leaves under GSH-conditions (the null hypothesis). The statistical tests indicate that the number of plants producing leaves in the presence of GSH is not less than or equal to the number producing leaves in the absence of GSH. Because the calculated P value is low, we must reject the null hypothesis in support of the alternative hypothesis that the number of plants producing leaves under GSH+ conditions is greater than GSH-conditions. This indicated that the root-to-shoot signal is not GSH.

Stress symptom analyses
Necrotic lesion formation was assessed by a visual inspection of bps1 and wild type seedlings. All organs of the investigated seedlings were examined on alternate days. To visualize patterns of H2O2 in seedlings (wild type and bps1), we used 3,3’-diaminobenzidine (DAB) staining as described [49,50]. We infiltrated 0.1% (W/V) DAB (Sigma), pH3.8, and allowed staining to progress for 4-6 hours. After staining, samples were cleared in 70% ethanol, and then transferred to 40% w/v glycerol, mounted on glass slides, and examined on Olympus BX50 and Olympus SZX16 microscopes. Visualization of superoxide patterns used nitroblue tetrazolium staining protocols as described [51,52].

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Authors’ contributions
JMVN carried out rml1 and BSO root arrest experiments and the apl bps1, shr bps1, and scl bps1 double mutant analyses. CM carried out analyses of ROS, characterized the lesion formation phenotype, and analyzed shoot phenotypes following resumption of root development. LES carried out the pCYC81:1-GUS analyses. LES and JMVN planned the project together, and the manuscript was primarily written by LES with assistance from JMVN. All authors read and approved the final manuscript.

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